Substrate Specificity of the Heparin Lyases from Flavobacterium heparinum[1,2]

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A detailed knowledge about the substrate specificities of the heparin lyases is necessary when using these enzymes as tools for elucidating the sequence of heparin and heparan sulfate. The substrate specificity of heparin lyases I, II, and III have been profiled with structurally defined, heparin-derived oligosaccharides. The primary substrate specificities of heparin lyases I and III require the presence of 2-*O*-sulfated α-L-idopyranosyluronic acid and β-D-glucopyranosyluronic acid residues, respectively, at the linkages being cleaved. Heparin lyase II demonstrates an intriguingly broad primary specificity for oligosaccharides, acting at linkages containing α-L-idopyranosyluronic and β-D-glucopyranosyluronic acid as well as at linkages containing α-L-galactopyranosyluronic acid residues. In addition to their primary specificities, each lyase also demonstrates secondary specificities under forcing conditions. Differences in the sulfation pattern within uronic acid residues and sulfation of adjacent residues has profound impact on the ease of lyase cleavage of a glycosidic linkage. Specifically, heparin lyases I and III exhibit secondary specificity for oligosaccharides containing an unsulfated α-L-idopyranosyluronic acid residue. The lack of sulfation on residues adjacent to the linkage undergoing cleavage increases the action of heparin lyase III on a glycosidic linkage. In contrast, reduced sulfation on adjacent residues make glycosidic linkage resistant to heparin lyase I. The primary and secondary specificity can be rationalized on the basis of most favorable solution conformation of the uronic acid residues. © 1993 Academic Press, Inc.

Heparin and heparan sulfate are sulfated, polydisperse, microheterogeneous, linear copolymers of alternating 1→4-linked 2-deoxy-2-aminoglucopyranose and hexopyranosyluronic acid residues. Although heparin has been used as an anticoagulant for the past 50 years, its precise structure remains unknown. Much of the biological activity of heparin and heparan sulfate (for reviews see 1, 2) stems from the polyelectrolyte nature of these polysaccharides.

Heparin lyases, isolated from *Flavobacterium heparinum* (3–5) (also called *Cytophaga heparina* (6)), are important members of a class of enzymes called polysaccharide lyases (EC 4.2.2) that depolymerize certain acidic polysaccharides (7). Heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (heparitinase II, no assigned EC number), and heparin lyase III (heparitinase I, EC 4.2.2.8) have recently been purified to apparent homogeneity in our laboratory (8). Although these enzymes have been used for a number of purposes, the precise mode of their action and many aspects of their substrate specificities are still unclear.

Enzymatic degradation studies of the heparin and heparan sulfate polymers and identification of resulting disaccharide products have been the principal methods used for studying substrate specificities of heparin lyases. Studies by a number of workers (7, 9–11) demonstrate that the cleavage site by heparin lyase I is →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→; that by heparin lyase III is →4)-α-D-GlcNp2Ac(or 2S)6OH(or 6S)(1→4)-β-D-GlcAp(1→. Heparin lyase II cleaves the linkages containing either (1→4)-α-L-idopyranosyluronic or (1→4)-β-D-glucopyranosyluronic acid residues (12–15). In addition, heparin lyase II can degrade many chemically modified polymers to a significant extent (16, 17). Rice and Linhardt (18) demonstrated that heparin lyases I and III tolerate 3-*O*-sulfation on the α-D-2-deoxy-2-aminoglucopyranose residue and the earlier report of 6-*O*-sulfate group on the α-D-2-deoxy-2-aminoglucopyranose moiety being an impediment to heparin lyase III (15) was not substantiated. Recent studies in our laboratories using chemically modified polymers have shown that heparin lyase III can degrade chains containing α-L-idopyrano-

syluronic acid residue (17). Studies by Yamada *et al.* (19) suggest that tetrasaccharides containing 3-*O*-sulfated 2-deoxy-2-aminoglucopyranose reducing end moiety are resistant toward the heparin lyases.

The analysis of substrate specificity has been intriguingly difficult due to contrasting results presented by many researchers. Enzyme preparations of varying purity have been used by different workers. The situation is also made more complicated by the use of different names for these enzymes, ill-defined polymeric substrates, and a variety of enzymatic reaction conditions. The present work utilizes homogeneous preparations of the three heparin lyases, defined oligosaccharide substrates, and optimized conditions for these enzymatic reactions (8).

Although the principal sites of action for the heparin lyases I and III comprise more than 80% of the heparin and heparan sulfate polymers, respectively, the lyase-based sequencing of heparin and heparan sulfate (20) requires a more complete understanding of specificities of these enzymes. The influence of sulfation on residues adjacent to linkage undergoing cleavage has also not been explored. The present paper examines the different sites of lyase action on structurally defined oligosaccharides and exposes some of the complexities of using these lyases for sequence analysis. An understanding of heparin lyase specificity on the basis of the favored solution conformational preference of the uronic acid residue undergoing cleavage is also presented.

EXPERIMENTAL PROCEDURES

Materials

Disaccharides 1–4 (Fig. 1) were purchased from Grampian Enzymes (Aberdeen, Scotland). Disaccharide 5 was prepared by semipreparative SAX–HPLC[4] fractionation of oligosaccharides afforded by heparin lyase II digestion of a chemically modified heparin (21). Oligosaccharides 6–19 (Fig. 1) were purified from oligosaccharides prepared by heparin lyase I depolymerization of porcine intestinal heparin using SAX–HPLC as previously described (18, 22–24). Heparin lyase I (heparinase, EC 4.2.2.7), heparin lyases II (no assigned EC number), and heparin lyase III (heparitinase, EC 4.2.2.8) were purified from *F. heparinum* (8) to apparent homogeneity. Heparin lyase I had enzymatic activity of 130 units/mg (μmol min^{-1} mg^{-1}) (1 unit of lyase activity is defined as 1 μmol of product formed per minute using 1 mg/ml of substrate at appropriate temperature) against heparin. Heparin lyase II had enzymatic activity of 19 units/mg against heparin and 36.5 units/mg against heparan sulfate, while heparin lyase III had an activity of 63.5 units/mg against heparan sulfate. Dialysis membranes with defined molecular weight cutoffs (MWCO) were obtained from Spectrum Medical Industries (Los Angeles, CA). HPLC was performed on dual constametric II pumps connected through a gradient mixer from LDC Milton Roy (Riveria Beach, FL). Fixed-loop injector 7125 from Rheodyne (Cotati, CA) and variable-wavelength UV-5 detector from ISCO (Lincoln, NE) were used. SAX–HPLC was carried out using a Spherisorb (4.6 × 250 mm, 5 μm particle size) column from Phase Separations (Norwalk, CT). Capillary electro-

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[4] Abbreviations used: NMR, nuclear magnetic resonance; SAX, strong anion exchange; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; AUFS, absorbance units full scale; MWCO, molecular weight cutoff.

phoresis was performed with Dionex Capillary Electrophoresis System from Dionex Corporation (Sunnyvale, CA). Electrophoresis-grade sodium borate and boric acid were from Fischer Scientific (Fair Lawn, NJ). All other chemicals were reagent grade.

Methods

Treatment of substrates with enzymes. The reactions were performed at the appropriate temperatures (heparin lyase I, 30°C; heparin lyase II, 30°C; heparin lyase III, 43°C) until reaching completion in 24 h. For heparin lyase I, oligomeric substrate (10 μg/50 μl) in 5 mM sodium phosphate containing 200 mM sodium chloride, pH 7.0, was treated with 25 mIU lyase. Heparin lyase II and III reactions were performed in 50 mM sodium phosphate, pH 7.0, utilizing similar ratios of lyase and substrate. The reactions were terminated by addition of hydrochloric acid, resulting in a solution of pH 3.0. The reaction mixture was poured onto an SP-Sephadex microcolumn (100-μl volume), preequilibrated with hydrochloric acid, pH 3.0, to remove the enzyme. After washing the column with hydrochloric acid (2 ml, pH 3.0), the combined eluent and washes were adjusted to pH 7.0 with sodium hydroxide and freeze–dried.

SAX–HPLC analysis of heparin lyase-treated substrates. The heparin lyase-treated heparin oligosaccharide was injected in amounts between 4 and 40 μg onto an analytical SAX–HPLC column (5-μm particle size). The sample was eluted from the column with a linear gradient [concentration (y, M) at any time (x, s) = 0.0002x + 0.2] of sodium chloride, pH 3.5, and a flow rate of 1.5 ml/min. The elution profile was monitored by the absorbance at 232 nm at 0.01 AUFS. The amount of the resulting oligosaccharide products was assessed by the computerized integration of peak area using a standard curve. Peaks were assigned by either coelution with an authentic sample or retention time.

Capillary electrophoresis of heparin lyase-treated substrates. For capillary electrophoresis analysis a fresh capillary (69 cm total length, 75 μm internal diameter) was activated by extensively washing sequentially with 0.1 M phosphoric acid, 0.5 M sodium hydroxide, deionized, distilled water, and buffer in that order. The buffer consisted of 10 mM sodium borate containing 50 mM boric acid, pH 8.8, or 10 mM sodium borate containing 50 mM sodium dodecylsulfate, pH 8.8. The analysis employed detection at 232 nm. The samples (2 mg/ml), prepared in deionized, distilled water, were injected into the capillary using hydrostatic pressure. Each injection used 14 nl sample. Electrophoresis was performed at a constant voltage of 20 kV for a period of 30 min.

NMR spectroscopy of oligosaccharides. The [^{1}H]NMR experiments for tetrasaccharides 12 and 14 were performed using a Bruker AMX 600 spectrometer at the operating frequency of 600 MHz. The samples were dissolved in deionized, distilled water and filtered through a 0.45-μm membrane. The solution was lyophilized and the resulting solid exchanged in ^{2}H$_2$O (99.9% atom ^{2}H) by repeated lyophilization. After three exchanges, the solid was dissolved in ^{2}H$_2$O (99.96% atom ^{2}H, 500 μl) for NMR experiments. The [^{1}H]NMR spectra were at recorded 298 K with a digital resolution of 0.123 Hz/point. The HO^{2}H signal was suppressed with a low-power pulse applied for 2.5 s.

RESULTS

Degradation of defined oligosaccharide substrates. A number of conditions for enzymatic depolymerization by heparin lyases are reported. These include an ethylenediamine–acetate buffer system (15), a sodium acetate-calcium acetate buffer system (25), and a sodium phosphate buffer system (14, 16, 26). Optimum conditions for the three pure heparin lyases have recently been elucidated by Lohse and Linhardt (8) and these conditions are used in this study.

The action of heparin lyases on oligosaccharide was followed quantitatively by the use of SAX–HPLC or by

CE. Each purified, structurally characterized, oligosaccharide substrate was analyzed by analytical SAX–HPLC (see Methods) both before and after enzymatic digestion. Oligosaccharide standards corresponding to the expected products of the lyase treatment were coinjected to confirm the identity of the product(s). Tetrasaccharide 11, for example, formed disaccharides 1 and 3 on treatment with heparin lyase I (Fig. 1, Table I). About 20% of tetrasaccharide 11 was converted to these products under the reaction conditions used. Treatment of tetrasaccharide 11 with heparin lyase II gave 1 and 3 with almost complete conversion of substrate 11, while heparin lyase III converted the substrate 11 to disaccharides 1 and 3 in ~70% yield. Thus all three enzymes can act with different facility to cleave the →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→ linkage present in 11.

Seven tetrasaccharides, four hexasaccharides, and one octasaccharide (Fig. 1) were used to profile the substrate specificity of the three heparin lyases and the results are tabulated in Table I. Oligosaccharides 10, 18, and 19 are completely cleaved into disaccharide 1 with heparin lyase I. While tetrasaccharides 6, 7, and 11, having α-L-idopyranosyluronic acid, are partially degraded (~20–50%), tetrasaccharides 8, 9, and 14 are completely resistant to heparin lyase I.

Heparin lyase II acted on all the oligosaccharides studied. In addition, heparin lyase II leads to almost complete degradation of the oligosaccharides within 24 h. Heparin lyase III cleaved substrates 8, 9, 11, and 15 in good yields. Table I suggests that oligosaccharides that are the best substrates for heparin lyase I (i.e., substrates 10, and 18) were most resistant to heparin lyase III.

Hexasaccharide 16 comprises a portion of the AT III binding pentasaccharide sequence (27, 28) that has an unique 3-O-sulfate group in one of the internal α-D-2-deoxy-2-aminoglucopyranose residues. Hence, it was important to examine the behavior of hexasaccharide 16 toward the heparin lyases. Only 50% of hexasaccharide 16 was converted to products on treatment with heparin lyase II. The digestion mixture on capillary electrophoresis showed two products, one of which was identified as disaccharide 1 by comigration. The second product eluting with a longer migration time did not correspond to any available oligosaccharide standard. This product was prepared by a large-scale enzymatic digestion of 16 with heparin lyase II, followed by semipreparative SAX–HPLC. The [¹H]NMR spectrum for this new oligosaccharide showed anomeric signals at δ 5.47 (d, H-1 of ΔUAp residue), 5.46 (d, H-1 of reducing end GlcNp2S3S6S residue), 4.64 (d, H-1 of internal GlcAp residue), and 5.17 (d, H-1 of internal GlcNAcp6S residue). A doublet at δ 5.81 ppm could be assigned to 4-H of ΔUAp residue. A signal at δ 4.56, characteristic for the 3-H of reducing end α-D-2-deoxy-2-aminoglucopyranose residue, indicated that the structure of the new product is 12.

Heparin lyases I and III do not act on hexasaccharide 16. Hexasaccharide 16 was reported to be degraded by heparin lyase III (18, 22), and heparin lyases I and II (14). The apparent contradiction with our earlier reports may be the result of lack of the homogeneity of earlier heparin lyase preparations. Contamination with 3-O-sulfoesterase may result in the desulfation at the 3 position of the reducing end, producing hexasaccharide 15 that is susceptible to heparin lyase III. Because of the high concentrations of enzyme and the long incubation time required for digestion of these oligomeric substrates, the heparin lyase preparations need to be extremely pure. This study utilizes only the apparently homogeneous heparin lyase preparations recently purified in our laboratory.

Hexasaccharide 15 is composed of α-L-idopyranosyluronic and β-D-glucopyranosyluronic acid residues and differs from hexasaccharide 16 only in the absence of 3-O-sulfate group in the reducing end α-D-2-deoxy-2-aminoglucopyranose residue. Yet its behavior toward the heparin lyases is strikingly different when compared to that of substrate 16. Heparin lyases II and III degrade substrate 15 into disaccharides 1, 3, and 4 in greater than 75% yield, while heparin lyase I yields disaccharide 1 and a new product that could not be identified as one of the oligosaccharide standards. The [¹H]NMR spectrum of new saccharide resembled that of tetrasaccharide 12 except for the absence of a multiplet at δ 4.56. This suggested that the new product in the heparin lyase I treated hexasaccharide 15 is tetrasaccharide 13.

Tetrasaccharides 14 and hexasaccharide 17 (21) having an unnatural internal α-L-galactopyranosyluronic acid residue, were also examined as substrates for all three heparin lyases. Heparin lyases I and III did not act on either of these oligosaccharides. Enzymatic digestion of either 14 or 17 with heparin lyase II resulted in the nearly complete disappearance of substrates as determined by capillary electrophoresis. One of the products was easily identified as disaccharide 1 its by comigration with standard disaccharide. A second faster moving product was identified as (1R,2S,3S)ΔUAp(1→4)-D-GlcNp2S6S (5) by its isolation and characterization using [¹H]NMR (17). Thus, heparin lyase II also acts at the →4)-α-D-GlcNp2S6S(1→4)-α-L-GalAp(1→ linkage.

DISCUSSION

The discovery of numerous and important biological activities of heparin has resulted in an intensive research effort toward developing methods of sequencing this acidic polysaccharide. The application of chemical methods for polysaccharide structure determination has been exploited by a number of workers (29, 30) with limited success. Chemical methods are not highly specific and often lead to modification of the polysaccharide chain, resulting in loss of important structural information. Heparin lyases have been used in oligosaccharide mapping of heparin
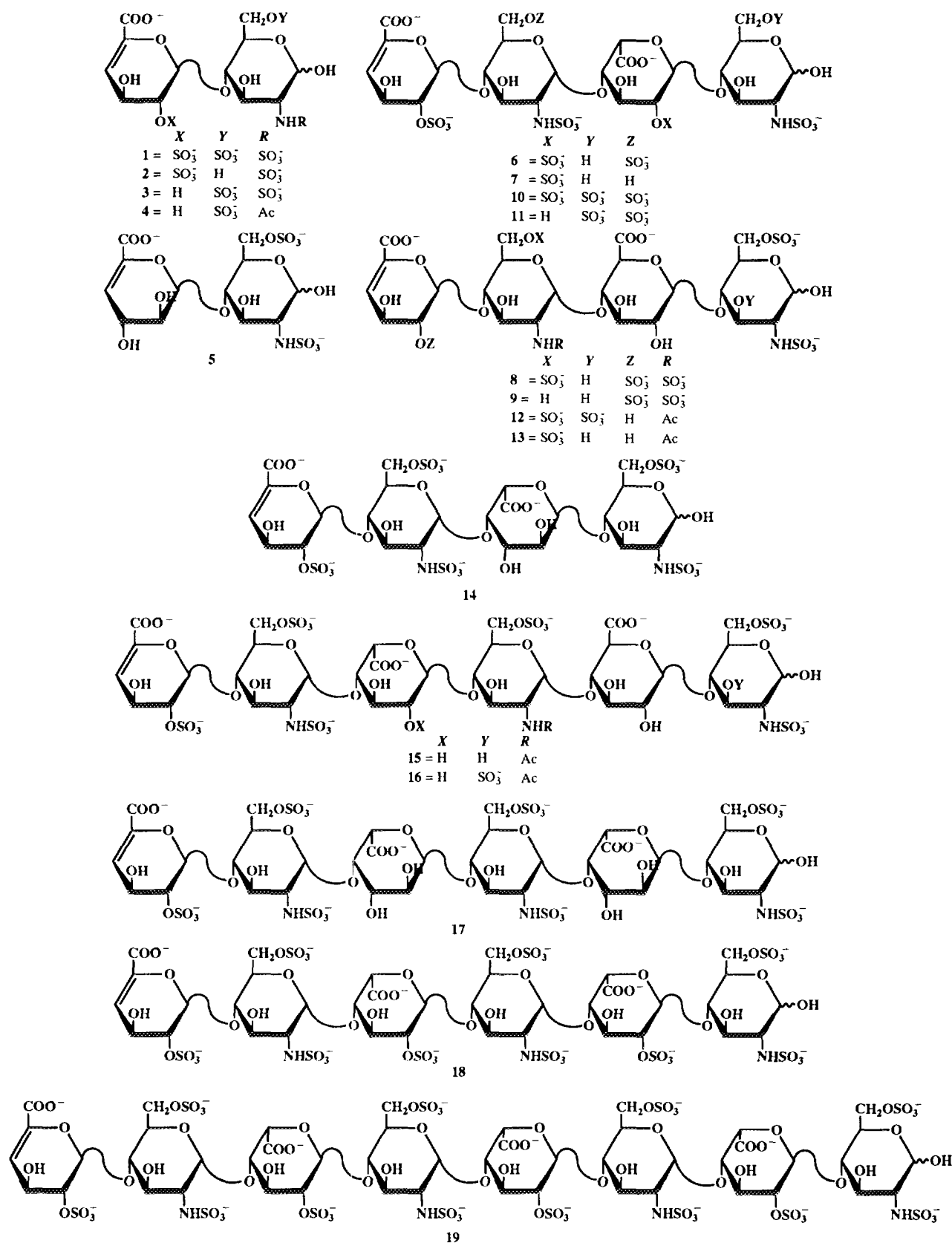
FIG. 1. Structures of oligosaccharides used for specificity studies.

TABLE I

Degradation[a] of Oligosaccharides (6–19) by Heparin Lyase I, II, and III

Substrates	Heparin lyase I		Heparin lyase II		Heparin lyase III	
	Products formed	Percentage cleavage[b]	Products formed	Percentage cleavage[b]	Products formed	Percentage cleavage[b]
6	1 and 2	20	1 and 2	100	No rxn	—
7	2 and 2	30	2 and 2	100	No rxn	—
8	No rxn[c]	—	1 and 3	100	1 and 3	50
9	No rxn	—	2 and 3	95	2 and 3	100
10	1 and 1	100	1 and 1	100	No rxn	—
11	1 and 3	20	1 and 3	100	1 and 3	70
14	No rxn	—	1 and 5	85	No rxn	—
15	1 and 13	80	1, 3, and 4	90	1, 3, and 4	75
16	No rxn	—	1 and 12	80	No rxn	—
17	No rxn	—	14 and 5	100	No rxn	....
18	1, 1, and 1	100	1, 1, and 1	100	No rxn	—
19	1, 1, 1, and 1	100	np[d]	—	np	—

[a] Degradation of oligosaccharides performed as described under Methods.
[b] Calculated from the proportion of starting oligosaccharide remaining in the depolymerization mixture.
[c] No reaction observed within 24 h.
[d] Not performed.

(14, 23, 24). Such enzymatic approaches are attractive because of their presumed high specificity, the conservation of information, and because of the reduced amounts of substrates required.

Polymeric substrates, such as heparin and heparan sulfate, contain multiple heparin lyase-cleavable sites and are more susceptible to enzymatic depolymerization than oligomeric substrates that have one or two sites at which heparin lyase can act (18). For heparin lyase I, the V_{max}/K_m (apparent) ratio was more than 10,000-fold higher for heparin than for a typical oligomeric substrate containing a single lyase cleavable site. For heparin lyase III, the V_{max}/K_m (apparent) ratio was 50-fold higher for heparan sulfate than for an oligosaccharide (18). The observation of reduced sensitivity of oligosaccharide substrates toward heparin lyases requires the use of a higher concentration of lyases for studies on oligomeric substrates than are typically used for polymeric substrates. The high concentrations of heparin lyases used in such studies unmask secondary activities that result in cleavages difficult to observe when studying the primary specificity with polymeric substrates. In this study, the reactivity of small quantities of heparin lyase toward a sensitive polymeric substrate is defined as a primary specificity, while the reactivity of large amounts of heparin lyase toward a resistant oligomeric substrate is referred to as secondary specificity.

The percentage of substrate converted to products, in the heparin lyase-catalyzed degradation of these oligosaccharides, represents a reasonable estimate of the sensitivity of the linkages being examined. Although it is conceivable that the enzyme may become thermally in-

activated before reaction completion, control experiments over the time period suggest that the heparin lyases are stable to the reaction conditions.

A cursory glance at Table I indicates that heparin lyase I acts preferentially on oligosaccharides containing α-L-idopyranosyluronic acid 2-O-sulfate residues and has a primary specificity for →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→ linkages. Heparin lyase I catalyzed cleavage of a linkage containing a nonsulfated α-L-idopyranosyluronic acid residue to a lesser extent. In addition, oligosaccharides that lack one and two sulfate groups at the 6 position of internal and/or reducing end D-2-deoxy-2-aminoglucopyranose residues also show reduced sensitivity toward heparin lyase I. This indicates the importance of both sulfation at the 2 position of L-idopyranosyluronic acid residue of the linkage being cleaved as well as the sulfation at the 6 position of the D-2-deoxy-2-aminoglucopyranose residues adjacent to the linkage being cleaved. These results show that while 2-O-sulfation of α-L-idopyranosyluronic acid residue and 6-O-sulfation of D-2-deoxy-2-aminoglucopyranose residue are not absolute requirements for substrate cleavage by heparin lyase I, these modifications result in more resistant sites and represent secondary specificities for heparin lyase I.

Heparin lyase II has the broadest specificity of the heparin lyases and has primary specificity for linkages to either L-idopyranosyluronic acid (with or without 2-sulfation) or D-glucopyranosyluronic acid residues. Variation in the sulfation pattern on adjacent residues (i.e., tetrasaccharides 7 and 11) and variation in the substitution of amino group (N-sulfation or N-acetylation) on D-2-deoxy-2-aminoglucopyranose (i.e., hexasaccharide 16)
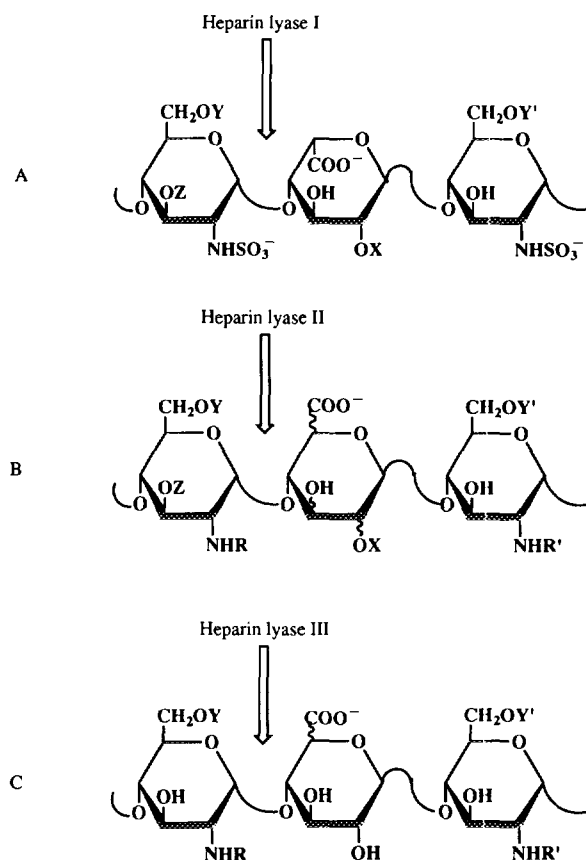
FIG. 2. Primary and secondary substrate specificities of the heparin lyases. (A) Heparin lyase I: Primary specificity for X, Y, and Y' = SO_3^-; Z = H or SO_3^-; secondary specificity when X = H, Y and Y' = SO_3^- or X = SO_3^-, Z = H; or Y = H, Y' = SO_3^-, Z = H; or X and Y = SO_3^-, Y' = H. (B) Heparin lyase II: Primary specificity for X, Y, and Z = SO_3^- or H; R = Ac or SO_3^-; (C) Heparin lyase III: Primary specificity for UAp = GlcAp, Y and Y' = H, R and R' = Ac or SO_3^-; secondary specificity for UAp = IdoAp, R and R' = Ac or SO_3^-, Y and Y' = H or UAp = IdoAp, R and R' = Ac, Y = SO_3^-, Y' = H. Primary specificity sites contained within small oligosaccharide substrates (having ≤6 saccharide residues) showed increased resistance to heparin lyase.

does not seem to affect the reactivity of a linkage toward heparin lyase II. No subspecificity was observed for heparin lyase II under the conditions examined, since heparin lyase II had nearly equal activity toward all linkages studied.

Heparin lyase III acts at D-glucopyranosyluronic acid residues and has a primary specificity for →4)-α-D-GlcNp2S(or GlcNpAc)(1→4)-β-D-GlcAp(1→ linkage. Sulfation of the 6 position of D-2-deoxy-2-aminoglucopyranose residue (i.e., tetrasaccharide 8) decreases the yield by 50%, suggesting that a low degree of sulfation is a requirement for the primary specificity of heparin lyase III. One of the most interesting subspecificities of heparin lyase III is the cleavage of →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→ linkage (tetrasaccharide 11 and hexasaccharide 15). These experiments on oligosaccharides 11

and 15 demonstrate definitively that heparin lyase III can cleave linkages to α-L-idopyranosyluronic acid and confirms recent studies on chemically modified heparins, suggesting an interesting secondary specificity for heparin lyase III (17).

The isolation of disaccharide 1 and tetrasaccharide 12 on the heparin lyase II treatment of hexasaccharide 16 demonstrates that the cleavage occurred at →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→ linkage. In addition, this also indicates that heparin lyase II did not cleave →4)-α-D-GlcNp2Ac6S(1→4)-β-D-GlcAp(1→ linkage. Although heparin lyases I and III usually act on linkages composed of L-idopyranosyluronic and D-glucopyranosyluronic acid residues, respectively, the resistance of hexasaccharide 16 shows that these heparin lyases may fail to act on certain linkages containing these uronic acid residues. The presence of 3-O-sulfate group on the reducing end D-2-deoxy-2-aminoglucopyranose residue of substrate 16 probably determines the susceptibility of these linkages to heparin lyase-catalyzed cleavage, since hexasaccharide 15, lacking the 3-O-sulfate group, was converted to the expected products. The resistance of tetrasaccharide 12, obtained from the heparin lyase II cleavage of hexasaccharide 16, to undergo cleavage with heparin lyases also been reported by Yamada et al. (19). However, the concentration of the heparin lyases used by these workers is far below what we have observed to effect cleavage of 16.

Substrates 14 and 17 were derived from a chemically modified heparin (21) and are composed of D-galactopyranosyluronic acid residues. Cleavage of the →4)-α-D-GlcNp2S6S(1→4)-α-L-GalAp(1→ linkage (not found in heparin or in heparan sulfate) by heparin lyase II indicates a much broader specificity than previously reported.

Substrate specificity of enzymes is a function of recognition of substrate by the enzyme (binding) and the induction of an appropriate substrate conformation in the active site of enzyme resulting in conversion to products (catalysis). Although the conformation of the substrate in the transition state is certainly different from its ground state conformation, heparin oligosaccharides, by the virtue of their high charge, may have similar global conformations in the ground state and in the transition state. As a first approximation, the substrate specificity of the heparin lyases (Fig. 2) might, therefore, be analyzed on the basis of the most favorable solution conformation of the uronic acid residue at the linkage undergoing cleavage.

The conformational preferences of the uronic acids of heparin have been the subject of intensive research by numerous groups (31–33). The L-idopyranosyluronic acid residue exhibits a number of conformational preferences (Fig. 3) depending upon the type of substitution within the ring as well as the substitution on the adjacent rings. Sulfation of the 2-position within the L-idopyranosyluronic acid ring stabilizes the $^1C_4$ conformation. Sulfation at the 2 and 6 positions of adjacent 2-deoxy-2-aminoglu-
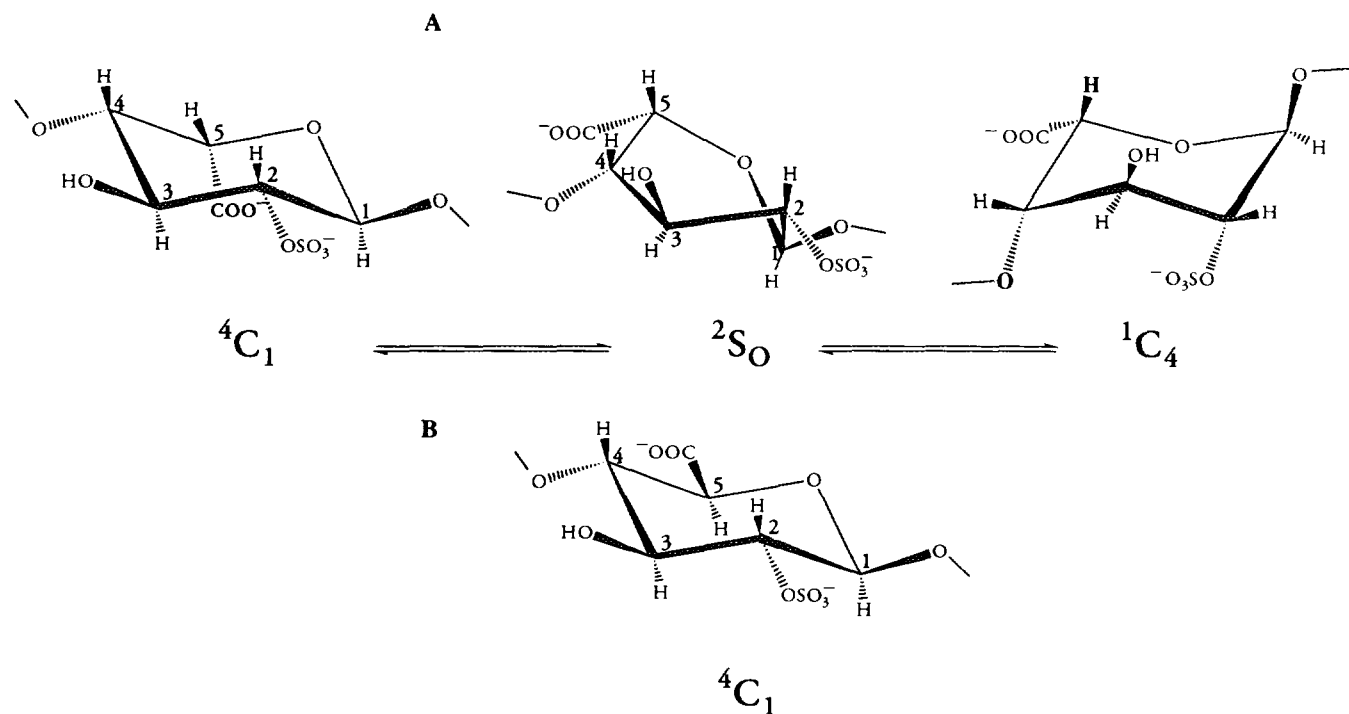
A

B

FIG. 3. Conformational preferences of α-L-idopyranosyluronic acid (A) and β-D-glucopyranosyluronic acid residues (B).

copyranose rings enhance the stability of the $^1C_4$ form of L-idopyranosyluronic acid. An unsulfated L-idopyranosyluronic acid residue gives an equilibrium of the skew (^2S_O) and the $^1C_4$ form. A reduction of sulfation on adjacent rings results in shift of conformational bias toward the skew (^2S_O) form.

The $^1C_4$ form of L-idopyranosyluronic acid residue is characterized by the *antiperiplanar* disposition of the 5α-H and the glycosidic bond at the 4 position appropriate for E2 concerted elimination. Neither ^2S_O nor $^4C_1$ conformations exhibit this antiperiplanar orientation. The D-glucopyranosyluronic acid residue tends to favor exclusively the $^4C_1$ chair form that is characterized by the equatorial disposition of substituents at the 2, 3, and 5 positions. The $^1C_4$, ^2S_O, $^4C_1$ forms, or the higher energy boat forms of D-glucopyranosyluronic acid residue, do not orient the 5α-H and the glycosidic bond at the 4 position in an *antiperiplanar* configuration necessary E2 elimination.

The preference for 2-O-sulfated L-idopyranosyluronic acid residues together with higher sulfation on residues adjacent to cleavage site by heparin lyase I suggests that this enzyme requires antiperiplanar disposition of the 5α-H and the interglycosidic bond at 4 position. Thus, oligosaccharides that stabilize the $^1C_4$ form of L-idopyranosyluronic acid residue are best cleaved by heparin lyase I (i.e., substrates 10, 18, and 19). Reduced sulfation on or near the linkage undergoing cleavage brings about a greater conformational mobility, resulting in reduced

reactivity to the heparin lyase I (e.g., substrates 6, 7, 11, and 15).

The preference of heparin lyase III for oligosaccharides containing D-glucopyranosyluronic acid residue (substrates 9, 8, and 15), which do not exhibit a conformational preference for the $^1C_4$ form, suggests that heparin lyase III does not require antiperiplanar disposition of the 5α-H and the interglycosidic bond at 4 position. Also, oligosaccharides containing L-idopyranosyluronic acid residues which are conformationally biased away from the $^1C_4$ form are susceptible to heparin lyase III. These data support a different mechanism for heparin lyase III from heparin lyase I.

The overall global conformation of hexasaccharide 16 is not yet known. Modeling studies suggest that 3-O-sulfation at the reducing terminal D-2-deoxy-2-aminoglucopyranose residue may decrease the accessibility of 5α-H of β-D-glucopyranosyluronic acid residue. This might explain the resistance of one of the susceptible linkages in hexasaccharide 16 all to three heparin lyases. The inability of heparin lyase I and III to cleave the →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→ linkage in 16 (the same linkage is cleaved in 15) appears to be attributable to the effect of 3-O-sulfation on the global conformation of the hexasaccharide 16.

In conclusion, the substrate specificity of the heparin lyases can be broadly distinguished as primary and secondary specificities. Although the structural requirements for each lyase to act appear to be stringent at low con-

centrations of the heparin lyases (primary specificity), secondary specificity under forcing conditions results in a surprising reduction in enzymatic selectivity and might pose problems in the use of these lyases for the sequencing heparin and heparan sulfate.

ACKNOWLEDGMENT

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