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Examination of the Substrate Specificity of Heparin and Heparan Sulfate Lyases†

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Received September 8, 1989; Revised Manuscript Received November 15, 1989

ABSTRACT: We have examined the activities of different preparations of heparin and heparan sulfate lyases from *Flavobacterium heparinum*. The enzymes were incubated with oligosaccharides of known size and sequence and with complex polysaccharide substrates, and the resulting degradation products were analyzed by strong-anion-exchange high-performance liquid chromatography and by oligosaccharide mapping using gradient polyacrylamide gel electrophoresis. Heparinase (EC 4.2.2.7) purified in our laboratory and a so-called Heparinase I (Hep I) from a commercial source yielded similar oligosaccharide maps with heparin substrates and displayed specificity for di- or trisulfated disaccharides of the structure →4)-α-D-GlcNp2S(6R)(1→-4)-α-L-IdoAp2S(1→ (where R = *O*-sulfo or OH). Oligosaccharide mapping with two different commercial preparations of heparan sulfate lyase [heparitinase (EC 4.2.2.8)] indicated close similarities in their depolymerization of heparan sulfate. Furthermore, these enzymes only degraded defined oligosaccharides at hexosaminidic linkages with glucuronic acid: →4)-α-D-GlcNpR(1→4)-β-D-GlcAp(1→ (where R = *N*-acetamido or *N*-sulfo). The enzymes showed activity against solitary glucuronate-containing disaccharides in otherwise highly sulfated domains including the saccharide sequence that contains the antithrombin binding region in heparin. A different commercial enzyme, Heparinase II (Hep II), displayed a broad spectrum of activity against polysaccharide and oligosaccharide substrates, but mapping data indicated that it was a separate enzyme rather than a mixture of heparinase and heparitinase/Hep III. When used in conjunction with the described separation procedures, these enzymes are powerful reagents for the structural/sequence analysis of heparin and heparan sulfate.

Heparin and heparan sulfate are complex, sulfated copolymers of alternating 1→4-linked glucosamine and hexuronic acid. Both biopolymers have been implicated in a diverse range of biological functions and therapeutic uses [for reviews, see Gallagher et al. (1986) and Linhardt and Loganathan (1989)].

Polysaccharide lyases (EC 4.2.2) are a class of enzymes that depolymerize certain acidic polysaccharides through an eliminative mechanism. This enzymatic reaction results in an unsaturated uronic acid residue at the nonreducing terminal sugar in the resulting oligosaccharide product (Linhardt et al., 1986a). *Flavobacterium heparinum* is a soil isolate (Payza & Korn, 1956) capable of utilizing either heparin or heparan sulfate as its sole carbon and nitrogen source (Galliher et al., 1981). The growth of this organism on these polymeric substrates is dependent on its production of a variety of enzymes including lyases, glucuronidases, sulfoesterases, and sulfamidases (Galliher et al., 1982). These polysaccharide lyases

† Supported by the Burroughs Wellcome Fund in the form of a Research Travel Grant, Cancer Research Campaign (J.T.G.), and by National Institutes of Health Grants HL29797 and GM38060 (R.J.L.). J.E.T. acknowledges receipt from Christie Hospital of a Research Travel Grant.

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0006-2960/90/0429-2611$02.50/0

include heparinase (EC 4.2.2.7), which has been purified to homogeneity (Yang et al., 1985), and heparitinase (EC 4.2.2.8). Heparinase acts at the →4)-α-D-GlcNp2S6S(1→-4)-α-L-IdoAp2S(1→ linkage, heparin's major disaccharide repeating unit. Heparinase does not require 6-sulfation to act and can tolerate 3-sulfation in the glucosamine residue (Rice & Linhardt, 1989). The heparitinase enzymes, initially described as heparitinase 1 and 2 (Linker & Hovingh, 1972), have not yet been purified to homogeneity. The commercially available heparitinase (EC 4.2.2.8) is described by the manufacturer (Seikagaku Kogyo Co.) as capable of cleaving → 4)-α-D-GlcNp(2-acetamido or 2-sulfate)(1→4)-β-D-GlcAp-(1→ and can tolerate 6-sulfation in the glucosamine residue. There is, however, only limited data available on the activity of heparan sulfate lyase against low molecular weight substrates (Rice & Linhardt, 1989), and there is some controversy regarding the precise number of different polysaccharide lyases produced by *F. heparinum* that are capable of acting on either heparin or heparan sulfate.

Recently, several polysaccharide lyases have been introduced onto the market which are capable of acting on heparin and/or heparan sulfate. These enzymes have demonstrated utility in the mapping and sequencing of heparin and heparan sulfate (Linhardt et al., 1988a; Turnbull & Gallagher, 1988) and in the preparation of potentially useful therapeutic agents (Linhardt et al., 1986b; Linhardt & Loganathan, 1989).

In the present paper we examine all of the commercially available polysaccharide lyases capable of acting on either heparin or heparan sulfate. These enzymes are applied to polymeric substrates in an effort to understand their range of specificities. The products of these reactions are analyzed by both gradient PAGE (Turnbull & Gallagher, 1988; Rice et al., 1987) and SAX-HPLC (Linhardt et al., 1988a) oligosaccharide mapping techniques.

Experimental Procedures

Materials

Heparin, sodium salt, was obtained from Hepar (Porcine Mucosal, lot ST 82261, 145 units/mg). Heparan sulfate, sodium salt, from porcine intestine (ORG 553) was a gift from Organon, Oss, The Netherlands. Heparinase (heparin lyase, EC 4.2.2.7) was purified from *Flavobacterium heparinum* [5 units/mg (μmol min^{-1} mg^{-1})] (Yang et al., 1985) or prepared by Seikagaku Kogyo Co. Ltd., Tokyo, Japan, and obtained from ICN Immunobiologicals, Lisle, IL. Heparinase (Heparinase I) was also obtained from Sigma (1.5 units/mg) or as Heparinase I directly from Grampian Enzymes, Aberdeenshire, Scotland. Heparitinase from *F. heparinum* (heparan sulfate lyase, EC 4.2.2.8) was prepared by Seikagaku Kogyo Co. An alternative source of heparitinase (designated as Heparinase III or Hep III) was obtained from Sigma or directly from Grampian Enzymes. Heparinase II (no assigned EC number) was obtained from Sigma or directly from Grampian Enzymes. Chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4) from *P. vulgaris* was obtained from ICN Immunobiologicals. Dialysis tubing Spectropore 3500 was 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.

Gradient control and data collection used an Apple IIe microcomputer running Chromatochart software from Interactive Microware, State College, PA. Strong-anion-exchange (SAX) HPLC was performed on a Spherisorb (5-μm particle size) column of dimensions 0.46 × 25 cm from Phase Separations, Norwalk, CT. UV spectroscopy used a Shimadzu Model UV-160 spectrophotometer equipped with a thermostated cell. A 32-cm vertical slab gel unit (SE620) was supplied by Hoefer Scientific Instruments (San Francisco, CA). Tris and SP-Sephadex C-50 were supplied by Sigma Chemical Co. Acrylamide and *N,N'*-methylenebis(acrylamide) (Electran grade) were obtained from BDH Chemicals. Bio-Gel P-2 (fine) and Coomassie dye reagent were from Bio-Rad, Richmond, CA. All other chemicals were reagent grade.

Methods

Preparation of Polymer Substrates. Heparin was prepared by dissolution of approximately 20 mg into 1 mL of water and dialysis (in M_r 3500 cutoff bags at 4 °C) first against 10 volumes of 1 M sodium chloride followed by 3 × 1000 volumes of deionized water. After dialysis the heparin was removed from the dialysis bags and syringe filtered into a preweighed vial, freeze-dried, and stored desiccated over anhydrous calcium chloride for 2 days. The heparin sample was then carefully weighed, and the appropriate volume of distilled water was added to obtain a 20 mg/mL stock of solution. Heparan sulfate (11 mL at 50 mg/mL) was prepared in 0.25 M sodium acetate and 2.5 mM calcium acetate solution, adjusted to pH 7.0, and 10 IU of chondroitinase ABC was added. The reaction mixture was incubated for 24 h at 37 °C after which the pH was adjusted to 3.0 with concentrated hydrochloric acid. The entire reaction mixture was added to a 15 × 0.5 cm SP-Sephadex column (preequilibrated with hydrochloric acid at pH 3.0). The column was washed with 15 mL of pH 3.0 hydrochloric acid, and the combined eluent was adjusted to pH 7.0 with 1 M sodium hydroxide and dialyzed overnight at 4 °C against 1000 volumes of distilled water. The nondiffusible material was freeze-dried, stored desiccated for 2 days, and prepared in distilled water at 20 mg/mL.

Preparation of Oligomeric Substrates. Heparin (100 mg at 16 mg/mL) was depolymerized at 30 °C with 62 mIU/mL heparinase (prepared and purified in our laboratories) in a solution of 0.2 M sodium chloride and 5 mM sodium phosphate at pH 7.0. The reaction was monitored by removing aliquots and measuring A_{232nm} after a 1:100 dilution in 30 mM hydrochloric acid. At 98% reaction completion, the reaction was terminated by heating at 100 °C for 1 min. The sample was desalted on a 35 × 2.5 cm Bio-Gel P-2 column and fractionated on a semipreparative SAX-HPLC column as previously described (Rice & Linhardt, 1989). Five major oligosaccharide products were collected. A second fractionation of these oligosaccharides on the same column followed by desalting and freeze-drying resulted in their recovery at >95% purity (Rice & Linhardt, 1989). The structures of these five oligosaccharides (F1–F5) representing 48, 8.8, 13, 12, and 5.5 wt % of the heparin starting material were determined as described previously (Linhardt et al., 1988a) and are shown in Figure 1.

Heparan sulfate (60 mg at 4 mg/mL) was similarly depolymerized with 90 mIU of heparitinase (Seikagaku Kogyo), and two disaccharides were prepared having the structures (**6**) ΔUA(1→4)-α-D-GlcNpAc and (**7**) ΔUA(1→4)-α-D-GlcNp2S, where ΔUA is 4-deoxy-α-L-*threo*-hex-4-enopyranuronic acid (Linhardt et al., 1989).

Determination of Enzymatic Activities. Heparin or heparan sulfate (40 μL of 20 mg/mL) was added to 350 μL of 5 mM sodium phosphate and 200 mM sodium chloride solution, adjusted to pH 7.0, at 30 °C in a microcuvette. Enzyme, approximately 15 mIU in 10 μL, was added to substrate and

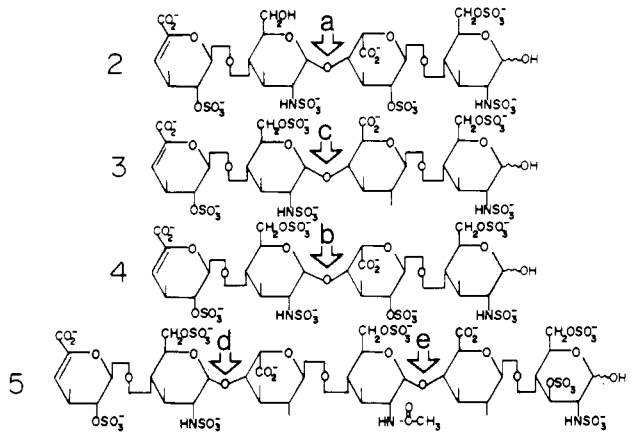

FIGURE 1: Structure of oligosaccharide substrates **2–5**. Linkages labeled a–e with arrows designate those cleaved by the enzymes as described under Results and in Table II.

the change in absorbance was measured continuously at 232 nm in a thermostated UV spectrophotometer. The initial rate [dA_{232} (1-cm path length/dt) (min)] was measured over the first 9 min of the reaction, and the final absorbance at 232 nm was measured directly after 100 min.

Treatment of Substrates with Enzymes. Polymeric substrate, heparin or heparan sulfate, was added to 425 μL of 0.2 M sodium chloride and 5 mM sodium phosphate (50 μL containing 20 mg/mL substrate), pH 7.0. Enzyme (25 μL, 15 mIU) was added to make the total volume of the solution 500 μL. The reactions were run at the appropriate temperatures (heparinase/Hep I, 30 °C; heparitinase/Hep III, 43 °C; Hep II, 30 °C) to completion in 8 h. The depolymerization reactions were terminated by heating at 100 °C for 1 min (Rice et al., 1987). Aliquots (4, 40, and 180 μL) were removed and added to 1 mL of 0.03 M hydrochoric acid to obtain a total volume of 1 mL, and the absorbance at 232 nm was determined.

Oligomeric substrate in 0.2 M sodium chloride and 5 mM sodium phosphate (50 μL containing 12 μg of substrate), pH 7.0, was treated with enzyme (25 mIU), and the reaction was run at the appropriate temperature for 24 h. The reactions were terminated by addition of concentrated hydrochloric acid, resulting in a solution at pH 3.0. The reaction mixture was poured onto an SP-Sephadex microcolumn (100-μL volume) equilibrated with hydrochloric acid at pH 3.0 to remove protein. After the column had been washed with 2 mL of pH 3.0 hydrochloric acid, the combined eluent and washes were adjusted to pH 7.0 with 1 M sodium hydroxide and freeze-dried.

Analytical SAX-HPLC Analysis of Enzyme-Treated Substrates. Enzyme-treated heparin, heparan sulfate, or heparin–oligosaccharide was injected in amounts between 4 and 40 μg onto an analytical Spherisorb, 5-μm particle size (0.46 × 25 cm) SAX-HPLC column equilibrated with 0.2 M sodium chloride, pH 3.5. 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 at pH 3.5 and a flow rate of 1.5 mL/min. The elution profile was monitored by the absorbance at 232 nm at 0.02 absorbance unit full scale (AUFS). The amount of the resulting oligosaccharide products was assessed by computer integration of peak area using a standard curve. Peaks were tentatively identified by either coelution with an authentic sample or by retention time and confirmed by comparison of each with purified oligosaccharide standards analyzed on a gradient polyacrylamide gel (Rice et al., 1987).

Gradient PAGE of Glycosaminoglycan Oligosaccharides. Polyacrylamide linear gradient resolving gels (T 20–30% acrylamide) were prepared as described by Turnbull and Gallagher (1988). A linear gradient was formed between the glass plates (32 cm × 16 cm × 0.75 mm) from the bottom (maximum gel concentration, T 30%/C 5%) upward to the top (minimum gel concentration, T 20%/C 0.5%).

Immediately before electrophoresis the gel surface was rinsed three times with stacking-gel buffer and stacking-gel solution (T 5%/C 0.5% acrylamide in stacking-gel buffer) was applied to the top of the resolving gel. A 15 × 5 mm well former comb was inserted between the glass plates. After polymerization (15 min) the comb was removed, and the wells were rinsed three times with electrophoresis buffer, after which the gel unit was placed into the electrophoresis tank.

Oligosaccharide samples (5–30 μL) containing approximately 10% (v/v) glycerol and a trace quantity of Phenol Red were carefully layered onto the bottom of the wells with a microsyringe. Marker samples (10 μL) containing trace quantities of Bromophenol Blue and Phenol Red in 10% (v/v) glycerol were also run on each gel. Electrophoresis was then performed as follows. Samples were initially run into the gel at 150 V for 30 min, followed by electrophoresis at 300 V for 16 h, and finally 1000 V for 1 h (total approximately 6000 V·h). Under these conditions the Phenol Red marker dye migrates to within approximately 3 cm of the bottom of the resolving gel. Throughout the run heat was dissipated by use of a heat exchanger with circulating tap water (10–15 °C). Oligosaccharides were visualized by staining with Alcian blue and Azure A as described previously (Rice et al., 1987).

RESULTS

The particular porcine mucosal heparin used as a polymeric substrate was chosen because it contained no detectable dermatan/chondroitin sulfates (Linhardt et al., 1988a). The mucosal heparan sulfate preparation was selected because of its availability in sufficient quantities to permit the characterization of its two major disaccharide products and because of its similarity (by oligosaccharide mapping) to commercially available bovine kidney heparan sulfate. Contaminating dermatan/chondroitin sulfates were removed from this preparation by exhaustive treatment with chondroitinase ABC followed by dialysis.

Selective Depolymerization of N-Sulfated Polysaccharides. The activities of the commercially available polysaccharide lyases obtained from *Flavobacterium heparinum* were measured with heparin and heparan sulfate under saturating conditions (Table I). This measurement facilitated the use of equivalent enzymatic treatment (mIU·h) per mole of substrate in subsequent experiments. Both heparin and heparan sulfate (1 mg/500 μL) were treated with 15 mIU for 8 h, and the reaction was followed kinetically to demonstrate that it was complete. The results of this study are presented in Table I. These results clearly identify heparin as the primary substrate for heparinase and Hep I, while heparan sulfate is the primary substrate for heparitinase and Hep III. Hep II acts to a significant degree on both heparin and heparan sulfate. The polymeric substrates, containing multiple cleavable sites, are substantially better substrates than are the oligomeric substrates having only a single site at which the enzyme can act (Rice & Linhardt, 1989). Kinetic studies involving heparinase (our preparation) demonstrate a V_{max}/apparent K_m that was 10000-fold higher for the polymeric substrate heparin (containing on the average 10 cleavable sites) than for oligomeric substrates containing a single cleavable site (Rice & Linhardt, 1989). A similar but less pronounced difference of

Table I: Susceptibility of Heparin and Heparan Sulfate to Flavobacterial Polysaccharide Lyases

enzyme[a]	substrate[b]	act. (IU/vial)[c] measured	act. (IU/vial)[c] specified	μmol of product/100 μg of substrate[d]	av no. of sites cleaved[e]
heparinase	H	0.100	0.100	0.050	7
	HS	nd	nd	0.025	5
Hep I	H	0.118	0.200	nd	nd
	HS	nd	nd	nd	nd
Hep II	H	1.686	1.666	0.101	14
	HS	nd	nd	0.117	25
heparitinase	H	nd	nd	0.007	1
	HS	0.324	0.200	0.094	20
Hep III	H	nd	nd	0.007	1
	HS	1.136	0.800	0.108	23

[a] Substrate is at 1 mg/500 μL, and enzyme is at 15 mIU/500 μL. [b] H is heparin; HS is heparan sulfate. [c] Heparinase prepared in our laboratory by the method of Yang et al. (1986) has been demonstrated to be identical with Seikagaku Kogyo heparinase purchased from ICN Immunobiologicals (Linhardt et al., 1988a; Rice & Linhardt, 1989). Hep I and Hep II prepared by Grampian Enzymes were obtained from Sigma. Heparitinase prepared by Seikagaku Kogyo was obtained from ICN Immunobiologicals. Heparinase, Hep I, and Hep II activities were measured with heparin as the polymeric substrate at 30, 30, and 25 °C, respectively. Heparitinase and Hep III were assayed with heparan sulfate as the polymeric substrate at 43 °C. Specified activity is the manufacturer's activity converted to international units (IU = 1 mmol/min). None of the enzymes examined had measurable amounts of glycuronidase or sulfatase activity (Yang et al., 1986). nd is not determined. [d] Calculated from A_{232} of sample diluted in 30 mM hydrochloric acid with a molar absorptivity of 5200 (Linhardt et al., 1988a). [e] Calculated from micromoles of product per micromoles of substrate. The estimated M_r for H is 14000 and for HS is 21600.

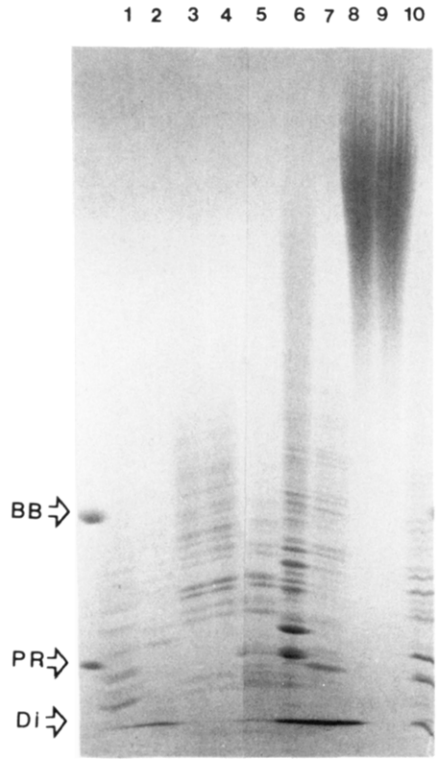

FIGURE 2: Gradient PAGE oligosaccharide maps of polysaccharide lyase depolymerized heparin and heparan sulfate. Polymeric substrates were depolymerized and the resulting oligosaccharide mixtures (100 μg each) separated by gradient PAGE and visualized by staining with Azure A, as described under Methods. Heparan sulfate was depolymerized with (1) heparinase (Sigma), (2) Hep II, (3) Hep III, (4) heparitinase, and (5) simultaneous digestion with heparinase (Sigma) and Hep III. Heparin was depolymerized with (6) heparinase (Sigma), (7) Hep II, (8) Hep III, (9) heparitinase, and (10) heparinase (Sigma) followed by Hep III. The migration positions of Bromophenol Blue (BB), a contaminating product (PR) in BB, and disaccharides (Di) were as indicated. The sample in lane 2 showed no banding between PR and Di, and the samples in lanes 8 and 9 ran above BB, showing no discrete banding.

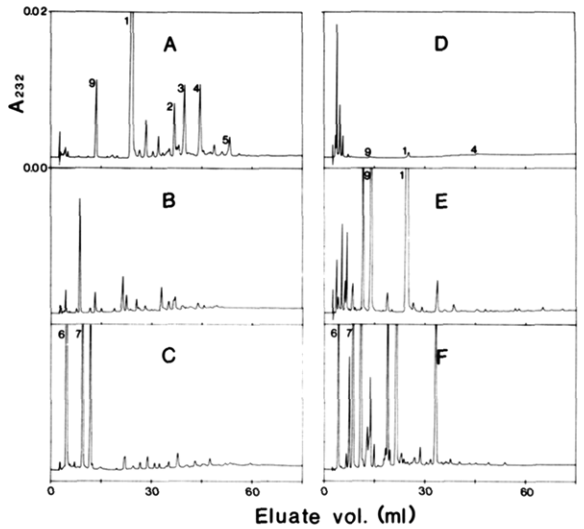

FIGURE 3: SAX-HPLC oligosaccharide maps of polysaccharide lyase depolymerized polymeric substrates. Chromatograms plotted as elution volume (mL) versus A_{232} (0.02 AUFS) are of oligosaccharide products formed by (A) heparinase acting on heparin, (B) heparinase acting on heparan sulfate, (C) heparitinase acting on heparan sulfate, (D) heparitinase acting on heparin, (E) Hep II acting on heparin, and (F) Hep II acting on heparan sulfate.

50-fold was observed for heparitinase (Seikagaku Kogyo) examined on heparan sulfate and oligomeric substrates.

Oligosaccharide Mapping. The products of these reactions were then examined by oligosaccharide mapping using SAX-HPLC and gradient PAGE oligosaccharide mapping as described under Methods (Figures 2 and 3). In all cases, multiple bands or peaks representing polysaccharide depolymerization products were observed. Heparitinase and Hep III yielded identical PAGE maps with either heparan sulfate (Figure 2, tracks 3 and 4) or heparin (tracks 8 and 9) as substrates, indicating that these are the same enzymes. Clearly, heparinase has quite distinct activity from that of the heparitinases on both substrates (tracks 1 and 6). The oligosaccharide maps obtained for Hep II were intriguing. This enzyme brought about extensive degradation of heparin (track 7) and heparan sulfate (track 2) to predominantly disaccharides. The maps with heparan sulfate as substrate were not reproduced by the combined actions of heparinase and heparitinase whether used simultaneously (track 5) or sequentially in either order (results not shown). Similar results were obtained with heparin as substrate (track 10), confirming the distinctive action of this enzyme. Hep II must have a broad specificity (see Table II), but the resistant linkages in the polymeric substrates have not been defined. Recent work suggests that the Hep II resistant oligosaccharides in heparan

Table II: Susceptibility of Defined Oligosaccharide Substrates to Polysaccharide Lyases

enzyme tested	substrate used	linkage cleaved	products recovered[f,g]
heparinase	**2**	*a*	**1, 9**
	3		none
	4	*b*	**1**
	5		none
Hep I	**2**	*a*	**1, 9**
	3		none
	4	*b*	**1**
	5		none
Hep II	**2**	*a*	**1, 9**
	3	*c*	**1, 10**
	4	*b*	**1**
	5	*d, e*	**1, 8, 11**
heparitinase	**2**		none
	3	*c*	**1, 10**
	4		none
	5	*e*	**12, 11**
Hep III	**2**		none
	3	*c*	**1, 10**
	4		none
	5	*e*	**12, 11**

[a] →4)-α-D-GlcNp2S(1→4)-α-L-IdoAp2S(1→. [b] →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→. [c] →4)-α-D-GlcNp2S6S(1→4)-β-D-GlcAp(1→. [d] →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→. [e] →4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→. [f] **1** is ΔUAp2S(1→4)-D-GlcNp2S6S; **8** is ΔUAp(1→4)-D-GlcNpAc6S; **9** is ΔUAp2S(1→4)-D-GlcNp2S; **10** is ΔUAp(1→4)-D-GlcNp2S6S; **11** is ΔUAp(1→4)-D-GlcNp2S3S6S; **12** is ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→4)-D-GlcNpAc6S. [g] Where one product is observed, it is obtained as 2 equiv, and where two or three products are observed, each is obtained in 1 equiv with respect to the substrate.

sulfate from human skin fibroblasts are largely composed of sequences of the type →4)-α-D-GlcNpAc(1→4)-β-D-GlcAp-(1→ (Turnbull and Gallagher, unpublished data).

Quantitative data on the oligosaccharide breakdown products were acquired with SAX-HPLC mapping procedures (see Methods). The action of heparinase on heparin gave the identified major oligosaccharides, **1**–**5** and **9**, and unidentified minor oligosaccharide components (Figure 3A), and Hep I gave an identical result (result not shown). Heparitinase acting on heparan sulfate gave two disaccharides, **6** and **7**, together with additional unidentified oligosaccharide components (Figure 3C), and Hep III produced an identical result (result not shown). Hep II acts extensively on heparin, giving a mixture of primarily disaccharide products (Figure 3E). Hep II acts on heparan sulfate giving disaccharides **6** and **7**, along with a large number of unidentified products (Figure 3F). The patterns obtained for Hep II are clearly unlike those obtained with heparinase/Hep I or heparitinase/Hep III. No combination (simultaneous or sequential in either order) of heparinase (or Hep I) and heparitinase (or Hep III) on either polymeric substrate can be used to obtain the same product distribution resulting from the action of Hep II (results not shown).

Degradation of Defined Oligosaccharide Substrates. Heparin oligosaccharides of known composition and sequence (Figure 1) prepared as described under Methods were used as substrates for the heparin and heparan sulfate lyases. Three of these oligosaccharides are tetrasaccharides that contain just one potentially susceptible hexosaminidic linkage. In all of the tetrasaccharides the hexosamine is N-sulfated; the adjacent hexuronate is α-L-IdoAp2S in **2** and **4** and β-D-GlcAp in **3**. The other oligosaccharide, **5**, is a hexasaccharide with two internal hexosamines, one being α-D-GlcNp2S6S linked to iduronate and the other being α-D-GlcNpAc6S linked to glucuronate.

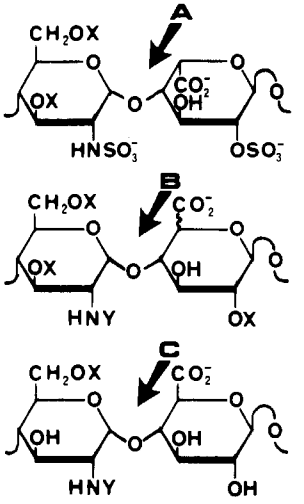

FIGURE 4: Glycosidic linkages that can be cleaved by (A) heparinase and Hep I, by (B) Hep II, and by (C) heparitinase and Hep III, where X = SO_3^- or H and Y = SO_3^- or $COCH_3$. From the defined oligomeric substrates available, it is unclear which enzymes, if any, are capable of acting at a linkage containing a 2-sulfated glucuronic acid or a free glucosamine residue.

Heparinase and Hep I only cleave tetrasaccharides **2** and **4**, which have internal α-L-IdoAp2S. These structures were resistant to heparitinase and Hep III (Table II). The latter enzymes degraded **3** and **5**, but these were poor substrates requiring high enzyme concentrations and long reaction times. Degradation of **5** produced tetrasaccharides and disaccharides of the structure expected if the hexosaminidic linkage was cleaved adjacent to glucuronic acid, i.e., →4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→ (Table II). Clearly, these enzymes can tolerate 6-O-sulfation of glucosamine (N-sulfated or N-acetylated) as shown by cleavage of **3** and **5**, respectively.

The linkage specificities of the enzymes examined, based on our data (both current and previous), are summarized in Figure 4. Details of permissible variations, as far as they have been established, are also given.

Discussion

The biological importance of heparin and heparan sulfates has led a number of research groups to undertake efforts to map and ultimately to sequence these polymers. Several approaches have been applied to these ends. Much has been learned about the structure of heparin and heparan sulfate from studying their biosynthesis in cell culture (Lindahl et al., 1986; Lindahl & Kjellén, 1987). Alternatively, nitrous acid can be used to chemically depolymerize either heparin or heparan sulfate into oligosaccharides, and following fractionation, their structure can be determined (Bienkowski & Conrad, 1985). We have primarily exploited polysaccharide lyases to enzymatically depolymerize heparin and heparan sulfate. By use of this approach, oligosaccharides have been prepared, and their structure has been defined (Merchant et al., 1985; Linhardt et al., 1986b, 1989; Loganathan et al., 1990). In addition to preparing oligosaccharide standards, polysaccharide lyases and nitrous acid have been used to determine patterns of depolymerization in both heparin (Linhardt et al., 1988a; Loganathan et al., 1990) and heparan sulfate (Gallagher & Walker, 1985; Turnbull & Gallagher, 1988) with oligosaccharide mapping techniques.

Heparinase (EC 4.2.2.7) and heparitinase (EC 4.2.2.8) first became commerically available in the early 1980s. In the past 2 years, Hep I, II, and III made their commercial debut and a series of heparitinase [I and II (from *F. heparinum*) and IV and V (from *Flavobacterium* sp. Hp206)] may soon be

available (Seikagaku Kogyo Co.). Of these enzymes, only heparinase has been purified to homogeneity (Yang et al., 1985).

Oligosaccharide mapping using gradient PAGE (Gallagher & Turnbull, 1988; Rice et al., 1987) and SAX-HPLC (Linhardt et al., 1988a) were developed for the analysis of the molecular fine structure of heparan sulfate and heparin. The present study demonstrates the value of these methods for comparing the specificities of polysaccharide lyases. Heparinase and Hep I are revealed by SAX-HPLC as identical enzymes because they produce identical oligosaccharide maps with heparin substrates (Figure 3) and display the same selective activities against oligosaccharide substrates (Table III). As expected from previous studies the enzymes show specificity for glycosidic linkages between α-D-GlcNp2S and α-L-IdoA2S and will tolerate C-6- and/or C-3-sulfation of the hexosamine unit: →4)-α-D-GlcNp2S(3S,6S)(1→4)-α-L-IdoAp2S(1→.

The enzymes therefore extensively degrade heparin substrates but bring about only limited fragmentation of heparan sulfate (Figures 2 and 3). We would point out that the commercial preparation of heparan sulfate is likely to be contaminated with heparin byproducts that have weak anticoagulant activities (Gallagher & Walker, 1985). Nevertheless, the preparation is useful to us because sufficient heparan sulfate-like polymers were present to enable us to generate oligosaccharide maps with heparan sulfate lyases. The degrees of depolymerization by heparinase and heparitinase (Table I) of this heparan sulfate preparation were estimated to be 20 and 80%, respectively.

The present results clearly demonstrate that heparitinase and Hep III from commercial sources are separate enzymes from heparinase. They produce different oligosaccharide maps (Figure 2 and 3) and have different activities against polymeric (Table I) and oligomeric substrates (Table II). It must be emphasized, however, that heparin contains a very small number of linkages that are susceptible to heparitinase, and likewise, there are linkages in heparan sulfate that are cleaved by heparinase. It has been known for many years that heparitinase will degrade the glycosidic link between α-D-GlcNpAc/α-D-GlcNp2S and β-D-GlcAp [reviewed by Linhardt et al., (1986a)] and that glucuronate is the most common hexuronate residue in heparan sulfate (Gallagher & Walker, 1985). Heparitinase is an efficient means of removing heparan sulfate chains in proteoglycans because an 8–9 disaccharide sequence of →4)-α-D-GlcNpAc(1→4)-β-D-GlcAp is contiguous with the protein linkage region (Lyon et al., 1987). It is difficult to make comparisons between different studies on the substrate specificities of heparitinase because some workers have reported that two forms of the enzyme exist, heparitinase I, which is inhibited if the amino sugar is sulfated at C-6, and heparitinase II, which tolerates C-6 sulfation of α-D-GlcNp2S (Dietrich et al., 1980).

Homogeneous preparations of heparitinase are not available, and the commercial heparitinases we have used might contain two separate enzyme activities (Otatoni & Yosizawa, 1981). However, the similarity in the oligosaccharide maps produced by heparitinase and Hep III suggests a single enzyme. Our results clearly demonstrate that C-6-sulfated amino sugars are substrates for these two commercial enzymes, and they confirm the requirement for hexosamine to be linked to glucuronic acid. The →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→ linkage in **5** was not degraded by heparitinase or Hep III. The use of defined oligosaccharides as substrates has demonstrated the hitherto unrecognized ability of heparitinase and Hep III to cleave the →4)-α-D-GlcNp(R)(1→4)-β-D-GlcAp(1→ (where R = *N*-acetamido or *N*-sulfo) linkage in otherwise highly sulfated environments (as found in structures **3** and **5**), suggesting that such areas of heparin and heparan sulfate chains might be conformationally distinct from the rest of the polymer. It is especially notable that these enzymes will degrade polysaccharides in the antithrombin III binding sequence (partly represented in **5**), and they might be useful for removing the anticoagulant activity from heparin preparations that might be used in the treatment of inflammation (Linhardt et al., 1988b) or lipaemia, when this activity may not be required or be undesirable. From an analytical viewpoint, the ability of heparitinases to degrade glucuronate-containing disaccharides in solitary locations as well as in extended sequences indicates their value as reagents for estimating the glucuronate content of heparan sulfate and heparin.

Hep II represents an unusual enzymatic activity as it is capable of cleaving at glycosidic linkages containing either a glucuronic or iduronic acid residue. If this activity is associated with a single enzyme, this enzyme would then be capable of catalyzing both anti (diaxial) and syn (axial–equatorial) elimination (Linhardt et al., 1986a). Only one other lyase displays such catalytic dexterity, chondroitinase ABC (EC 4.2.2.4) from *Bacteroides* sp. or *Protease vulgaris* (Linhardt et al., 1986a). Since the purification of Hep II to homogeneity has not been reported, it was necessary to rule out the possibility that Hep II was simply a mixture of heparinase and heparitinase. The oligosaccharide maps of Hep II acting on the polymeric substrates heparin and heparan sulfate are distinctly different from the maps obtained for a mixture of heparinase and Hep III (Figures 2 and 3) and suggest that Hep II is a distinct enzyme species. In studies using defined oligomeric substrates, Hep II also demonstrates its distinctive characteristics by cleaving hexasaccharide **5** at two sites (Figure 4, Table III). One of these is cleavable by heparitinase (or Hep III) but not heparinase (or Hep I), but the second linkage, containing an unsulfated iduronic acid residue, is not cleavable by any other enzyme examined.

In conclusion, the results presented in this paper suggest that there are at least three basic types of polysaccharide lyases which can act on linkages within heparin and heparan sulfate. Heparinase (or Hep I) and heparitinase (or Hep III) are enzymes whose major structural requirements for acting on glycosidic linkages are the presence of iduronic acid 2-sulfate and glucuronic acid, respectively. Heparinase requires an N-sulfated glucosamine residue at this glycosidic linkage while heparitinase tolerates both N-sulfated and N-acetylated derivatives. Hep II, on the other hand, can cleave glycosidic linkages containing iduronic acid, iduronic acid 2-sulfate, and glucuronic acid. However, Hep II does not completely degrade heparin or heparan sulfate to disaccharides so there are some constraints on its degradative activity. As mentioned under Results, it is likely that this enzyme has weak activity against unsulfated disaccharide sequences. In the case of heparinase, permissible variations of other groups at the basic cleavable linkage are well established, whereas these remain to be completely defined for heparitinase/Hep III or Hep II.

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Direct Measurement of Agonist Binding to Genetically Engineered Peptides of the Acetylcholine Receptor by Selective T_1 NMR Relaxation†

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Received June 30, 1989; Revised Manuscript Received September 25, 1989

ABSTRACT: Interactions of four ligands of the nicotinic acetylcholine receptor with genetically engineered peptides have been studied by NMR. A recombinant cholinergic binding site was prepared as a fusion protein between a truncated form of the bacterial protein trpE and a peptide corresponding to the sequence α184–200 from the *Torpedo californica* receptor. This construct binds α-bungarotoxin while the trpE protein alone does not, and thus serves as a negative control [Aronheim, A., Eshel, Y., Mosckovitz, R., & Gershoni, J. M. (1988) *J. Biol. Chem. 263*, 9933–9937]. In this study agonist binding to α184–200 is demonstrated by monitoring the T_1 relaxation of the ligand's protons in the presence and absence of the recombinant binding site. This binding is specific as it can be competed with α-bungarotoxin. Quantitative analyses of such competitions yielded the concentration of binding sites, which corresponded to 3.3% and 16.5% of the total protein, for partially purified and affinity-purified α184–200 constructs, respectively. The K_D values for the binding of acetylcholine, nicotine, *d*-tubocurarine, and gallamine to the affinity-purified construct were 1.4, 1.4, 0.20, and 0.21 mM, respectively, while K_D's with the nontoxin binding protein were all above 10 mM. Thus, this is a direct demonstration that the toxin binding domain α184–200 may comprise a major component of the cholinergic agonist site.

With the advent of recombinant DNA technologies much has been learned about the structure of the nicotinic acetylcholine receptor (AChR)[1] [for recent reviews, see Popot and Changeux (1984), Hucho (1986), McCarthy et al. (1986), and Lentz and Wilson (1988)]. Since 1982, the complete amino acid sequences of a wide variety of AChR's have been published (Ballivet et al., 1983; Numa et al., 1983; Boulter et al., 1985, 1988; Bossy et al., 1988). From these studies, not only has the subunit composition of the AChR been confirmed, but it has become clear that all the receptor subunits thus far analyzed are common to one gene family. Furthermore, new types of subunits have been discovered (Takai et al., 1985). The "next step" in studying the structure and function of the

† This work was supported in part by grants to J.M.G. by the Minerva Foundation and the Israel Institute of Psychobiology.
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[1] Abbreviations: ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; BTX, α-bungarotoxin; GA, gallamine; NMR, nuclear magnetic resonance; TC, *d*-tubocurarine.

0006-2960/90/0429-2617$02.50/0