Sequence Variation in Heparin Octasaccharides with High Affinity for Antithrombin III†

Donald H. Atha, Andrew W. Stephens,‡ Abraham Rimon,§ and Robert D. Rosenberg*

ABSTRACT: We have isolated from nitrous acid cleavage products of heparin two major octasaccharide fragments which bind with high affinity to human antithrombin. Octasaccharide S, with the predominant structure iduronic acid→*N*-acetylglucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 3,6-di-*O*-sulfate→iduronic acid 2-*O*-sulfate→N-sulfated glucosamine 6-*O*-sulfate→iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate, is sensitive to cleavage by *Flavobacterium* heparinase as well as platelet heparitinase and binds to antithrombin with a dissociation constant of (5–15) × 10^{-8} M. Octasaccharide R, with the predominant structure iduronic acid 2-*O*-sulfate→N-sulfated glucosamine 6-*O*-sulfate→iduronic acid→*N*-acetylglucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 3,6-di-*O*-sulfate→iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate, is resistant to degradation by both enzymes and binds antithrombin with a dissociation constant of (4–18) × 10^{-7} M. The occurrence of a 15–17% replacement of N-sulfated glucosamine 3,6-di-*O*-sulfate with N-sulfated glucosamine 3-*O*-sulfate and a 10–12% replacement of iduronic acid with glucuronic acid in both octasaccharides indicates that these substitutions have little or no effect on the binding of the oligosaccharides to the protease inhibitor. When bound to antithrombin, both octasaccharides produce a 40% enhancement in the intrinsic fluorescence of the protease inhibitor and a rate of human factor Xa inhibition of 5 × 10^5 M^{-1} s^{-1} as monitored by stopped-flow fluorometry. This suggests that the conformation of antithrombin in the region of the factor Xa binding site is similar when the protease inhibitor is complexed with either octasaccharide.

Heparin is a sulfated polysaccharide that functions as an anticoagulant by binding to antithrombin and accelerating the rate at which this protease inhibitor inactivates the proteolytic enzymes of the hemostatic mechanism (Rosenberg, 1977). An earlier report from our laboratory provided evidence that only a small fraction of a given heparin preparation interacts with antithrombin and is responsible for virtually all of the anticoagulant activity for the polysaccharide (Lam et al., 1976). The antithrombin binding domain of highly active heparin is known to contain a tetrasaccharide sequence with a nonsulfated iduronic acid residue on the nonreducing end and a glucuronic acid moiety on the opposite side of an *N*-acetylglucosamine 6-*O*-sulfate group as well as an N-sulfated glucosamine 6-*O*-sulfate residue at its reducing end (Rosenberg et al., 1978; Rosenberg & Lam, 1979; Lindahl et al., 1979). Subsequently, Leder et al. (1980) isolated a sulfatase that specifically removes 3-*O*-sulfate substituents from glucosamine residues of heparin and postulated that this unique substituent might be present within the antithrombin binding domain of highly active heparin. Data provided by Lindahl et al. (1980), utilizing this exoglycosidase, have confirmed this supposition and placed the 3,6-di-O-sulfated glucosamine moiety on the reducing end of the glucuronic acid residue which lies within the tetrasaccharide region of the protease inhibitor binding domain. Thus, the ability of heparin to bind to antithrombin and accelerate the action of the protease inhibitor appears to require the presence of the tetrasaccharide sequence iduronic acid→N-acetylated glucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 3,6-di-*O*-sulfate. This conclusion is supported by the NMR studies of Casu et al. (1981). Riesenfeld et al. (1981), Choay et al. (1980), and Oosta et al. (1981) have isolated octasaccharide fragments of heparin which contain the above tetrasaccharide structure and exhibit a high affinity for antithrombin as well as the capacity to accelerate the inhibition of factor Xa. In this study, we have isolated two major octasaccharides from the above pool which contain the unique tetrasaccharide sequence on the reducing end or in the center of the molecule. This separation of the active octasaccharide fraction into discrete structures which differ in monosaccharide sequence has permitted us to directly examine the effect that the latter parameter plays in the ability of an oligosaccharide to bind antithrombin and accelerate the neutralization of factor Xa. In this regard, we present evidence that residues within the octasaccharide which are important in the binding of the oligosaccharide to antithrombin may be of little consequence in the activation of the protease inhibitor with respect to the neutralization of factor Xa.

Materials and Methods

Preparation of Octasaccharide. Porcine mucosal heparin (7.5 g of batch 41681 at 169 USP units/mg: Diosynth Inc., Chicago, IL) was dissolved in 150 mL of cold 0.2 M citric acid and the pH adjusted to 1.5 with concentrated sulfuric acid. Sodium nitrite was added to a final concentration of 0.05 M. The reaction proceeded for 9 min at 0 °C and was quenched with excess ammonium sulfamate (0.075 M). The reaction mixture was precipitated with a final concentration of 80% (v/v) cold ethanol, centrifuged at 10000*g* for 15 min, dissolved in a minimum volume, and gel filtered at a flow rate of 270 mL/h on a polyacrylamide P-10 (Bio-Rad) column (4.7 × 200

† From the Department of Biology and Whitaker College, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (D.H.A., A.R., and R.D.R.), the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115 (D.H.A. and R.D.R.), and Beth Israel Hospital, Boston, Massachusetts 02115 (A.W.S. and R.D.R.). *Received March 6, 1984.* This investigation was supported by National Institutes of Health Grant HL28625 and the National Foundation for Cancer Research. A preliminary account of this work was presented at the Annual Meeting of the American Society of Biological Chemists, St. Louis, MO, June 1984.

* Correspondence should be addressed to this author at Whitaker College, Massachusetts Institute of Technology, Cambridge, MA 02139.

‡ Present address: Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Science Center, Denver, CO 80262.

§ Present address: Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel.

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cm) in 0.5 M ammonium bicarbonate. The octasaccharide fraction was selected from well-resolved peaks ranging from disaccharide to dodecasaccharide as monitored by the absorbance at 254 nm and a colorimetric assay of uronic acid (Bitter & Muir, 1962) and rechromatographed on the same column to remove traces of residual hexasaccharide and decasaccharide (Oosta et al., 1981). The octasaccharides from four preparations as described above were combined for a total yield of ~1.5 g.

Affinity Fractionation. Bovine antithrombin (1.5 g) was added to octasaccharide (1.3 g) at final concentrations of 450 μM and 10 mM, respectively, in 0.01 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and 0.15 M NaCl, pH 7.5. The solution, in four 13-mL additions, was applied to polyacrylamide P-100 columns (2.5 × 100 cm), equilibrated in the same buffer, and gel filtered at 30 mL/h. The column effluent was monitored by the absorbance at 280 nm and by colorimetric assay of uronic acid (Bitter & Muir, 1962). The antithrombin–octasaccharide complex, which completely separated from the free mucopolysaccharide, was freeze-dried, redissolved in 0.01 M Tris-HCl–3 M NaCl, pH 7.5, and loaded on identical columns equilibrated in the high-salt buffer. The octasaccharide was desalted by using polyacrylamide P-2 columns (2.5 × 100 cm) equilibrated with 0.5 M ammonium bicarbonate. The affinity-fractionated fragment was rechromatographed on a polyacrylamide P-10 column (1.6 × 200 cm) equilibrated in 0.5 M ammonium bicarbonate, and peak fractions were pooled and freeze-dried. The active octasaccharide pool (~50 mg) had an anti-factor Xa activity of 200 USP units/mg as measured by chromogenic assay compared to 2 USP units/mg for the octasaccharide pool before affinity fractionation.

NMR. High-field proton and carbon-13 NMR measurements were made at 25 °C by using Bruker Model HFX 270 and HFX 250 spectrophotometers equipped with 5-mm proton and carbon-13 probes. Measurements were conducted at 250 MHz for proton spectra and at 67.9 MHz for carbon-13 spectra. Chemical shifts were measured with reference to 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propionate ([2,2,3,3-$^2H_4$]TSP) for proton NMR and with methanol [50.1 ppm relative to tetramethylsilane (Me_4Si)] for ^{13}C spectra. Deuterium oxide (99.9%, Aldrich) was used as the solvent.

Radiolabeling. Heparin octasaccharide (1 mg) was N-desulfated by incubation of the pyridinium salt with dimethyl sulfoxide containing H_2O (5%) for 1.5 h at 50 °C in a flame-sealed tube (Inoue & Nagasawa, 1976). The N-desulfation reaction was stopped by the addition of 0.5 M ammonium bicarbonate followed by gel filtration on a polyacrylamide P-2 column (0.6 × 200 cm) equilibrated in 0.5 M ammonium bicarbonate. N-Resulfation (^{35}S) was conducted according to the method of Lloyd et al. (1971). The reaction mixture contained 70 μg of the N-desulfated fragments in 15 μL of 0.5 M sodium bicarbonate, pH 9.5, 5 μL of 20 mg/mL (trimethylamine)[^{35}S]sulfur trioxide at 67 mCi/mmol (Amersham), and 15 μL of saturated solution of unlabeled (trimethylamine)sulfur trioxide [synthesized as described by Lloyd et al. (1971)] in 0.5 M sodium bicarbonate. The reaction mixture was incubated at 53 °C for 18 h. Approximately 30% of the octasaccharide was recovered after polyacrylamide P-2 chromatography and affinity fractionation as described above. The labeled octasaccharide had a specific activity of (6–8) × 10^7 ^{35}S cpm/mg.

Oligosaccharides were labeled by sodium [^3H]borohydride reduction of the reducing-end anhydromannose on the basis of previously described methods (Shively & Conrad, 1970, 1976). The heparin fragment (0.01–1 mg) was dissolved in 20 μL of 1 M sodium bicarbonate. Ten microliters of 0.25 M sodium [^3H]borohydride at 0.3–8 Ci/mmol (New England Nuclear) was added and allowed to react for 30 min at 50 °C before acidifying with 10 μL of 10 N acetic acid followed by the addition of saturated sodium carbonate to pH 7.0. The reaction mixture was applied to Whatman 1 paper separated from excess [^3H]borohydride by using an ethyl acetate/acetic acid/formic acid/water (18:3:1:4) solvent system, and rechromatographed on a polyacrylamide P-2 column (0.6 × 200 cm) in 0.5 M ammonium bicarbonate. Essentially all of the oligosaccharide was recovered with a specific activity ranging from ~1 × 10^7 to 10 × 10^7 ^3H cpm/mg.

Hydrazinolysis. Deacetylation by hydrazinolysis was performed as described (Thunberg et al., 1982; Dmitriev et al., 1975). Heparin octasaccharide (300 μg) was dried over phosphorus pentoxide under vacuum and incubated in 150 μL of 12.5 mg of hydrazine sulfate in 1.0 mL of anhydrous hydrazine in a flame-sealed tube at 104 °C for 3 h. The reaction was quenched by the addition of toluene followed by evaporation to dryness.

Nitrous Acid Degradation. The heparin octasaccharide was cleaved by either low pH (1.5) or high pH (3.9) nitrous acid treatment (Shively & Conrad, 1970), radiolabeled with sodium [^3H]borohydride, and gel filtered as described above.

Measurement of Protein or Mucopolysaccharide Concentration. Antithrombin and factor X concentrations were determined by absorbance measurements at 280 nm using absorptivity values ($A^{1\%,1cm}$) of 6.5 and 11.6, respectively (Nordenman et al., 1977; Discipio et al., 1977). Endoglycosidase concentrations were calculated by absorbance measurements at 280 nm assuming an absorptivity of 10.0 (Oosta et al., 1982). Mucopolysaccharide concentrations were estimated colorimetrically by assay of uronic acid as described (Bitter & Muir, 1962).

Proteins. Human and bovine antithrombins were prepared in homogeneous form by chromatography on heparin–Sepharose and DEAE-cellulose (Damus & Rosenberg, 1976; Jordan et al., 1982). Human activated factor X was isolated as described in a previous publication from this laboratory (Jordan et al., 1980). The α-iduronidase was obtained from bovine kidney by chromatography on concanavalin A–Sepharose, heparin–Sepharose, hydroxylapatite, CM-cellulose, and polyacrylamide P-100 (Rome et al., 1978). The final product was physically homogeneous as judged by disc gel electrophoresis and contained no detectable iduronate sulfatase or β-glucuronidase activities when assayed with the radiolabeled disaccharides iduronic acid 2-*O*-sulfate→[^3H]-anhydromannitol 6-*O*-sulfate, iduronic acid 2-*O*-sulfate→[^3H]anhydromannitol, and glucuronic acid→[^3H]anhydromannitol 6-*O*-sulfate by methods analogous to those described by Lim et al. (1974). β-Glucuronidase, purified from placental tissue, was generously provided by William Sly (Brot et al., 1978). This enzyme was homogeneous when analyzed by immunodiffusion and possessed no detectable α-iduronidase or iduronate sulfatase activities when assayed as described above. The *N*-acetylglucosamine sulfatase was prepared from bovine kidney by chromatography on concanavalin A–Sepharose, heparin–Sepharose, DEAE-cellulose, CM-cellulose, and Sephacryl S200. Purification and assay methods were similar to those described by Basner et al. (1979). The *N*-acetylglucosaminidase was isolated from human placental tissue by chromatography on concanavalin A–Sepharose, heparin–Sepharose, hydroxylapatite, CM-cellulose, and Sephacryl S300. Purification and assay methods were similar

to those described by von Figura (1977). These enzymes were homogeneous and free of trace contamination by other exoglycosidases when assayed as described above. Human platelet heparitinase was obtained in homogeneous form as outlined in a recent publication from our laboratory (Oosta et al., 1982). *Flavobacterium* heparinase was prepared from *Flavobacterium heparinum* by protamine sulfate precipitation, hydroxylapatite chromatography, cellulose phosphate chromatography, and Sephacryl S200 chromatography according to the method of Hovingh & Linker (1970) as modified by Castellot et al. (1981).

Enzymatic Degradation. Digestion of octasaccharides with *Flavobacterium* heparinase was carried out in 0.1 M sodium acetate–10 mM calcium acetate, pH 7.2, for 3 h at 30 °C. Digestion with human platelet heparitinase was carried out in 0.1 M sodium phosphate, pH 6.3, for 24 h at 37 °C. α-Iduronidase and β-glucuronidase digestions were carried out for 18 h at 37 °C in 0.1 M sodium formate–0.2 M sodium chloride, pH 3.55, and in 0.05 M sodium acetate, pH 4.3, respectively (Oosta et al., 1982). *N*-Acetylglucosamine sulfatase and *N*-acetylglucosaminidase digestions were carried out for 18 h at 37 °C in 0.08 M sodium acetate, pH 5.5, and in 0.5 M sodium citrate, pH 4.2, respectively.

High-Performance Liquid Chromatography (HPLC). HPLC was performed on a Waters system including two 6000A pumps, a U6K injector, and a 660 solvent programmer. Ion exchange was conducted on a Whatman Partisil PXS-1025 SAX column at a flow rate of 1 mL/min with various concentrations of potassium phosphate, pH 4.3 (Delaney et al., 1980). Monosulfated disaccharides were eluted isocratically with 40 mM KH_2PO_4. Disulfated disaccharides and tetrasaccharides were separated by using a 30-min convex gradient from 40 to 400 mM KH_2PO_4 and a second 30-min linear gradient from 400 to 1000 mM. Gel filtration was carried out at a flow rate of 0.5 mL/min on a Toya Soda TSK G-2000SW column (0.75 × 60 cm) equilibrated with either 1 M NaCl or 1 M ammonium acetate, pH 6.5.

Fluorescence Measurements. Fluorescence measurements were made by using a Perkin-Elmer MP-44A spectrofluorometer equipped with a thermostated sample compartment and a differential corrected spectra accessory. Heparin octasaccharide at a concentration of 20 μM was added with a 10-μL syringe to 2.75 mL of human antithrombin at a concentration of 12 nM in 0.01 M Tris, pH 7.5, and 0.15 M NaCl. The solution was carefully stirred with the syringe needle after each addition. The binding of heparin fragments to antithrombin was monitored by measuring the fluorescence enhancement of tryptophan residues. The wavelengths of excitation and emission were 280 and 330 nm, respectively. The dissociation constants for the mucopolysaccharide–protein complexes were calculated by a nonlinear least-squares fit of the data to a single-site binding model as described previously (Jordan et al., 1979).

Equilibrium Dialysis. The binding of heparin fragments to human antithrombin was measured by using 1-mL equilibrium dialysis cells (Technilab Instruments Inc., Pequannock, NJ) and a Spectrapor 2 (12000–14000 molecular weight cutoff) membrane (Spectrum Medical Industries, Los Angeles, CA). A solution of antithrombin (1 nM–0.5 mM) and labeled heparin fragment (5000 cpm or approximately 10 nM) in 0.01 M Tris, pH 7.5, and 0.15 M NaCl was added to one compartment of the dialysis cell whereas buffer alone was added to the other compartment. The cells were mounted on a rotator at 6 °C and equilibrated at approximately 1 revolution/s. Samples (0.1–0.2 mL) were withdrawn from both cell compartments, mixed with 3.0 mL of Ultrafluor scintillation cocktail (National Diagnostics, Somerville, NJ), and counted for 10 min each. To ensure that the solutions had reached equilibrium, samples were taken at time intervals from 1 to 10 days and compared to measurements in which the labeled heparin fragment had been added to the non-protein-containing compartment. After constant readings were established, the ratio of bound to total heparin fragment (HAT/H) was calculated from the difference in concentrations (in cpm per milliliter) between the protein-containing and non-protein-containing compartments divided by the concentration of fragment in the protein-containing compartment. This ratio (HAT/H) was determined in separate cells as a function of protein concentration. The dissociation constants for the mucopolysaccharide–protein complexes were calculated by a nonlinear least-squares fit of the equilibrium dialysis data to a single-site binding model as described above for fluorescence data.

Stopped-Flow Measurements. The rate of inhibition of human factor Xa by antithrombin was measured by fluorescent probe displacement as previously outlined for thrombin (Evans et al., 1982; Olson & Shore, 1982). A Durrum stopped-flow fluorescence spectrophotometer (Model D-110) and a Digital PDP 11-03 computer were used to record changes in fluorescence after equal volumes of a solution containing 2.3 μM factor Xa and 400 μM *p*-aminobenzamidine were mixed with a solution of 21 μM human antithrombin and 100 μM octasaccharide. The displacement of the probe from the active site of factor Xa resulted in an exponential decrease in fluorescence with a rate constant k_{obsd}. Under these conditions of a 10-fold excess of antithrombin, the bimolecular rate constant k for the inhibition of factor Xa is related to k_{obsd} by the following equation (Evans et al., 1982):

$$k_{obsd} = \frac{k[AT]_0}{1 + [P]_0/K_p}$$

where $[AT]_0$ and $[P]_0$ are the initial antithrombin and probe concentrations, respectively, and K_p is the dissociation constant of the probe. The constant K_p was measured for factor Xa by fluorescence titration as described by Evans et al. (1982) and resulted in a value of 115 μM. Both the stopped-flow and the fluorescence titration measurements were made at 25 °C in 0.01 M Tris, pH 7.5, containing 0.15 M NaCl and 0.1% poly(ethylene glycol) (PEG) 6000. Dialysis was performed on a mixture of *p*-aminobenzamidine and active octasaccharide in order to detect a possible interaction between the two under the conditions of the stopped-flow experiments. The rate of equilibration of the probe, measured by fluorescence, was not affected by the presence of octasaccharide. Therefore, it was concluded that these components do not interact to any significant extent.

Chromogenic Assays. The activity of mucopolysaccharide fractions with respect to human factor Xa inactivation was measured by using a two-stage chromogenic assay. A known amount of mucopolysaccharide was added to a solution containing factor Xa and human antithrombin each at a final concentration of 0.227 μM in 0.01 M Tris–0.15 M NaCl, pH 7.5. The mixture was incubated at 37 °C for 45 s. The second stage of the assay was initiated by the addition of polybrene and S-2222 (Ortho Diagnostics) in water to final concentrations of 0.31 and 0.375 mg/mL, respectively. After the reaction mixture was incubated for 90 s, the reaction was quenched by the addition of 200 μL of glacial acetic acid. The extent of amidolysis was quantitated by measuring the absorbance at 405 nm. A heparin preparation of known United

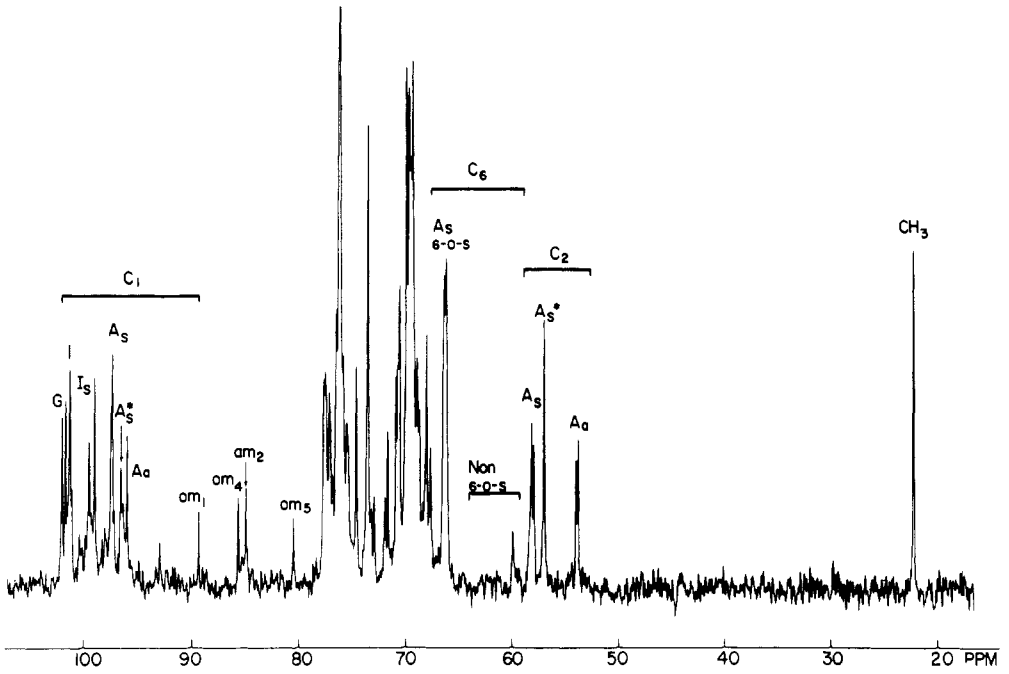


FIGURE 1: Carbon-13 NMR of the total active octasaccharide pool. Measurements were made at 25 °C using 21K pulses of 7.5-μs duration (70°). The measurements were broad-band decoupled by using a receiver delay, acquisition time, and relaxation delay of 30 μs, 0.475 ms, and 2.0 ms, respectively. Data points (16K per scan) were averaged, exponentionally multiplied with a decay constant of 3.0, and Fourier transformed. The mucopolysaccharide concentration was 90 mg/mL. All abbreviations except for am (=anhydromannitol) are given in Table I.

States Pharmacopeia (USP) potency was employed as the reference standard.

Results

Structural Studies of Heparin Octasaccharide. (A) Carbon-13 NMR. We have examined the total active octasaccharide pool by high-field carbon-13 NMR and have assigned resonances according to the previous measurements of Casu et al (1981). As seen in Figure 1, the majority of assignments can be made with the exception of the 68–75 ppm region in which considerable overlapping of peaks occurs. Integration of assigned peak areas yields the compositional information given in Table I. Here we compare our data to molar ratios based on the previously described active octasaccharide structure: iduronic acid→N-acetylated glucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 3,6-di-*O*-sulfate→iduronic acid 2-*O*-sulfate→N-sulfated glucosamine 6-*O*-sulfate→iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate (Rosenberg & Lam, 1979; Lindahl et al., 1979, 1980; Casu et al., 1981). The measured compositions are within a 12% deviation from the literature values with the exception of N-sulfated glucosamine 6-*O*-sulfate which is slightly higher and the non-6-*O*-sulfated N-sulfated glucosamine which is present in small amounts. The carbon-13 value for *N*-acetylglucosamine was considerably lower than the literature value. However, the proton NMR measurements, which we consider to be more accurate, yielded values within the 12% deviation from literature values.

(B) Nitrous Acid Degradation. Nitrous acid treatment of heparin causes deaminative ring conraction of glucosamine residues with concomitant glucosamine–uronic acid glycosidic bond cleavage. This results in the formation of a new anhydromannose reducing end and leaves the uronic acid unmodified. N-Sulfated glucosamine moieties are uniquely sensitive to cleavage at pH 1.5 while unsubstituted glucosamine moieties are rapidly cleaved at pH 3.9 (Shiveley & Conrad, 1970, 1976). *N*-Acetylglucosamine residues are completely resistant to nitrous acid under these conditions (Shiveley & Conrad, 1970, 1976). We have analyzed the total active octasaccharide pool by these methods as well as two octasaccharide fractions, S and R (see Chart I under Discussion), which have been isolated from the octasaccharide pool by affinity fractionation and enzyme cleavage. In addition, we have analyzed the resulting nitrous acid degradation products by a combination of gel filtration, HPLC ion-exchange chromatography, and exoglycosidase treatment to elucidate the structure and heterogeneity of the active octasaccharides.

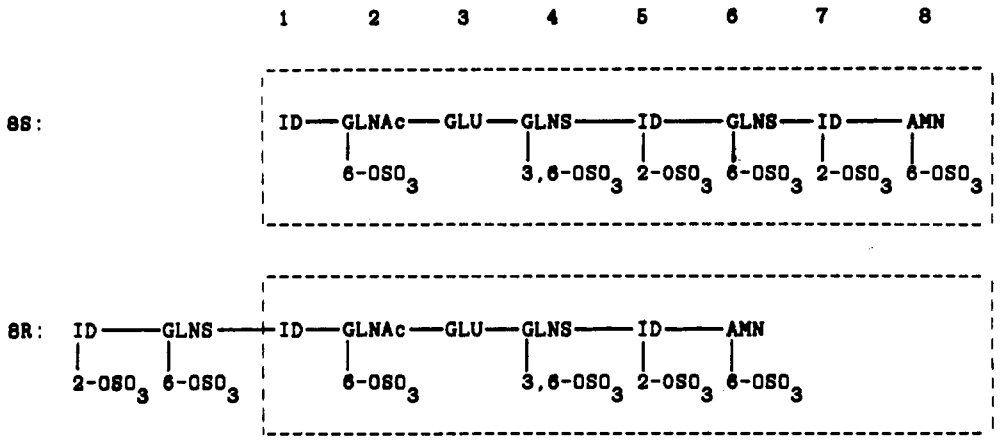
Initially the total active octasaccharide pool was degraded by low-pH (1.5) nitrous acid immediately followed by [^{3}H]-borohydride reduction. This results in incorporation of ^{3}H into both preexisting and newly formed anhydromannose residues. The resulting elution profile (solid line in Figure 2A) revealed

Table I: NMR Measurements of Active Octasaccharides

hexose[a]	composition (molar ratio) obsd[b] ^{13}C	obsd[b] ^{1}H	lit.[c]
uronic			
I + G + I_s	0.53		0.50
I + G	0.27		0.25
I_s	0.26		0.25
glucosamine			
A_s + A_{s*} + A_a (6-O-sulfated)	0.35		0.375
A_s + A_{s*} + A_a (non-6-O-sulfated)	0.025		0.0
A_s	0.15		0.125
A_{s*}	0.14		0.125
A_a	0.08	(0.11, 0.14)	0.125

[a] Abbreviations: I, iduronic acid; G, glucuronic acid; I_s, iduronic acid 2-*O*-sulfate; A_s, N-sulfated glucosamine 6-*O*-sulfate; A_{s*}, N-sulfated glucosamine 3,6-di-*O*-sulfate; A_a, *N*-acetylglucosamine 6-*O*-sulfate. [b] Carbon-13 values were determined from C_1 resonances with the exception of non-6-*O*-sulfated glucosamine and of the individual ratios of A_s, A_{s*}, and A_a which were determined from C_6 and C_2 regions, respectively. ^{1}H values were determined from the acetyl resonance at 2 ppm. The values in parentheses correspond to octasaccharides S and R, respectively (see results). [c] Molar ratios are based on the previously described active octasaccharide structure (see Results).

Chart I[a]



[a] Abbreviations: ID, iduronic acid; GLU, glucuronic acid; GLNAc, *N*-acetylglucosamine; GLNS, N-sulfated glucosamine; AMN, anhydromannitol.

two major peaks corresponding to tetra- and disaccharides and a minor peak (~5%) corresponding to hexasaccharide. Integration of these peaks including the 5% hexasaccharide gave a di- to tetrasaccharide ratio of 1.7. This indicates the presence of approximately two N-sulfated glucosamine residues per octasaccharide. To test whether or not equal incorporation of tritium was occurring in the tetra- and disaccharide fractions, the experiment was repeated with HPLC gel filtration monitored by the refractive index. This resulted in two major peaks of approximately the same size (data not shown). Integration and correction for the expected molecular weight gave a di- to tetrasaccharide ratio of 1.7, which is in excellent agreement with the results obtained by radiolabeling. High-pH nitrous acid treatment (pH 3.9) did not further degrade the tetrasaccharide fraction nor did dilute acid hydrolysis performed according to the method of Bienkowski (1984). This showed that the glucosamine moieties of the tetrasaccharides are completely N-acetylated and do not contain free amino groups or possess deaminative ring contractures (2-aldehydro-D-pentofuranose).

In another set of experiments, [^{3}H]borohydride reduction of the octasaccharide pool prior to low-pH nitrous acid treatment resulted in the formation of disaccharide with almost no tetrasaccharide and no hexa- or octasaccharide (<10% total). This demonstrated that the tetrasaccharide sequence containing *N*-acetylglucosamine either is on the nonreducing end of the active octasaccharide or is internal to disaccharides on both the reducing and nonreducing ends. These studies show that the active octasaccharide contains essentially two N-sulfated glucosamines and one N-acetylated glucosamine and that the glucosamine residue proximal to the reducing end can be acetylated only to a very small extent (<10%).

In order to determine the structure of the nonreducing end, we treated the active octasaccharide pool with α-iduronidase followed by low-pH nitrous acid degradation and [^{3}H]borohydride reduction. The products were analyzed by gel filtration on polyacrylamide P-4. As can be seen in Figure 2B (solid line), the octasaccharide was degraded approximately 50% by the α-iduronidase as evidenced by equal amounts of tetra- and trisaccharides.

(*C*) *HPLC Ion Exchange of Nitrous Acid Fragments.* Products from nitrous acxid degradation and borohydride reduction previously described were analyzed for structural heterogeneity by using HPLC ion-exchange chromatography. Analysis of the tetrasaccharide fraction resulted in three major peaks, B, D, and E, as shown by the solid line in Figure 3A. These fractions were of sufficient purity and amount to determine their structures by exoglycosidase treatment (see below). The first residue of fraction C could also be determined. Fraction A appeared to be the contaminating disaccharide iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate.

The disaccharide peak from nitrous acid degraded active octasaccharide was analyzed under conditions which could clearly resolve monosulfated disaccharides (40 mM KH_2PO_4) as well as disulfated disaccharides (40–400 mM, convex gradient). Under these conditions, 95% of the disaccharides eluted as the disulfated disaccharide [iduronic acid 2-*O* sulfate→anhydromannitol 6-*O*-sulfate (peak 4 in Figure 3B)], and only 5% eluted as an equal mixture of all three monosulfated disaccharides [glucuronic acid→anhydromannitol 6-*O*-sulfate, iduronic acid→anhydromannitol 6-*O*-sulfate, and iduronic acid 2-*O*-sulfate→anhydromannitol (peaks 1–3 in Figure 3B)]. Furthermore, when the octasaccharide was reduced with cold borohydride prior to nitrous acid degradation and [^{3}H]borohydride reduction, the resulting disaccharide was essentially all iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate and contained no measurable monosulfated disaccharide.

(*D*) *Enzyme Degradation of Tetrasaccharides.* The structures of the tetrasaccharides derived from the active octasaccharide pool and isolated by HPLC ion exchange were determined by sequential cleavages using α-iduronidase, β-glucuronidase, glucosamine sulfatase, and α-*N*-acetylglucosaminidase. Degradations of the predominant E tetrasaccharide are shown in Figure 4. This tetrasaccharide degrades completely to a trisaccharide with α-iduronidase yet not at all with β-glucuronidase (Figure 4A). This shows that the non-reducing-end residue of the E tetrasaccharide is a nonsulfated iduronic acid. When the trisaccharide was incubated with a combination of glucosamine sulfatase and *N*-acetylglucosaminidase, it was completely converted to disaccharide (Figure 4B). If the same fragment was incubated with *N*-acetylglucosaminidase alone, no degradation occurred. In addition, the glucosamine sulfatase treated trisaccharide eluted substantially earlier on HPLC ion exchange than the untreated trisaccharide (not shown). These two experiments demonstrate that the second residue of the E tetrasaccharide is a 6-*O*-sulfated *N*-acetylglucosamine. Furthermore, treatment of the resulting disaccharide with either α-iduronidase or β-glucuronidase resulted in the conversion of 85% of the substrate with the latter enzyme but none with α-iduronidase (Figure 4C). These data show that the third residue of the E tetrasaccharide is glucuronic acid.

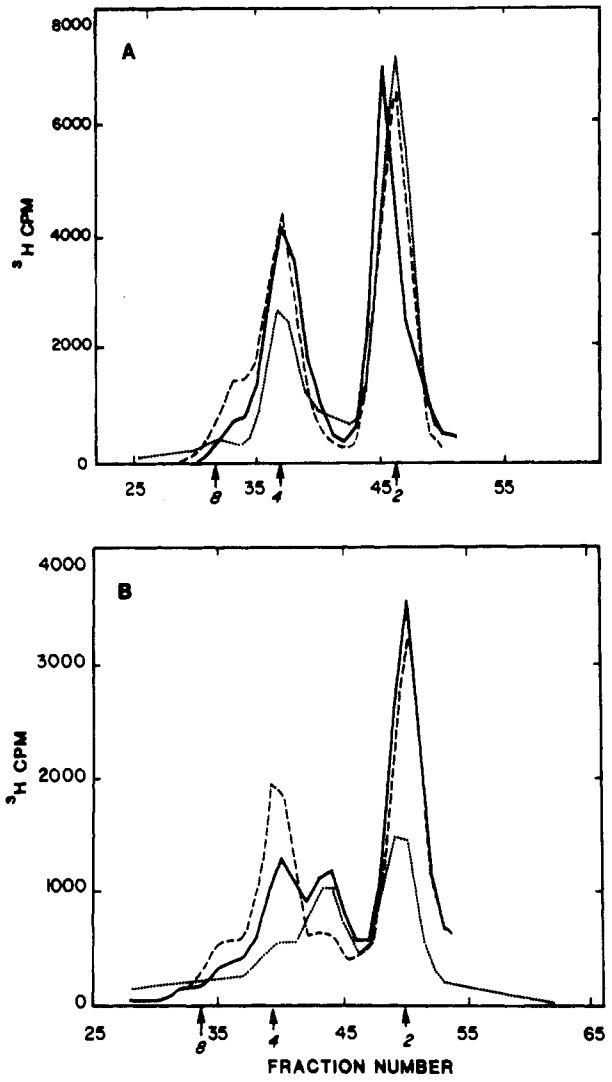


FIGURE 2: Nitrous acid degradation of active octasaccharides. Oligosaccharides were treated with low-pH nitrous acid, radiolabeled with sodium [^{3}H]borohydride, and gel filtered on a polyacrylamide P-4 column (0.6 × 200 cm). Fractions of 0.6 mL were collected and evaluated by scintillation counting. Column calibration points corresponding to the elution volumes for octasaccharide (8), tetrasaccharide (4), and disaccharide (2) are indicated in the lower margins. (A) Comparison of the active octasaccharide pool (—), octasaccharide R (---), and octasaccharide S (···). (B) Comparison of the α-iduronidase-treated active octasaccharide pool (—), octasaccharide R (---), and octasaccharide S (···).

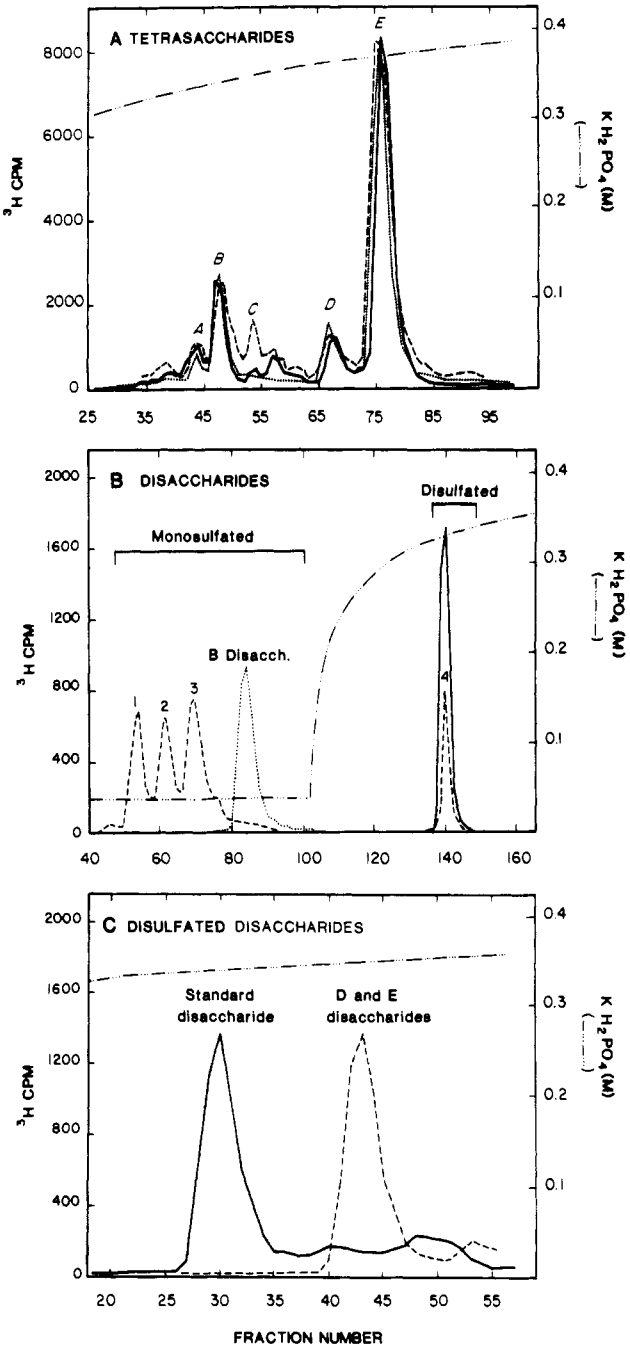


FIGURE 3: HPLC ion-exchange chromatography of tetra- and disaccharide fragments produced from nitrous acid and enzyme degradation of active octasaccharides. Tetra- and disaccharides obtained from active octasaccharides were applied to an HPLC ion-exchange column and compared to standards as indicated. (A) Comparison of tetrasaccharides obtained by low-pH nitrous acid degradation of the active octasaccharide pool (—), octasaccharide R (---), and octasaccharide S (···). Major components, labeled B, C, D, and E, were isolated and structurally analyzed as described (see text). (B) Disaccharides obtained from low-pH nitrous acid degradation of the active octasaccharide pool (—) and from exoglycosidase treatment of the B tetrasaccharide (···) are compared to monosulfated and disulfated disaccharide standards (---). Monosulfated standards (peaks 1–3) are glucuronic acid→anhydromannitol 6-*O*-sulfate, iduronic acid→anhydromannitol 6-*O*-sulfate, and iduronic acid 2-*O*-sulfate→anhydromannitol, respectively. The disulfated standard (peak 4) is iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate. (C) Separation of D and E disaccharides from the disulfated disaccharide standard iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate.

The remaining three pools of tetrasaccharides were treated in a manner identical with tetrasaccharide E treatment. Sequential degradation of tetrasaccharide B yielded results similar to those for tetrasaccharide E except that the trisaccharide was 70% degraded by *N*-acetylglucosamine sulfatase and *N*-acetylglucosaminidase. Tetrasaccharide D was 70% degraded to trisaccharide by glucuronidase and not at all by α-iduronidase. This showed that tetrasaccharide D contains glucuronic acid in place of iduronic acid at its nonreducing end. The results of subsequent degradation steps were identical with those of tetrasaccharide E. Tetrasaccharide C was 90% degraded to trisaccharide by α-iduronidase but could not be degraded at all by β-glucuronidase. Trisaccharide C could not be obtained in sufficient quantity for further analysis.

Disaccharides from the previous enzyme digestions were subjected to HPLC ion-exchange chromatography. Disaccharide B eluted in the region of monosulfated disaccharides as a discrete homogeneous peak. It was, however, retained longer than any of the known monosaccharide standards (see Figure 3B). Disaccharide B could be 65% degraded by β-glucuronidase but not at all with α-iduronidase. Since the undegraded glucuronidase-treated disaccharide was analyzed

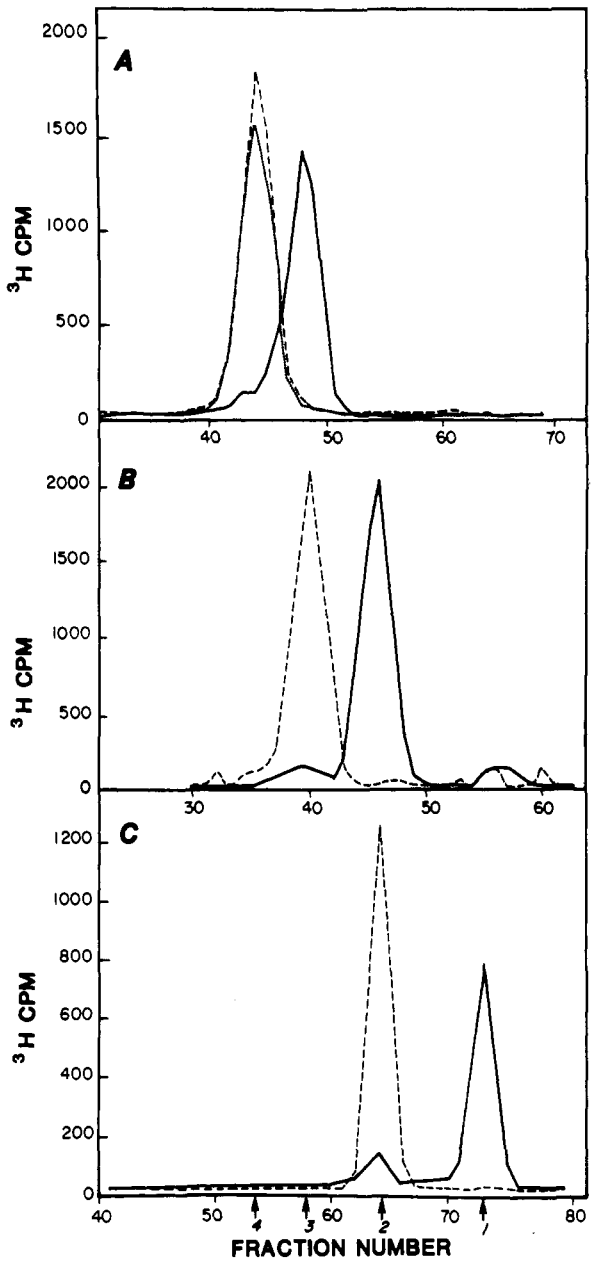


FIGURE 4: Exoglycosidase digestion of the major tetrasaccharide from nitrous acid treatment of the active octasaccharide pool. Radiolabeled [^{3}H]heparin fragments were treated with exoglycosidases as indicated and applied to polyacrylamide P-4 columns (0.6 × 200 cm) as described. Fractions of 0.6 mL were collected and evaluated by scintillation counting. Column calibration points corresponding to the elution volumes for tetrasaccharide (4), trisaccharide (3), disaccharide (2), and monosaccharide (1) are indicated in the lower margin. (A) E tetrasaccharide before (---) and after (—) treatment with α-iduronidase or with β-glucuronidase (···). (B) E trisaccharide treated with *N*-acetylglucosaminidase (---) or a combination of *N*-acetylglucosamine sulfatase and *N*-acetylglucosaminidase (—). (C) E disaccharide treated with α-iduronidase (---) or β-glucuronidase (—).

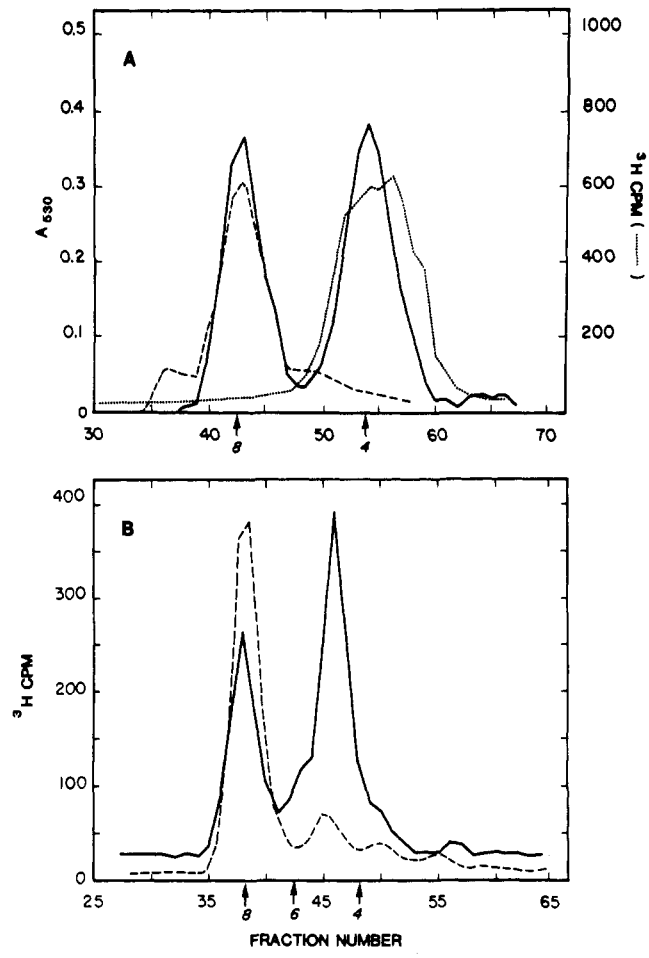


FIGURE 5: Endoglycosidase treatment of active octasaccharides. Active octasaccharides were treated with endoglycosidases and gel filtered on a polyacrylamide P-4 column (0.6 × 200 cm). Fractions of 0.6 mL were collected and evaluated by scintillation counting (^{3}H cpm) or assay of uronic acid (A_{530}). Column calibration points corresponding to the elution volumes for octasaccharide (8), hexasaccharide (6), and tetrasaccharide (4) are indicated in the lower margins. (A) The active octasaccharide pool (—), octasaccharide R (---), or octasaccharide S (···) was treated with *Flavobacterium* heparinase as described (see Materials and Methods). (B) The active octasaccharide pool (—) and octasaccharide R (---) were treated with platelet heparitinase.

by HPLC and found to elute in the same position as the original material, we suspect that the reason for this incomplete digestion is that there is a requirement for a 6-*O*-sulfate on the anhydromannitol residue which renders the B disaccharide a poor substrate for β-glucuronidase. Similar effects may be responsible for the other incomplete (70%) digestions. Disaccharides from D and E initially eluted in the same position by HPLC as iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate using the 40–400 mM 30-min convex gradient. When the same gradient was run from 220 to 364 mM, D and E disaccharides still could not be separated from each other whereas they could be clearly resolved from the disaccharide standard (see Figure 3C). Both D and E disaccharides were quantitatively reduced to monosaccharide by digestion with β-glucuronidase.

Data obtained from ^{13}C NMR (Table I) demonstrate that there must be one 3-*O*-sulfated glucosamine sulfate residue in each active octasaccharide. Therefore, we conclude that disaccharides B, D, and E all must contain a 3-*O*-sulfated anhydromannitol. The structure of the monosulfated B disaccharide is glucuronic acid→anhydromannitol 3-*O*-sulfate, and the disulfated D and E disaccharides are glucuronic acid→anhydromannitol 3,6-di-*O*-sulfate. On the basis of these observations and the results described above, the structures and percentages of the individual tetrasaccharides which make up the nitrous acid resistant tetrasaccharide pool are given in Table II.

(*E*) *Identification and Separation of Two Species within the Active Octasaccharide Pool.* The active octasaccharide pool was treated with *Flavobacterium* heparinase in order to produce the unique tetrasaccharide binding sequence with an N-sulfated glucosamine on the reducing end. This digestion resulted in approximately 60% conversion of the octasaccharides to tetrasaccharides without producing hexa- or

Table II: Structure and Percentage Composition of Tetra- and Disaccharides from Nitrous Acid Degradation of Active Octasaccharides

	% composition[a]			
	8P[c]	8S	8R	structure[b]
tetrasaccharide				
E	63	64	61	ID→GLNAc-6-O-SO_3→GLU→-AMN-3,6-O-$(SO_3)_2$
B	16	17	15	ID→GLNAc-6-O-SO_3→GLU→-AMN-3-O-SO_3
D	10	12	10	GLU→GLNAc-6-O-SO_3→GLU→-AMN-3,6-O-$(SO_3)_2$
disaccharide	95	97	93	ID-2-O-SO_3→AMN-6-O-SO_3

[a] Percentage compositions were determined by HPLC ion-exchange chromatography (see Figure 3). [b] Abbreviations: ID, iduronic acid; GLU, glucuronic acid; GLNAc, *N*-acetylglucosamine; AMN, anhydromannitol. [c] 8P = active octasaccharide pool.

disaccharide (solid line in Figure 5A). The remaining octasaccharide was treated in a manner similar to the enzyme, but no further degradation occurred. This result demonstrated that the active octasaccharide pool contains a molecular species (octasaccharide R) which is resistant to degradation by *Flavobacterium* heparinase. Fractions from the initial column profile were assayed for their ability to enhance the anti-factor Xa activity of antithrombin using the chromogenic substrate S-2222. This measurement revealed an initial plateau of approximately 60 USP units/mg for the resistant octasaccharide compared to 200 USP units/mg for the active octasaccharide pool prior to enzyme treatment as well as a subsequent plateau of approximately 4 USP units/mg through the region of the tetrasaccharide (data not shown). This trace amount of activity in the region of the tetrasaccharide was due entirely to contaminating octasaccharide which could be removed by rechromatography of the latter component.

Octasaccharide R possessed only about one-third the activity of the original active octasaccharide pool. This suggested to us that octasaccharide R might be separable from the more active component by affinity fractionation. Therefore, we subjected the octasaccharide pool (6.5 mg) to affinity fractionation for a second time but at an antithrombin concentration of 10 μM rather than the 450 μM previously employed. By this method, we were able to remove the lower affinity octasaccharide R and obtain an octasaccharide S ($\sim$1 mg) with higher affinity for antithrombin. The octasaccharide R could not be degraded with *Flavobacterium* heparinase whereas the octasaccharide S was completely cleaved by *Flavobacterium* heparinase (Figure 5A). Treatment of the active octasaccharide pool with platelet heparitinase also resulted in only 60% degradation. Octasaccharide R was resistant to degradation (Figure 5B) whereas octasaccharide S was completely cleaved to penta- and trisaccharides (data not shown). The reason for the resistance of octasaccharide R to degradation is not known. However, a similar effect has been observed by Fransson (1968) in a reduced octasaccharide from dermatan sulfate which is completely resistant to degradation by hyaluronidase even though it contains a β-hexosaminidic bond to a D-glucuronic acid residue.

Structure Studies of Octasaccharides S and R. The octasaccharides S and R were degraded by nitrous acid followed by [^{3}H]borohydride reduction (Figure 2A). The degradation of octasaccharide S resulted in a di- to tetrasaccharide ratio of 2.0 with only a trace amount of hexasaccharides produced ($\sim$2%). This indicated a total of two N-sulfated glucosamine residues within the molecule. When the octasaccharide R was degraded in a similar manner, it exhibited a ratio of di- to tetrasaccharides of approximately 1.7 and produced approximately 10% hexasaccharides. This indicated an average of 1.4 N-sulfated glucosamine residues in octasaccharide R.

HPLC ion-exchange chromatography of the tetrasaccharides produced from low-pH nitrous acid degradation of octasaccharides S and R yielded profiles which were almost identical in the proportions of the three major peaks as shown in Figure 3A. The disaccharides coeluted with iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate as indicated in Figure 3B. These data suggested to us that the octasaccharides S and R differ only in the positioning of the unique tetrasaccharide. In octasaccharide S, this acetylated tetrasaccharide is positioned at the nonreducing end of the molecule whereas in octasaccharide R it is centrally placed between the two disaccharides: iduronic acid 2-*O*-sulfate→N-sulfated glucosamine 6-*O*-sulfate and iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate (see Chart I under Discussion). To test this hypothesis, we treated octasaccharides S and R with α-iduronidase followed by low-pH nitrous acid degradation and [^{3}H]borohydride reduction. The products were analyzed by gel filtration on polyacrylamide P-4. As shown in Figure 2B, octasaccharide S degraded aproximately 90% by α-iduronidase, resulting in primarily tri- and disaccharides with only a small amount of residual tetrasaccharide. The octasaccharide R was not degraded at all by the enzyme and gave only tetra- and disaccharides upon nitrous acid treatment. These experiments show that octasaccharide S almost exclusively contains a nonsulfated iduronic acid residue on its nonreducing end. Likewise, octasaccharide R contains no nonsulfated iduronic acid on its nonreducing end. The small amount of nondegraded octasaccharide S can be attributed to the 12% glucuronic acid corresponding to the D tetrasaccharide as described above.

The preexisting reducing ends of octasaccharides S and R were radiolabeled with sodium [^{3}H]borohydride and then subjected to low-pH nitrous acid degradation. This resulted in essentially all disaccharide for octasaccharide S and 25% hexasaccharide for octasaccharide R (data not shown). This indicated that 25% of the octasaccharide R preparation contains a non-N-sulfated glucosamine residue on the reducing end of the unique tetrasaccharide. The hexasaccharide was treated by high-pH nitrous acid and found to degrade approximately 50% to tetra- and disaccharides. This showed that approximately half of these non-N-sulfated glucosamine residues are not N-acetylated. To determine the extent of N-acetylation of the penultimate residue at the nonreducing end, octasaccharide R was reduced with cold borohydride prior to low-pH nitrous acid treatment. The mixture was radiolabeled and gel chromatographed as previously described and yielded an equal ratio of tetra- and disaccharides and approximately 5% hexasaccharide (data not shown). The hexasaccharide obtained by this procedure could not be degraded by high-pH nitrous acid treatment. This demonstrated that the original octasaccharide R preparation contains no more than about 5% *N*-acetylglucosamine adjacent to the nonreducing end of the molecule. The *N*-acetylglucosamine contents of octasaccharides S and R were individually measured by using high-field proton NMR. This was accomplished by comparing the integrated *N*-acetyl resonances of 2 mM solutions of octasaccharides S and R to that of a 2 mM standard solution of *N*-acetylglucosamine. The latter showed that octasaccharides R and S contain respectively 1.1 and 0.9 mol of *N*-acetylglucosamine per mol of octasaccharide (Table I). This difference in the total *N*-acetylglucosamine content of octasaccharides S and R as measured by NMR is in good

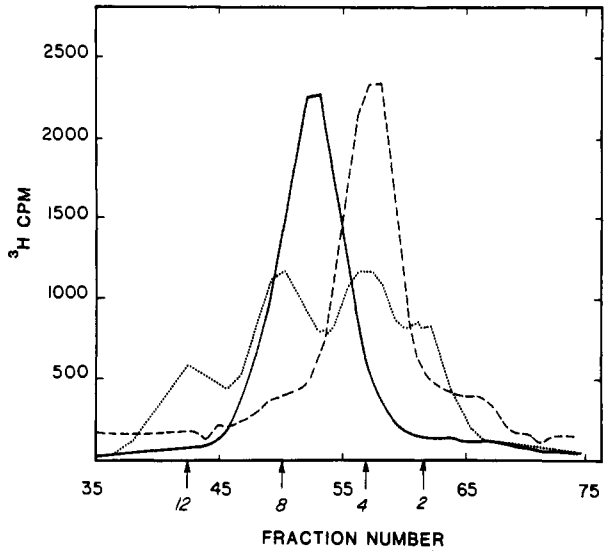


FIGURE 6: High-pH nitrous acid degradation of active octasaccharides S and R after hydrazinolysis. Radiolabeled octasaccharide S (—) and octasaccharide R (- - -) were deacetylated by hydrazine, treated with high-pH nitrous acid, and chromatographed on an HPLC G-2000 column. Fractions of 0.15 mL were collected, evaluated by scintillation counting, and compared to heparin size standards (···) indicated in the lower margin.

Table III: Antithrombin Binding Affinity and Biologic Activity of Octasaccharides S and R

octa-saccharide	fluorescence		equilibrium dialysis,	anti-factor Xa activity,
	K_{diss} (M)	ΔF_{max}	K_{diss} (M)	k_{assoc} (M^{-1} s^{-1})
8S	5.0×10^{-8}	0.40	1.5×10^{-7}	5.4×10^5
8R	4.1×10^{-7}	0.36	1.8×10^{-6}	5.4×10^5

agreement with the ~17% difference observed by high-pH nitrous acid degradation as described above.

The reducing end labeled octasaccharides S and R were also analyzed for positional variations of the unique tetrasaccharide sequence by hydrazinolysis and high-pH nitrous acid degradation. This method permits us to deacetylate *N*-acetylglucosamine residues and subsequently convert the resultant glucosamine moieties to anhydromannose groups with scissioning of the respective glycosidic bond. As expected, octasaccharide R yielded a labeled tetrasaccharide whereas octasaccharide S yielded a labeled hexasaccharide (Figure 6).

Functional Studies of Octasaccharides S and R. The binding of octasaccharides S and R to human antithrombin was analyzed by intrinsic fluorescence titration (Figure 7A) as well as equilibrium dialysis (Figure 7B). The dissociation constants derived by fitting the data to a single-site model and the maximum fluorescence change, ΔF_{max}, obtained at saturating concentrations of mucopolysaccharide are given in Table III. Although a 3–4-fold difference was observed in the dissociation constants obtained by these two methods, they showed an 8–12-fold higher affinity of octasaccharide S relative to octasaccharide R for antithrombin. Despite this difference in the binding affinity, both octasaccharides exhibited similar ΔF_{max} values when complexed with antithrombin. This suggests that the conformation of antithrombin is similar when octasaccharides S and R bind to the protease inhibitor (Olson & Shore, 1981). As an additional probe of the conformation of antithrombin, we have measured the rate at which factor Xa is inactivated by the protease inhibitor with saturating concentrations of octasaccharides S and R. This measurement was performed by monitoring the displacement

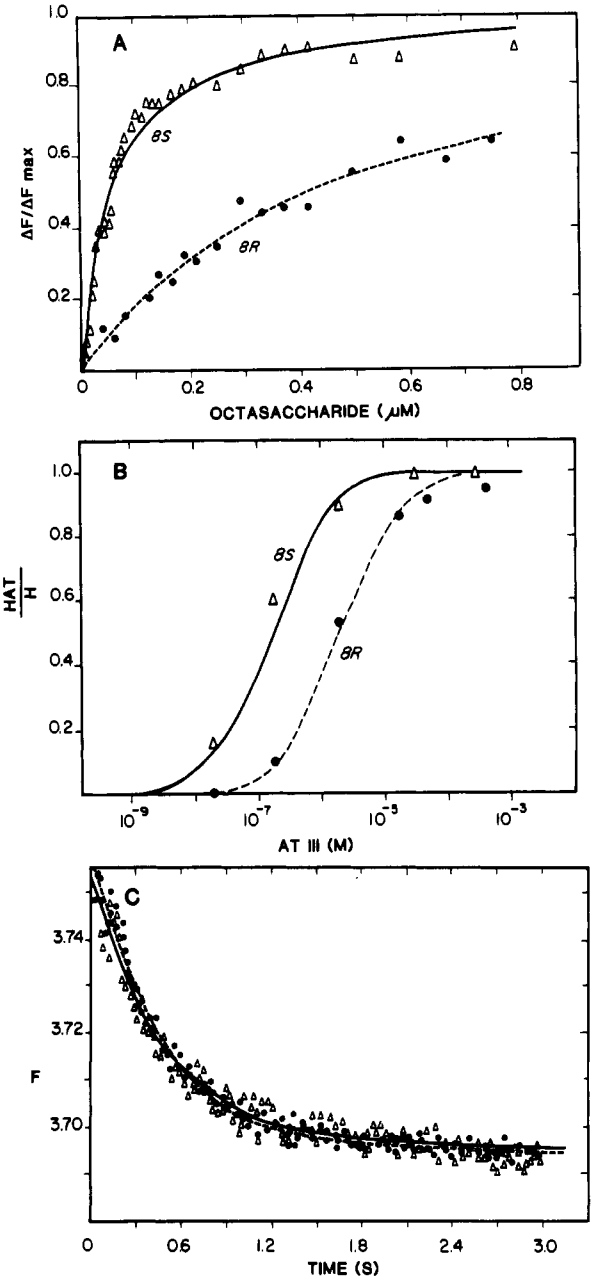


FIGURE 7: Comparison of antithrombin activity of octasaccharides S and R. Octasaccharide S (△) and octasaccharide R (●) were compared in 0.01 M Tris, pH 7.5, and 0.15 M NaCl. The solid and dashed lines represent the respective least-squares fits of octasaccharide S and octasaccharide R data to the one site heparin binding model or a single exponential rate expression for the inhibition of factor Xa (see text for details). (A) Intrinsic fluorescence enhancement of human antithrombin. Measurements were made at 6 °C at an antithrombin concentration of 12 nM with excitation and emission wavelengths of 280 and 330 nm, respectively. (B) Equilibrium dialysis of ^{3}H- and ^{35}S-radiolabeled octasaccharides. Measurements were made at 6 °C by using the indicated concentrations of human antithrombin (AT III) and an octasaccharide concentration of ~10 nM. (C) Time course of the inhibition of human factor Xa by human antithrombin. Fluorescence measurements were made at 25 °C at antithrombin, factor Xa, heparin, and *p*-aminobenzamidine concentrations of 10.5, 1.2, 50, and 200 μM, respectively. The excitation wavelength was 320 nm, and the emission was monitored above 340 nm with a cutoff filter.

of the active-site probe *p*-aminobenzamidine from factor Xa. This method has been developed by Evans et al. (1982) and Olson & Shore (1982) to monitor the rapid inactivation of thrombin by antithrombin (see Materials and Methods). If the conformation of antithrombin in the region of the factor

Xa binding site is similar in complexes with octasaccharides S and R, we would expect the same rate of inactivation. It is apparent from Figure 7C and Table III that there is no difference between the two octasaccharides in this respect.

Discussion

We have extracted from high-field carbon-13 NMR measurements of the active octasaccharide pool obtained from nitrous acid products of heparin compositional information consistent with the predominant structure iduronic acid→*N*-acetylglucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 3,6-di-*O*-sulfate→iduronic acid 2-*O*-sulfate→N-sulfated glucosamine 6-*O*-sulfate→iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate. Our treatment of the octasaccharide pool with high-pH and low-pH nitrous acid produced the correct proportions of tetrasaccharides and disaccharides to account for the two N-sulfated glucosamine residues and one *N*-acetylglucosamine residue in the above oligosaccharide. Furthermore, we have separated and analyzed these tetrasaccharide and disaccharide products by a combination of HPLC ion-exchange chromatography as well as exoglycosidase digestion. The results obtained for the sequences of the major tetrasaccharide and disaccharide components are consistent with the above structure. This structure is also in agreement with previous studies (Rosenberg & Lam, 1979; Lindahl et al., 1979, 1980; Casu et al., 1981).

The exhaustive digestion of the active octasaccharide pool with *Flavobacterium* heparinase demonstrated that about 40% of the oligosaccharide would not degrade. This heparinase-resistant fraction had a 3-fold lower anti-factor Xa activity than the original active octasaccharide pool as measured by using the chromogenic substrate S-2222. This demonstrated that the active octasaccharide pool contained two major components: a highly active fraction that is sensitive to heparinase degradation and a resistant fraction with lower activity. We separated these two major components of the active octasaccharide pool by an additional affinity fractionation step at an antithrombin concentration of 10 μM rather than the 450 μM previously employed with the initial material. These conditions have been shown to be selective for binding the higher affinity octasaccharide S (Atha et al., 1984).

HPLC analysis of the nitrous acid degradation products of octasaccharides S and R demonstrated that both oligosaccharides contain the predominant tetrasaccharide E structure iduronic acid→*N*-acetylglucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 3,6-di-*O*-sulfate as well as the trisulfated and disulfated disaccharides iduronic acid 2-*O*-sulfate→N-sulfated glucosamine 6-*O*-sulfate and iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate. Nitrous acid degradation of octasaccharides S and R after treatment with hydrazine or α-iduronidase yielded products which revealed that the two octasaccharides differ in sequence. The unique tetrasaccharide sequence is centrally positioned in octasaccharide R whereas in octasaccharide S it is located on the nonreducing end of the molecule. The results of nitrous acid treatment of the reducing end labeled octasaccharides S and R showed that there can only be a small amount (<10%) of either octasaccharide in which the unique tetrasaccharide is located on the reducing end.

There is about a 30% decrease in the level of N-sulfated glucosamine residues in the octasaccharide R preparation, compared to the octasaccharide S preparation. This is consistent with the increased amount of hexasaccharide produced by low-pH nitrous acid degradation of octasaccharide R. About 85% of these non-N-sulfated glucosamines are on the reducing end of the unique tetrasaccharide E sequence. The presence of this hexasaccharide in such large amounts in octasaccharide R and its absence in octasaccharide S indicate that the lack of an *N*-sulfate group at this position results in a somewhat lowered affinity for antithrombin. This is in agreement with Riesenfeld et al. (1981), who have observed decreases in the affinity of N-desulfated octasaccharides using affinity chromatography. Our fluorescence binding measurements have been conducted in a range of antithrombin concentrations which is not sufficiently high to accurately measure this low-affinity material. The octasaccharide used in the equilibrium dialysis measurements has been N-resulfated with ^{35}S and contains about 12% *N*-acetylglucosamine on the reducing end of the unique tetrasaccharide. This could explain the slight deviation at the upper end of the equilibrium dialysis data obtained with octasaccharide R which indicates the presence of a small amount of lower affinity material.

Structural variations in the tetrasaccharide E sequence were also apparent within the tetrasaccharide pool derived from nitrous acid degradation of the two octasaccharides. Tetrasaccharide B represents about 16% of the total tetrasaccharide pool and was found to contain 3-O-sulfated N-sulfated glucosamine in place of 3,6-di-O-sulfated N-sulfated glucosamine found within tetrasaccharide E. Given that 33% of the glucosamine residues of the octasaccharide lie within the tetrasaccharide sequence, 16% of these moieties would correspond to a lack of 6-*O*-sulfation of 5–6% of the total glucosamine residues of the oligosaccharide. This is consistent with the NMR measurements obtained with the octasaccharide pool in which approximately 7% of the glucosamine residues were observed to be non-6-O-sulfated. In addition, tetrasaccharide D, which is about 10% of the total tetrasaccharide pool, contains a glucuronic acid in place of iduronic acid found in tetrasaccharide E.

The presence of such a high concentration of oligosaccharides which contain tetrasaccharide B in the active octasaccharide pool indicates that this substitution has little or no effect on the binding of these species to antithrombin. Studies by Lindahl et al. (1980, 1983) provide indirect evidence for the presence of high concentrations of this structural variant in their preparations of antithrombin binding octasaccharides. On this basis, they claim that the variant 6-*O*-sulfate group is functionally unimportant. This supposition is supported in our studies by the fact that the intrinsic fluorescence and equilibrium dialysis data of both octasaccharide S and octasaccharide R can be fit with single dissociation constants. A computer analysis revealed that the presence of a contaminating octasaccharide at a level of 16% of the bulk species with a greater than 10-fold lower affinity for antithrombin would produce measurable distortions in these binding curves. A similar analysis of the binding affinity of the octasaccharide which contains tetrasaccharide D is more difficult since these oligosaccharides are present at a level of only 10–12% of the bulk species. The perturbations of the binding isotherms would be apparent only if the octasaccharide which contains tetrasaccharide D exhibited a 100-fold or greater reduction in affinity for antithrombin. However, the fact that the molar proportions of tetrasaccharides E, D, and B are nearly the same in octasaccharides S and R further supports the conclusion that both these substitutions have little or no effect on the antithrombin binding affinity of the various oligosaccharides.

The separation of the active octasaccharide fraction into the two major components as described above has allowed us to directly evaluate the importance of the position of the unique tetrasaccharide. If we assume that the unique tetrasaccharide sequence of octasaccharides S and R binds at the same position

in the heparin binding site on antithrombin, shown as dashed lines, the two octasaccharides will be aligned with respect to this binding site as shown in Chart I. In comparison to octasaccharide S, octasaccharide R lacks an N-sulfated glucosamine at position 6 as well as possible sites of interaction at positions 7 and 8. This could contribute to its 8–12-fold lower affinity. However, we would expect that the hexasaccharide on the reducing end of octasaccharide R would bind as well as octasaccharide R. In fact, our inability to affinity fractionate this hexasaccharide at an antithrombin concentration of 4.5×10^{-4} M indicates that this hexasaccharide must have a dissociation constant greater than 10^{-4} M. This is a reasonable upper limit for the affinity of the hexasaccharide, considering that the non-reducing-end tetrasaccharide from *Flavobacterium* heparinase digested octasaccharide S, consisting of residues 1–4, has a measured binding constant of approximately 10^{-4} M (Atha et al., 1984). Since octasaccharide R binds at about 10^{-6} M, a 100-fold higher affinity, the iduronic acid 2-*O*-sulfate and N-sulfated glucosamine residues on the nonreducing end of octasaccharide R must contribute approximately 3 kcal/mol additional binding energy. These additional sites of interaction would require that the binding site on antithrombin, as diagrammed above, be extended two residues to the left.

An alternative explanation for the observation that the binding affinity of octasaccharide R is reduced is that the tetrasaccharide sequence previously described is not aligned in the antithrombin binding site in the same way that it is for octasaccharide S. Instead, octasaccharide R could be shifted two residues to the right in order to completely fill the antithrombin binding site. However, the fact that only the last two residues on the reducing end of octasaccharide R would be identically positioned relative to octasaccharide S makes this alternative unlikely. Other alternatives in which the binding orientation of octasaccharide R is inverted relative to that of octasaccharide S seem even less likely in this respect.

Equilibrium dialysis has been used by Atha et al. (1984) to determine the importance of various residues of octasaccharide S in the binding of this oligosaccharide to antithrombin. In these studies, the binding energy of octasaccharide S was compared to those for fragments ranging from hepta- to tetrasaccharide which were produced by enzymatic degradation of the original octasaccharide. These measurements show that the two nonsulfated uronic acid residues and the N-sulfated glucosamine 3,6-di-*O*-sulfate in the unique tetrasaccharide do not contribute to the binding energy of the octasaccharide. However, the contribution of the glucuronic acid residue was determined by using the hexasaccharide which lacks the critical *N*-acetylglucosamine 6-*O*-sulfate. In an intact octasaccharide, the two nonsulfated residues may still be required to permit the critical *N*-acetylglucosamine 6-*O*-sulfate to interact with a specific region on the antithrombin molecule. In a similar manner, the contribution of the N-sulfated glucosamine 3,6-di-*O*-sulfate of the unique tetrasaccharide sequence was determined on the basis of a comparison of the binding energies of the non-reducing-end tetra- and trisaccharides. In an intact octasaccharide, the binding of this N-sulfated glucosamine 3,6-di-*O*-sulfate may be linked to the critical *N*-acetylglucosamine 6-*O*-sulfate (Atha et al., 1984). The present observation that the substitution of the N-sulfated glucosamine 3,6-di-*O*-sulfate by N-sulfated glucosamine 3-*O*-sulfate in octasaccharides S and R has little or no effect on the affinity for antithrombin indicates that the 6-*O*-sulfate group of this residue could not participate in such an effect to any significant extent.

The fact that the increase in intrinsic fluorescence and the rate of inactivation of factor Xa by antithrombin are identical in saturating concentrations of octasaccharides S and R suggests that the antithrombin conformation in the region of the factor Xa binding site is similar in the presence of either octasaccharide. In contrast, circular dichroism measurements of Stone et al. (1982) provide evidence that multiple conformations of antithrombin may occur upon binding of heparin fragments of varying size. These measurements of heparin–antithrombin complexes have shown two major types of chiral absorption spectra. One pattern is produced with heparin fragments ranging from octasaccharide to tetradecasaccharide. The other pattern is produced by fragments ranging from octadecasaccharide to high molecular weight heparin (M_r 22000). These patterns are most likely a reflection of different conformational states of the antithrombin. The two ranges in heparin size observed for the circular dichroism spectra correspond exactly with the range in heparin size which will or will not accelerate the neutralization of thrombin by antithrombin (Oosta et al., 1981). This suggests that the two major regions of the mucopolysaccharide interact with separate areas of the antithrombin and probably induce different conformations of the protease inhibitor. Our measurements of the rate of inactivation of factor Xa by antithrombin complexed with octasaccharides S and R indicate that multiple conformations in antithrombin do not occur within the factor Xa binding site when the protease inhibitor is complexed to octasaccharides of differing sequence and affinity.

The use of the stopped-flow technique has enabled us to conveniently monitor the inhibition of factor Xa by antithrombin which is completely complexed to octasaccharide. Since these measurements were made at saturating concentrations of oligosaccharide, it is unnecesary to take into account differences in the binding affinities of these octasaccharides for antithrombin. The chromogenic assay S-2222, which must be used at lower concentrations of mucopolysaccharide, resulted in an activity for octasaccharide R which was one-third that of the active octasaccharide pool. Under these conditions, the antithrombin is complexed to differing extents with the oligosaccharides, depending on the particular affinity of the octasaccharide being tested.

Given that our measurements using the stopped-flow technique do not reflect changes in the affinity of the mucopolysaccharide for antithrombin, we can use this method to directly probe the factor Xa activation site on the protease inhibitor. We believe that quantitation of the rate of factor Xa inhibition by antithrombin complexed to heparin fragments of differing structure and varying avidity for the protease inhibitor should allow us to determine which groups on the mucopolysaccharide are necessary for acceleration of factor Xa inhibition. Indeed, it should be obvious from the present study that monosaccharide residues which play an important role in regulating the affinity of the oligosaccharide for antithrombin may be of little importance in activating the protease inhibitor with respect to the neutralization of factor Xa. In a similar manner, we also hope to examine the thrombin activation site of antithrombin using heparin fragments which are large enough to enhance the neutralization of this enzyme by the protease inhibitor.

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