Contribution of Monosaccharide Residues in Heparin Binding to Antithrombin III†

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ABSTRACT: The importance of 3-O- and 6-O-sulfated glucosamine residues within the heparin octasaccharide iduronic acid$^{(1)}$→*N*-acetylglucosamine 6-*O*-sulfate$^{(2)}$→glucuronic acid$^{(3)}$→N-sulfated glucosamine 3,6-di-*O*-sulfate$^{(4)}$→iduronic acid 2-*O*-sulfate$^{(5)}$→N-sulfated glucosamine 6-*O*-sulfate$^{(6)}$→iduronic acid 2-*O*-sulfate$^{(7)}$→anhydromannitol 6-*O*-sulfate$^{(8)}$ was determined by comparing with synthetic tetra- and pentasaccharides its ability to bind human antithrombin. The octasaccharide had an affinity for antithrombin of 1×10^{-8} M (10.2 kcal/mol) measured by intrinsic fluorescence enhancement at 6 °C. The synthetic pentasaccharide, consisting of residues 2–6, had an affinity of 3×10^{-8} M (9.6 kcal/mol). The same pentasaccharide, except lacking the 3-*O*-sulfate on residue 4, had an affinity of 5×10^{-4} M (4.5 kcal/mol) measured by equilibrium dialysis. The tetrasaccharide, consisting of residues 2–5, bound antithrombin with an affinity of 5×10^{-6} M (6.8 kcal/mol). The tetrasaccharide, consisting of residues 3–6, had an affinity of 5×10^{-5} M (5.5 kcal/mol). Since the loss of either the 6-O-sulfated residue 2 or the 3-*O*-sulfate of residue 4 results in a 4–5 kcal/mol or a 40–50% loss in binding energy of the pentasaccharide, these two residues must be the major contributors to the binding and must be linked to the biologic activity of the octasaccharide.

Heparin is an anticoagulant that acts by binding to antithrombin and accelerating the rate of which this protease inhibitor neutralizes proteolytic enzymes of the hemostatic mechanism (Rosenberg, 1977). Only a small fraction of all heparin preparations exhibit high avidity for antithrombin and are responsible for essentially all of the anticoagulant properties of the polysaccharide (Lam et al., 1976). This subpopulation of anticoagulantly active heparin was found to possess an antithrombin binding domain with the unique tetrasaccharide sequence iduronic acid→*N*-acetylglucosamine 6-*O*-sulfate→glucuronic acid→N-sulfated glucosamine 6-*O*-sulfate (Rosenberg et al., 1978; Rosenberg & Lam, 1979; Lindahl et al., 1979). Subsequently, Leder (1980) isolated a sulfatase that specifically removes 3-*O*-sulfate groups from nonreducing end glucosamine residues of heparin and postulated that this unique substituent might be present within the antithrombin binding domain of heparin. Data provided by Lindahl et al. (1980) and Casu et al. (1981) have confirmed this supposition and placed the 3-*O*-sulfate on the glucosamine moiety at the reducing end of the unique tetrasaccharide sequence. Octasaccharide fragments that contain the tetrasaccharide binding region have also been isolated from deaminative as well as enzymatic cleavage products of heparin. These oligosaccharides exhibit a high affinity for antithrombin as well as the capacity to accelerate the neutralization of factor Xa (Choay et al., 1980; Casu et al., 1981; Oosta et al., 1981; Riesenfeld et al., 1981; Atha et al., 1984b). Studies of Choay et al. (1981) suggested that the major features of the antithrombin binding site of heparin are contained within a pentasaccharide sequence. This hypothesis was confirmed by the synthesis of a pentasaccharide that exhibited a significant affinity for antithrombin as well as the ability to accelerate factor Xa inhibition (Choay et al., 1983a).

Despite progress in elucidating the overall structure of this major binding domain of heparin, relatively little is known about the quantitative contribution of individual monosaccharide units within the octasaccharide sequence to interactions with antithrombin and the subsequent acceleration of factor Xa–protease inhibitor complex formation. Studies performed by Riesenfeld et al. (1981) and Thunberg et al. (1982) have provided evidence for an important role of certain sulfate groups. Moreover, Choay et al. (1983b) were able to produce a synthetic pentasaccharide devoid of the unique 3-*O*-sulfate that exhibited a reduced ability to bind to the protease inhibitor. In a previous study, Atha et al. (1984a) have determined the binding energies of heparin fragments produced by enzymatic and nitrous degradation with regard to their interaction with antithrombin. This investigation led to an indirect evaluation of the contribution of the unique 3-*O*-sulfate and the suggestion that the role of this substituent with respect to antithrombin binding must be linked to the 6-*O*-sulfate group on the N-acetylated glucosamine residue. In this study, we have used synthetically produced pentasaccharides in which the 3-*O*-sulfate group is present or absent in order to directly determine the contribution of this moiety to the binding energy of the heparin octasaccharide. In addition, we have examined synthetic tetrasaccharides in which the 6-O-sulfated glucosamine is present or absent so that we might establish the effect of this residue on antithrombin binding. The data obtained allow us to assign quantitative values to the various residues within the octasaccharide se-

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quence with respect to the binding to antithrombin.

Materials and Methods

Preparation of Octasaccharide. Porcine mucosal heparin (7.5 g of batch no. 41681 at 169 USP units/mg, Diosynth Inc., Chicago) 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 cold 80% (v/v) 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 cm) in 0.5 M ammonium bicarbonate. The octasaccharide fraction was selected from well-resolved peaks ranging from disaccharide to dodecasaccharide as monitored by absorbance at 254 nm and 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 octasaccharide fractions 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 absorbance at 280 nm and 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 and 3 M NaCl, pH 7.5, and loaded on identical columns equilibrated in the high-salt buffer. The octasaccharide was desalted on 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. The predominant active octasaccharide S was further purified of any lower affinity forms by an additional affinity fractionation step as described (Atha et al., 1984b). The structure was confirmed by a combination of methods including high-resolution proton and carbon-13 NMR as described (Atha et al., 1984b).

Preparation of Synthetic Tetra- and Pentasaccharides. Synthetic tetra- and pentasaccharides were prepared from D-glucose and D-glucosamine by a chemical synthesis that permits the sulfation of hydroxyl groups as required (Sinäy et al., 1984). The structures were confirmed by comparison of the high-resolution proton NMR spectra with those of a number of synthetic model compounds (Choay et al., 1983a; Sinäy et al., 1984) and by spin decoupling (Torri et al., 1985). The extent of O-sulfation was determined by the downfield shift of 0.6–1 ppm for adjacent protons of the glucosamine residues. The specific 3-O-sulfation of the central glucosamine of the pentasaccharides was measured by a downfield shift in H-3 resonance from 3.70 to 4.37 ppm (Torri et al., 1985). Integration of the anomeric signals in the different tetra- and pentasaccharides yielded an equimolar amount of all of the monosaccharide residues and demonstrated that the synthetic oligosaccharides are >90% pure. In addition, the homogeneity of the oligosaccharides was confirmed by high-pressure ion-exchange chromatography as described below.

Measurement of Protein or Mucopolysaccharide Concentration. Antithrombin concentrations were determined by absorbance measurements at 280 nm with an absorptivity value $A^{1\%,1cm}$ of 6.5 (Nordenman et al., 1977). Mucopolysaccharide concentrations were measured colorimetrically by assay of uronic acid and were found to agree with oligosaccharide concentrations based on dry weight (Bitter & Muir, 1962).

Proteins. Human and bovine antithrombin were prepared in homogeneous form by chromatography on heparin–Sepharose and DEAE-cellulose (Damus & Rosenberg, 1976; Jordan et al., 1982).

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 a gradient of potassium phosphate, pH 4.3, from 40 mM to 1 M.

Fluorescence Measurements. Fluorescence measurements were made with a Perkin-Elmer MP-44A spectrofluorometer equipped with a thermostated sample compartment and a differential corrected spectra accessory. Heparin oligosaccharide at a concentration of 20 μM–10 mM was added by using a 10-μL syringe to 2.75 mL of human antithrombin at a concentration of 10–100 nM in 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.5, and 0.15 M NaCl. The solution was carefully stirred with the syringe needle after each addition. The binding of the oligosaccharide to antithrombin was monitored by measurement of the tryptophan fluorescence F relative to the fluorescence determined in the absence of mucopolysaccharide F_0. 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 oligosaccharides to human antithrombin was measured by using 1-mL equilibrium dialysis cells (Technilab Instruments Inc., NJ) and Spectrapor No. 2 (M_r cutoff 12000–14000) membrane (Spectrum Medical Industries, Los Angeles). A solution of antithrombin (75–380 μM) and oligosaccharide (60–80 μM) 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 per second. Samples (0.2 mL) were withdrawn from both cell compartments and assayed for mucopolysaccharide concentration as described above except that protein-containing solutions were first precipitated with 10% trichloroacetic acid. To ensure that the solutions had reached equilibrium, samples were taken at time intervals from 1 to 14 days and compared to samples in which the oligosaccharide had been added to the non-protein-containing compartment. After constant readings were established, the ratio of bound to total oligosaccharide (HAT/H) was calculated from the difference in concentration between the protein-containing and non-protein-containing compartments divided by the concentration of oligosaccharide in the protein-containing compartment. This ratio (HAT/H) was determined in separate cells at different protein concentrations. The dissociation constants for the mucopolysaccharide–protein complexes were calculated by nonlinear

Table I: Equilibrium Dialysis of Heparin Oligosaccharides

saccharide[a]	residue[b]	protein, compt[c] 2 (μM)	oligosaccharide[d] compt 1 (μM)	oligosaccharide[d] compt 2 (μM)	HAT/H[e] expt	HAT/H[e] model	K_{diss} (M)
pentasaccharide	2–6	75	8 (8)	54 (54)	0.86 (0.86)	0.9	10^{-6}–10^{-7}
pentasaccharide (no 3-*O*-SO_3)	2–6	75	31 (28)	34 (37)	0.1 (0.2)	0.12	5×10^{-4}
		380	21 (26)	40 (40)	0.37 (0.35)	0.42	
		730	12 (10)	53 (56)	0.77 (0.80)	0.58	
tetrasaccharide	2–5	75	27 (26)	51 (52)	0.47 (0.50)	0.50	$\sim 10^{-5}$
tetrasaccharide	3–6	75	33 (34)	44 (44)	0.25 (0.24)	0.37	1×10^{-4}
		380	12 (12)	66 (66)	0.82 (0.82)	0.77	

[a] Structures are given in Table II. [b] Residue 2 of tetra- and pentasaccharides is N-sulfated glucosamine 6-*O*-sulfate. [c] compt = compartment. [d] Compartment 2 contains antithrombin at the indicated protein concentration. Oligosaccharide concentrations were determined as described by using the average of at least three carbohydrate determinations (see Materials and Methods). Values in parenthesis were obtained from identical experiments in which the oligosaccharide was initially added to compartment 1 instead of compartment 2 of the dialysis cell. [e] HAT/H is the fraction of total oligosaccharide in compartment 2 that is complexed to antithrombin. Data were analyzed by a single-site binding model to yield the dissociation equilibrium constant, K_{diss} (see Materials and Methods).

Table II: Binding of Heparin Oligosaccharides to Antithrombin

saccharide[a]	T (°C)	ΔF_{max} (%)	K_{diss} (M)	$\Delta G°$[b] (kcal/mol)
8S: ID→GLNAc-6-*O*-SO_3→GLU→GLNS-3,6-*O*-$(SO_3)_2$→ID-2-*O*-SO_3→GLNS-6-*O*-SO_3→ ID-2-*O*-SO_3→AMN-6-*O*-SO_3	37	48	2.3×10^{-8}	10.8
	6	35	1.0×10^{-8}	10.2
5: GLNS-6-*O*-SO_3→GLU→GLNS-3,6-*O*-$(SO_3)_2$→ID-2-*O*-SO_3→GLNS-6-*O*-SO_3	37	44	1.0×10^{-7}	9.9
	6	33	3.0×10^{-8}	9.6
5:[c] GLNS-6-*O*-SO_3→GLU→GLNS-6-*O*-SO_3→ID-2-*O*-SO_3→GLNS-6-*O*-SO_3	6		5×10^{-4}	4.5
4: GLNS-6-*O*-SO_3→GLU→GLNS-3,6-*O*-$(SO_3)_2$→ID-2-*O*-SO_3	37	35	1.7×10^{-5}	6.8
	6	15	4.6×10^{-6}	6.8
4: GLU→GLNS-3,6-*O*-$(SO_3)_2$→ID-2-*O*-SO_3→GLNS-6-*O*-SO_3	37	33	9.8×10^{-5}	5.7
	6	16	5.1×10^{-5}	5.5

[a] ID = iduronic acid; GLU = glucuronic acid; GLNS = N-sulfated glucosamine; GLNAc = *N*-acetylglucosamine; AMN = anhydromannitol. [b] $\Delta G°$ = standard-state free energy change based on 1 M concentrations of reactants. [c] Binding affinity of non-3-*O*-sulfated pentasaccharide was determined by using equilibrium dialysis (see Table I).

least-squares fit of the equilibrium dialysis data to a single-site binding model as described above for fluorescence data.

Results and Discussion

We have compared the ability of a number of heparin oligosaccharides to bind to antithrombin. To accomplish this, we have quantitated the avidity of these oligosaccharides with intrinsic fluorescence measurements as well as equilibrium dialysis. This examination has permitted us to ascertain the relative contributions of individual monosaccharide residues within the antithrombin binding domain of the polysaccharide.

Measurements of the Binding of Heparin Oligosaccharides to Antithrombin. The interaction of heparin with antithrombin is accompanied by about a 30–40% enhancement in intrinsic fluorescence. This spectral signal can be utilized to determine the avidity of heparin oligosaccharides for protease inhibitor provided that these species produce a measurable change in the fluorescence of the protein and exhibit binding constants in the range of 1 nM–100 μM. Heparin oligosaccharides ranging from tetrasaccharide to octasaccharide, with the exception of the pentasaccharide lacking the 3-*O*-sulfate group on residue 4, satisfy these conditions at temperatures as high as 37 °C and as low as 6 °C. Figure 1 shows that the data obtained for the natural octasaccharide S and three synthetic oligosaccharides could be closely fit to a single-site binding model (see Materials and Methods). The affinity of the synthetic pentasaccharide lacking the 3-*O*-sulfate group on residue 4 was too low to induce a fluorescence change in antithrombin, and its binding constant was determined by equilibrium dialysis. Under conditions of high protein and polysaccharide concentrations and low temperature where the extent of complex formation is increased, this pentasaccharide exhibited a measurable interaction. The binding constant for this oligosaccharide is given in Table I. The avidity of the pentasaccharide with the 3-*O*-sulfate group on residue 4 was also examined by equilibrium dialysis and was shown to be 1000-fold higher than that of the pentasaccharide lacking the 3-*O*-sulfate. The binding constant of this species is also given in Table I, but the error in this measurement is high since it approaches the limit at which dissociation constants of unlabeled oligosaccharides can be measured by this technique. The avidity of the natural octasaccharide S for antithrombin, which is even higher than that of the synthetic pentasaccharide with the 3-*O*-sulfate, could not be quantitated under these conditions. The avidities of synthetic tetrasaccharides for protease inhibitor were also determined by equilibrium dialysis and are listed in Table I. The data show that these species bind to antithrombin with about a 100–1000-fold lower affinity than the pentasaccharide containing the 3-*O*-sulfate group at residue 4 and that the synthetic tetrasaccharide that contains the 6-O-sulfated glucosamine residue interacts with protease inhibitor with about a 10-fold higher affinity than the tetrasaccharide that lacks this residue.

The dissociation constants obtained by fitting the fluorescence data to a single-site model and the maximum fluorescence change ΔF_{max} obtained at saturating concentrations of mucopolysaccharide are given in Table II. The data provided also include the dissociation constant obtained by equilibrium dialysis for the pentasaccharide lacking the 3-*O*-sulfate group at residue 4. The binding affinities range from 10 nM for the natural octasaccharide S to 0.5 mM for the synthetic pentasaccharide lacking the 3-*O*-sulfate group at residue 4. This is a range in binding constants of almost 10^5 and approaches the limits at which the intrinsic fluorescence and equilibrium dialysis methods are useful. The binding constants obtained by fluorescence are more accurate than those determined by equilibrium dialysis and were employed to calculate values for the Gibbs free energy listed in Table II. The intrinsic fluorescence change ΔF_{max} ranges from 48% for the natural octasaccharide S at 37 °C to 15–16% for the synthetic tet-

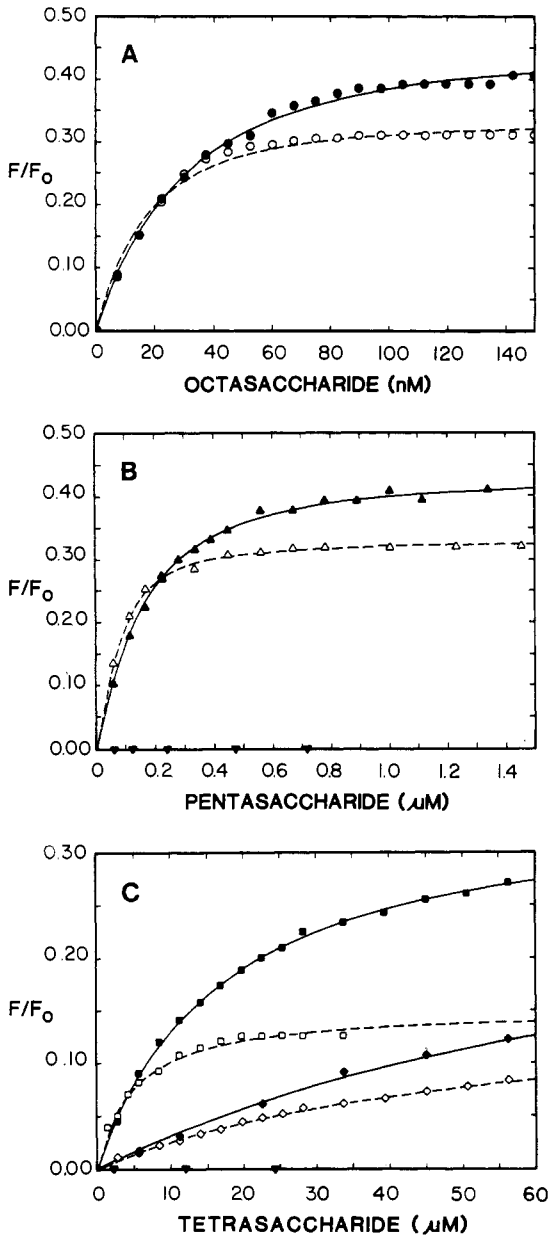

FIGURE 1: Comparison of oligosaccharide binding to antithrombin. The intrinsic fluorescence enhancement of human antithrombin was measured in the presence of natural octasaccharide S, residues 1–8 (●, ○), synthetic pentasaccharide, residues 2–6 (▲, △), synthetic pentasaccharide, residues 2–6, no 3-*O*-sulfate (▼), synthetic tetrasaccharide, residues 2–5 (■, □), and synthetic tetrasaccharide, residues 3–6 (◆, ◇). Measurements were made at 37 (solid symbols) and 6 °C (open symbols) with an antithrombin concentration of 100 nM except for the octasaccharide, which was measured at 10 nM. The solid and dashed lines represent least-squares fits of the data to a single-site binding model (see Materials and Methods). The resulting dissociation constants K_{diss} and values of maximum fluorescence at saturation ΔF_{max} are given in Table II.

rasaccharides at 6 °C. This result indicates that significant differences in the conformation of antithrombin exist when the protease inhibitor is complexed with heparin fragments ranging from octasaccharide to tetrasaccharide.

Evaluation of the Functional Contribution of Individual Residues of the Octasaccharide Binding Domain of Heparin. The binding energies obtained for synthetic oligosaccharides in this study as well as those observed for deaminative and enzyme cleavage fragments of the natural octasaccharide S in a previous investigation (Atha et al., 1984a) are summarized in Figure 2. The solid lines represent the various heparin oligosaccharides that have been examined for binding to antithrombin. The numbers under these lines are binding energies in kilocalories per mole that have been directly determined for these fragments. The numbers under the dotted lines have been calculated from differences in the binding energies of various oligosaccharides. On the basis of these data, the relative contributions of individual residues within the octasaccharide S have been estimated and are provided at the bottom of the figure.

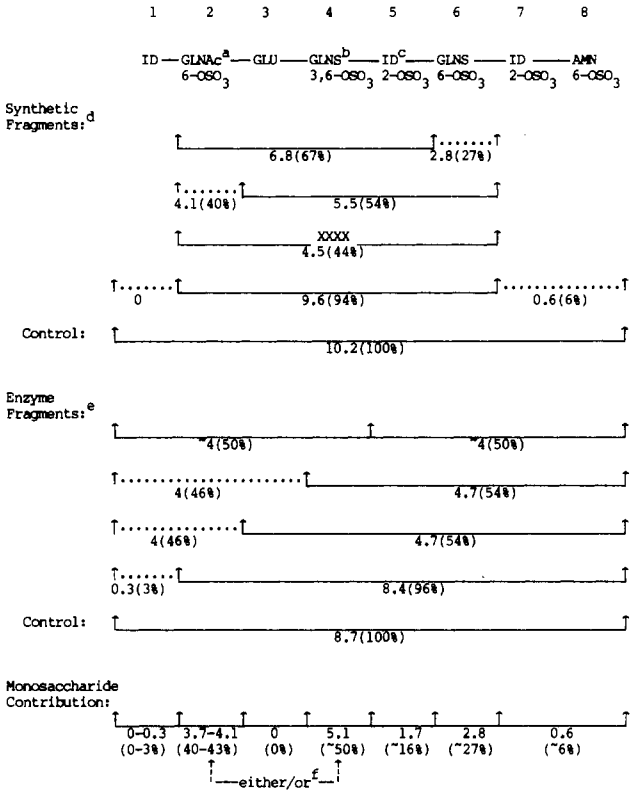

FIGURE 2: Contribution of individual residues within the antithrombin binding octasaccharide. Abbreviations: ID, iduronic acid; GLU, glucuronic acid; GLNAc, *N*-acetylglucosamine; GLNS, N-sulfated glucosamine; AMN, anhydromannitol. The numbers below solid brackets are the directly determined binding energies for the fragments in kilocalories per mole. The numbers below the dotted brackets have been calculated from the differences in the binding energies of the various fragments and are used to compute the relative contributions of the monosaccharides as given below. The numbers in parentheses are the percentages of the total binding energy of octasaccharide S (see Results and Discussion). Footnotes a–f designate the following: (a) Residue 2 of tetra- and pentasaccharides is N-sulfated glucosamine 6-*O*-sulfate in the synthetic tetra- and pentasaccharides. (b) Residue 4 is N-sulfated glucosamine 6-*O*-sulfate in the non-3-O-sulfated synthetic pentasaccharide as indicated by XXXX. (c) Residue 5 is unsaturated in the reducing end tetrasaccharide produced by using heparinase from *Flavobacterium heparinum*. (d) The binding energies of the synthetic tetra- and pentasaccharides were taken from Table II in this study. (e) The binding energies of oligosaccharides produced from enzymatic degradation of the octasaccharide were taken from Table I of Atha et al. (1984a). (f) The relative contributions of residues 2 and 4 can be linked to the conformational change in antithrombin such that the loss of either results in essentially a complete loss of binding energy expected for the nonreducing end tetrasaccharide (see Results and Discussion).

The contribution of the nonreducing end iduronic acid, residue 1, to the binding energy of the octasaccharide is minimal. This conclusion is based on a prior investigation in which the removal of this residue from the octasaccharide by treatment with α-iduronidase did not change the binding energy by more than 0.3 kcal/mol (Atha et al., 1984a). Previous studies by Choay et al. (1981) and Lindahl et al. (1983) have showed that the elimination of this moiety occurred with no qualitative effect on the binding of octasaccharide to anti-

thrombin. However, it should be noted that sulfation of this residue never occurs within the natural oligosaccharide and that nonsulfated glucuronic acid is occasionally found at this position (Atha et al., 1984b).

The contribution of the 6-O-sulfated *N*-acetylglucosamine, residue 2, to the binding energy of the octasaccharide is 4.1 kcal/mol. This estimate is reached by comparing the avidities of synthetic tetrasaccharide, residues 3–6, and synthetic pentasaccharide, residues 2–6, for antithrombin. The latter value agrees with previous measurements in which a loss of 3.7 kcal/mol in binding energy occurred when the 6-*O*-sulfate group was removed from residue 2 by treatment of a heptasaccharide with *N*-acetylglucosamine sulfatase (Atha et al. 1984a). This result also indicates that the contribution of this residue is mainly due to the 6-*O*-sulfate group. Previous studies by Lindahl et al. (1983) with natural oligosaccharides and Choay et al. (1983a) with the synthetic tetrasaccharide, residues 3–6, have shown that the absence of residue 2 is associated with a significant qualitative reduction in the binding of the oligosaccharide to antithrombin. The fact that residue 2 is N-acetylated in the natural oligosaccharides but N-sulfated in the synthetic pentasaccharides and tetrasaccharides should not alter our estimates of binding energy. Prior investigations of Lindahl et al. (1983) have shown that this variation has essentially no effect on the interaction of oligosaccharides with protease inhibitor.

The contribution of the glucuronic acid, residue 3, to the binding energy of the octasaccharide S is essentially zero since enzyme-produced pentasaccharide, residues 4–8, and hexasaccharide, residues 3–8, do not differ in their avidity for the protease inhibitor (Atha et al., 1984a). However, it should be noted that sulfation of this residue never occurs within the natural octasaccharide (Atha et al., 1984b).

The contribution of the 3-*O*-sulfate group of the N-sulfated, 6-O-sulfated glucosamine, residue 4, to the binding energy of the octasaccharide is 5.1 kcal/mol. This conclusion is reached by comparing synthetic pentasaccharides with and without the above functional group. A previous investigation by Atha et al. (1984a) led to an indirect estimate of the importance of the 3-*O*-sulfate group of residue 4 based on a comparison of the binding of the nonreducing end tetrasaccharide and the reducing end pentasaccharide to antithrombin. This evaluation did not indicate that residue 4 would contribute in a major way to the above interaction. However, Atha et al. (1984a) suggested that the 3-*O*-sulfate group of residue 4 could be functionally linked to the 6-*O*-sulfate group of residue 2 such that both of these moieties would be required for the binding of octasaccharide to the protease inhibitor. Data summarized in Figure 2 support this hypothesis by revealing that the loss of either the 6-*O*-sulfate group of residue 2 or the 3-*O*-sulfate group of residue 4 leads to the same 4–5 kcal/mol loss in binding energy (see below). We should emphasize that the lack of an apparent contribution by residues 1 and 3 to the interaction of the octasaccharide S with antithrombin does not indicate that these moieties are without importance. It is likely that these two nonsulfated uronic acid units function as critical spacers in orientating the 6-*O*-sulfate group of residue 2 and the 3-*O*-sulfate group of residue 4 and that sulfation of either residue would interfere with the binding of this region of the oligosaccharide to antithrombin.

The contribution of the 2-O-sulfated iduronic acid, residue 5, to the binding of octasaccharide S to antithrombin is about 1.7 kcal/mol. This estimate is generated by comparing synthetic tetrasaccharide, residues 2–5, which exhibits 6.8/10.2 or 67% of the binding energy of the octasaccharide, with the nonreducing end tetrasaccharide, residues 1–4, obtained from *Flavobacterium* heparinase degradation, which possesses about 50% of the binding energy of the octasaccharide. These observations are consistent with the fluorescence binding measurements of Lindahl et al. (1984) utilizing the nonreducing end hexasaccharide, residues 1–6, obtained from nitrous acid degradation of affinity-fractionated octasaccharide. This hexasaccharide, which contains an anhydromannitol on its reducing end, binds to the protease inhibitor with a dissociation constant of 2×10^{-5} M compared to a value of 1.7×10^{-5} M for the synthetic tetrasaccharide, residues 2–5.

The contribution of the N-sulfated glucosamine 6-*O*-sulfate, residue 6, to the binding of octasaccharide S to antithrombin is approximately 2.8 kcal/mol. This value is obtained by comparing synthetic tetrasaccharide, residues 2–5, which exhibits a binding energy of 6.8 kcal/mol, to synthetic pentasaccharide, residues 2–6, which possesses a binding energy of 9.6 kcal/mol. The fact that the nonreducing end hexasaccharide with a 6-O-sulfated anhydromannitol binds with the same affinity as the synthetic tetrasaccharide, residues 2–5, indicates that the *N*-sulfate group but not the 6-*O*-sulfate group of residue 6 is significantly involved in the interaction of the octasaccharide with antithrombin.

The contributions of residues 5 (1.7 kcal/mol) and 6 (2.8 kcal/mol) together with those of residues 7 and 8 (0.6 kcal/mol) to the binding of octasaccharide S to antithrombin is approximately 5.1 kcal/mol out of a total of 10.2 kcal/mol or 47% of the binding energy of the octasaccharide. This summed contribution is in excellent agreement with that estimated for the reducing end tetrasaccharide, which exhibited about 4 kcal/mol out of a total of 8.7 kcal/mol or 46% of the binding energy of the octasaccharide (Atha et al., 1984a).

In a previous study, we isolated a natural octasaccharide R that varies in sequence from octassaccharide S in that it contains an iduronic acid 2-*O*-sulfate→N-sulfated glucosamine 6-*O*-sulfate on the nonreducing end (Atha et al., 1984b). The tetrasaccharide sequence, corresponding to residues 1–4 of octasaccharide S, occurs in residues 3–6 of octasaccharide R followed by iduronic acid 2-*O*-sulfate→anhydromannitol 6-*O*-sulfate. This octasaccharide has an 8–12-fold lower binding affinity for antithrombin relative to octasaccharide S. The above findings could be explained, as suggested in this work, by the loss of about 4 kcal/mol in binding energy that would be expected in the absence of positions corresponding to residues 5–8 in octasaccharide S. This loss in avidity would be partially compensated by the additional binding of the first two residues on the nonreducing end of octasaccharide R. The latter set of additional interactions of octasaccharide R with antithrombin would amount to about 3 kcal/mol. This surmise is reasonable considering that the N-sulfated glucosamine of residue 6 has been shown to contribute 2.8 kcal/mol of binding energy within the interaction domain of octasaccharide S.

The contributions of individual residues were evaluated as outlined above by assuming that the free energy of connecting monosaccharides to each other is negligible compared to their free energy of binding to antithrombin. To determine the contributions of individual components A and B of a ligand A–B, the measured free energy of binding of the ligand must be corrected by a connection free energy ΔG^s, which represents the change in the probability of interaction that results from the connection of A and B. This parameter can be estimated by comparing the sum of the free energies of the individual components to that of the entire ligand (Jencks, 1981). The ΔG^s for connecting reducing and nonreducing end tetrasaccharides within the octasaccharide must be relatively small

since the sum of binding energies of the two tetrasaccharides is approximately equal to that of the entire ligand (Atha et al., 1984a). In addition, the ΔG^s for connecting monosaccharides within the reducing end tetrasaccharide must be minimal since the sum of the binding energies of the monosaccharides within the reducing end tetrasaccharide is approximately equal to that of the entire ligand. However, the sum of the binding energies of the monosaccharides within the nonreducing end tetrasaccharide is about twice that of the binding energy of the entire ligand (see Figure 2). As pointed out by Jencks (1981), ΔG^s includes any free energy needed to bring about a conformational change in the protein, the ligand, or both provided that this transition occurs during the interaction of A, B, and A–B with the protein. Thus, it seems likely that the binding of the 6-*O*-sulfate group of residue 2 is linked to the binding of the 3-*O*-sulfate of residue 4 by a conformational change that might take place within the protease inhibitor, the oligosaccharide, or both. Given that a conformational change is known to occur in antithrombin upon binding to heparin and that the oligosaccharide is a relatively rigid structure, it appears reasonable that the above linkage effect must be due to a transition within the protein. Furthermore, it is quite possible that the above alteration in antithrombin might be responsible for the acceleration of factor Xa–protease inhibitor interaction. Therefore, we have monitored conformational changes in antithrombin that are produced by synthetic oligosaccharides in order to determine whether these transitions are triggered by residues 2 and 4 within the nonreducing end tetrasaccharide. This can be accomplished by measuring the intrinsic fluorescence of the protease inhibitor saturated with various types of oligosaccharides (ΔF_{max}). It is believed that these conformational alterations in antithrombin are, in part, related to the mucopolysaccharide-induced transitions of the protease inhibitor that lead to the acceleration of factor Xa neutralization (Olson & Shore, 1981; Olson et al., 1981; Evans et al., 1982).

The synthetic pentasaccharide, residues 2–6, with the 6-*O*-sulfate group of residue 2 and the 3-*O*-sulfate group of residue 4 exhibits a value of ΔF_{max} that is about 93% of that produced by octasaccharide S. The synthetic pentasaccharide, residues 2–6, with the 6-*O*-sulfate group of residue 2 but without the 3-*O*-sulfate group of residue 4 shows no indication of intrinsic fluorescence enhancement despite a level of saturation of 5% which was obtained with all of the available oligosaccharide. It should be quite revealing to determine if any enhancement of fluorescence can be observed at saturating levels of the above synthetic pentasaccharide. One would expect that the maximum increase in this parameter would be that observed for the synthetic tetrasaccharide, residues 3–6, that does not possess the 6-*O*-sulfate group of residue 2 (see below). However, to attain a level of saturation of 80% in these measurements would require a 100-fold increase in concentration of pentasaccharide, which is about 100 times more oligosaccharide than is available at this time. The synthetic tetrasaccharide, residues 3–6, with the 3-*O*-sulfate group of residue 4 but without the 6-*O*-sulfate group of residue 2 and the synthetic tetrasaccharide, residues 2–5, with both of these groups exhibit values of ΔF_{max} that are 30–50% lower than that of octasaccharide S. These results partially support our hypothesis that the binding of the 6-*O*-sulfate group of residue 2 is functionally linked to the binding of the 3-*O*-sulfate group of residue 4 via a conformational change in antithrombin and that the loss of either residue leads to a substantial reduction in the extent of this transition. However, it should also be emphasized that synthetic tetrasaccharide, residues 3–6, induces a ΔF_{max} that is almost identical with that triggered by synthetic tetrasaccharide, residues 2–5. This observation suggests that the oligosaccharide-induced enhancement of antithrombin fluorescence may not constitute a completely accurate probe of the conformational changes of the protease inhibitor that lead to kinetic acceleration of factor Xa inactivation.

On the one hand, the above difficulty with fluorescence measurements could be due to spectral changes in exposed tryptophan residues that are induced by contact with monosaccharide residues that sum with alterations in the positioning of deep tryptophan residues which signal a major conformational transition in antithrombin. Olson et al. (1981) have demonstrated that the fluorescence yield of exposed tryptophan residues is unaffected by the binding of heparin. However, these investigators have also noted that the emission spectrum of these moieties is blue-shifted, which indicates that polysaccharides perturb the environment of these exposed residues. The proportion of the fluorescence enhancement that is caused by changes in the environment of the exposed tryptophan residues may be much larger for the smaller synthetic tetrasaccharide and hence might make the interpretation of ΔF_{max} more complex.

On the other hand, the above difficulty with fluorescence measurements might be caused by auxiliary conformational changes in antithrombin triggered by smaller oligosaccharides that are of minimal importance to the acceleration of factor Xa inactivation. The existence of multiple transitional states in the protease inhibitor is supported by the circular dichroism measurements of Stone et al. (1982). These investigators have observed two major types of chiral absorption spectra that occur when antithrombin is complexed with heparin fragments from octasaccharide to tetradecasaccharide and when protease inhibitor is bound to polysaccharide ranging from octadecasaccharide to polymers with an average M_r of 22 000. Both types of heparin have similar abilities to accelerate factor Xa–antithrombin interactions but differ greatly in their capacity to enhance thrombin–antithrombin interactions. Circular dichroism measurements of Lindahl et al. (1984) have also yielded a difference in the chiral absorption spectra of protease inhibitor complexed with heparin fragments of the two molecular weight ranges described above, as well as by hexasaccharide, residues 1–6, which has a binding constant equivalent to that of the synthetic tetrasaccharide, residues 2–5. This hexasaccharide also produced a much smaller ΔF_{max} which indicates an incomplete conformational change in antithrombin such as that produced by the synthetic tetrasaccharides in the present study.

We note that the results obtained are most consistent with the binding of heparin to multiple lysyl residues on antithrombin as initially suggested by Rosenberg & Damus (1973). Villanueva (1984) has recently hypothesized that the polysaccharide complexes with an unstable helical segment of the protease inhibitor composed of residues 281–292. On the basis of available NMR and X-ray data of the repeating units of heparin, the above investigator showed that the three lysine residues within the α-helix of this segment (Lys-282, Lys-286, and Lys-289) could be matched for maximal interaction with the 6-*O*-sulfate of residue 2, the *N*-sulfate of residue 4, and the *N*-sulfate of residue 6 of the octasaccharide. The present study demonstrates that the first and third of these sulfate groups on the octasaccharide are important for binding oligosaccharide to antithrombin but also reveals that the 3-*O*-sulfate group of residue 4 is critical for complex formation. This latter moiety appears to be out of range of Lys-286.

However, it is possible that the 3-*O*-sulfate group of residue 4 might interact with a different helical segment in the N-terminal region of antithrombin and thereby induce a major alteration in the conformation of the protease inhibitor. This hypothesis is consistent with the investigations of Blackburn et al. (1984) in which modification of Trp-49 near the N-terminal end of antithrombin blocks the heparin-enhanced inhibition of thrombin or factor Xa and would also explain the fluorescence-transfer studies of Pecon & Blackburn (1984) which indicate that 1–2 lysines essential for polysaccharide binding are located near Trp-49. If such is the case, the interactions of the 6-*O*-sulfate group of residue 2 and the 3-*O*-sulfate group of residue 4 with separate domains on antithrombin might induce a conformational change in the protease inhibitor that would functionally link the binding of these two residues and could also lead to the exposure of a protease complexing site that is responsible for the acceleration of factor Xa neutralization. This hypothesis is currently under examination in our laboratories by stopped-flow measurements of the rates of factor Xa inhibition by antithrombin saturated with the various types of oligosaccharides.

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Registry No. Pentasaccharide 2–6, 88096-19-9; pentasaccharide 2–6 (no 3-O-SO_3), 92745-16-9; tetrasaccharide 2–5, 92745-15-8; tetrasaccharide 3–6, 88096-18-8; octasaccharide 1–8, 90067-80-4; heparin, 9005-49-6; antithrombin, 9000-94-6.

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