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Evaluation of critical groups required for the binding of heparin to antithrombin

(mucopolysaccharide/oligosaccharide/anticoagulant function)

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ABSTRACT **We have examined the quantitative importance of various monosaccharide residues of an octasaccharide domain of heparin that are responsible for the binding of this oligosaccharide to antithrombin. Different fragments of the octasaccharide were prepared by enzymatic digestion and the avidities of these oligosaccharides for antithrombin were determined by equilibrium dialysis. The data show that the nonreducing-end and the reducing-end tetrasaccharides contribute equally to the binding energy of the octasaccharide. The O^6-sulfate group of the *N*-acetyl glucosamine moiety within the nonreducing-end tetrasaccharide is responsible for ≈45% of the binding energy of the octasaccharide. Neither the two nonsulfated uronic acid groups that flank this residue nor the *N*-sulfated glucosamine residue on the reducing end of this tetrasaccharide sequence that bears the unique O^3-sulfate substituent contribute significantly to the binding energy of the octasaccharide. We suggest that the lack of sulfation of the two uronic acid moieties within the nonreducing-end tetrasaccharide may be required to permit the *N*-acetyl glucosamine O^6-sulfate group to interact with a specific region on the antithrombin molecule. However, we cannot exclude the possibility that the O^3-sulfate group plays an important role in orienting this O^6-sulfate group within the nonreducing-end tetrasaccharide.**

Heparin 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 (1). 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 of the mucopolysaccharide (2). The existence of highly active and relatively inactive heparin species has been confirmed by Höök *et al.* (3). 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*-acetyl glucosamine O^6-sulfate group as shown by Rosenberg *et al.* (4, 5) and confirmed by Lindahl *et al.* (6). Subsequently, Leder (7) isolated an exoglycosidase that specifically removes O^3-sulfate substituents from glucosamine residues of heparin and postulated that this specific substituent might be present within the antithrombin binding domain of highly active heparin. Data provided by Lindahl *et al.* (8), using the exoglycosidase described above, have confirmed this supposition and placed the O^3,O^6-disulfated glucosamine moiety on the reducing end of the glucuronic acid residue that lies within the protease inhibitor binding domain of the mucopolysaccharide. This structural assignment is supported by the careful NMR studies of Casu *et al.* (9). Riesenfeld *et al.* (10), Choay *et al.* (11), and Oosta *et al.* (12) have isolated octasaccharide fragments of heparin that possess the tetrasaccharide-binding region, and exhibit a high affinity for antithrombin as well as a capacity to accelerate the neutralization of factor Xa. However, little is known concerning the contributions of the monosaccharides within this octasaccharide sequence to the strength of the interaction between the oligosaccharide and the protease inhibitor. In this communication we provide a quantitative examination of the importance of various residues of the octasaccharide sequence in the binding of this oligosaccharide to antithrombin.

MATERIALS AND METHODS

Human and bovine antithrombin were prepared in homogeneous form by chromatography on heparin Sepharose and DEAE cellulose (13, 14). Antithrombin concentrations were determined using an absorptivity value at 280 nm of 6.5 (15). The α-iduronidase and *N*-acetyl glucosamine sulfatase from bovine kidney and the *N*-acetyl glucosaminidase from human placental tissue were prepared by chromatography on Concanavalin A-Sepharose, heparin-Sepharose, hydroxylapatite, DEAE or CM cellulose, and polyacrylamide or Sephacryl S200. These enzymes were homogeneous by gel electrophoresis and free of trace contamination by the other exoglycosidases as monitored by their inability to degrade radiolabeled disaccharides and trisaccharides of known structure. Human platelet heparitinase was obtained in homogeneous form as outlined in a recent publication from our laboratory (16). *Flavobacterium* heparinase was obtained from *Flavobacterium heparinum* as described (17, 18). Concentrations of the above enzymes were determined assuming an absorptivity value at 280 nm of 10.0.

Porcine mucosal heparin (Diosynth, 7.5 g at 169 USP units/mg) was cleaved by nitrous acid degradation at pH 1.5 for 9 min at 0°C and separated by gel filtration as described (12). Fractions containing octasaccharide were selected from well resolved peaks, as monitored by colorimetric assay of uronic acid (19), and rechromatographed on the same column to remove traces of residual hexasaccharide and decasaccharide (12). The octasaccharide was then affinity-fractionated with bovine antithrombin using final concentrations of 10 mM and 450 μM, respectively, as outlined in an earlier communication (20). The octasaccharide product had a specific factor Xa inhibitory activity of 200 USP units/mg (12). This pool was shown to consist of two major oligosaccharides. The predominant form (≈60%) exhibited the specific tetrasaccharide sequence described in the introduction on the nonreducing end of the octasaccharide and possessed the highest affinity for the protease inhibitor. The minor form (≈40%) exhibited the tetrasaccharide at positions 3–6 of the octasaccharide and possessed a somewhat lower affinity for the pro-

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tease inhibitor. To obtain the predominant oligosaccharide free of the minor oligosaccharide, bovine antithrombin was added to the octasaccharide fraction at a final concentration of 10 μM. The reaction mixture was affinity-fractionated and the oligosaccharide was subsequently freed of protease inhibitor in a fashion similar to that described above.

Oligosaccharides obtained from nitrous acid degradation or octasaccharide isolated by affinity fractionation were radiolabeled by reduction with NaB^3H_4, as described by Shively and Conrad (21, 22). Octasaccharide was also radiolabeled with ^{35}S by *N*-desulfation of the pyridinium salt with dimethyl sulfoxide containing 5% H_2O (vol/vol) for 1.5 hr at 50°C (23) and subsequent *N*-resulfation with trimethylamine [^{35}S]sulfur trioxide (Amersham, England) in dimethylformamide for 18 hr at 53°C (24). The radiolabeled octasaccharides were rechromatographed on a polyacrylamide P2 column and affinity-fractionated with bovine antithrombin as described above. High-pressure liquid chromatography of ^3H- and ^{35}S-labeled oligosaccharides was carried out with a Waters system and a Whatman Particil PXS-1025 SAX column using various concentrations of potassium phosphate (pH 4.3) as outlined by Delaney *et al.* (25).

The binding of radiolabeled oligosaccharides to bovine or human antithrombin was quantitated by equilibrium dialysis using cells of 1 ml volume and Spectrophor no. 2 membranes with a 12,000–14,000 molecular weight cut-off (Spectrum Medical Industries, Los Angeles, CA). Various concentrations of antithrombin (1 nM to 0.5 mM) and a single concentration of oligosaccharide (5000 cpm or ≈10 nM) in 0.15 M NaCl/0.01 M Tris·HCl, pH 7.5, were placed in one compartment. Only the buffer was placed in the other compartment. The cells were rotated at ≈1 revolution per sec at 5°C. Samples of 0.1–0.2 ml were removed from both compartments, mixed with 3.0 ml of Ultrafluor scintillation fluid (National Diagnostics, Somerville, NJ), and assayed for 10 min. To ensure that the components had reached equilibrium, sample distributions were followed for 1–10 days and compared to measurements obtained when radiolabeled oligosaccharides were introduced into the chamber containing buffer rather than protein. The ratio of bound oligosaccharide to total oligosaccharide was calculated for each protein concentration from the measured oligosaccharide concentrations at equilibrium. The dissociation constants of the radiolabeled oligosaccharides were calculated by nonlinear, least-squares fit of the equilibrium dialysis data to a single-site binding model as described (20).

RESULTS

Affinity-fractionated octasaccharide, prepared as described above, was cleaved using nitrous acid degradation at pH 1.5. The fragments were radiolabeled by reduction with NaB^3H_4, freed of unincorporated ^3H by descending paper chromatography on Whatman no. 1 paper using a solvent of ethyl acetate/acetic acid/formic acid/water (18:3:1:4) and analyzed by gel filtration on polyacrylamide P4 columns (0.6 × 200 cm) equilibrated with 0.5 M ammonium bicarbonate (pH 8.8) at a flow rate of 2.5 ml/hr. Fig. 1*A* shows that the octasaccharide has been degraded to tetrasaccharide and disaccharide fragments in the expected ratio of approximately 1:2. The radiolabeled disaccharide pool was examined by HPLC using Particil PXS-1025 SAX columns (HPLC/Particil). It consisted predominantly of iduronosyl-2-sulfate → anhydromannitol 6-sulfate (≈97%). The radiolabeled tetrasaccharide pool was also analyzed by HPLC/Particil and the major component (≈70%) was observed to comigrate with the critical tetrasaccharide necessary for binding to antithrombin, which has been previously described (5). The sequence of this component is iduronic acid → *N*-acetyl glucosamine 6-sulfate → glucuronic acid → anhydromannitol 3,6-disulfate (5, 8). The minor tetrasaccharides separated by HPLC/Particil were shown to contain glucuronic acid in place of iduronic acid at the nonreducing end or anhydromannitol 3-sulfate in place of anhydromannitol 3,6-sulfate at the reducing end. The position of the predominant tetrasaccharide within the octasaccharide structure was determined by treatment of affinity-fractionated oligosaccharide with platelet endoglycosidase. This enzyme has been shown to cleave only glucuronosyl-glucosamine bonds (16). To this end, 0.16 unit of the endoglycosidase (16) was incubated for 36 hr with 100,000 cpm (≈10 μg) of octasaccharide that had been radiolabeled with NaB^3H_4 at the reducing-end group. The environmental conditions were maintained at 0.1 M sodium phosphate (pH 6.3) and 37°C. Subsequently, the reaction mixture was examined by gel filtration on a polyacrylamide P4 column. Fig. 1*B* indicates that the octasaccharide was almost completely degraded to a pentasaccharide. Based on this data, the predominant structure of the octasaccharide and pentasaccharide

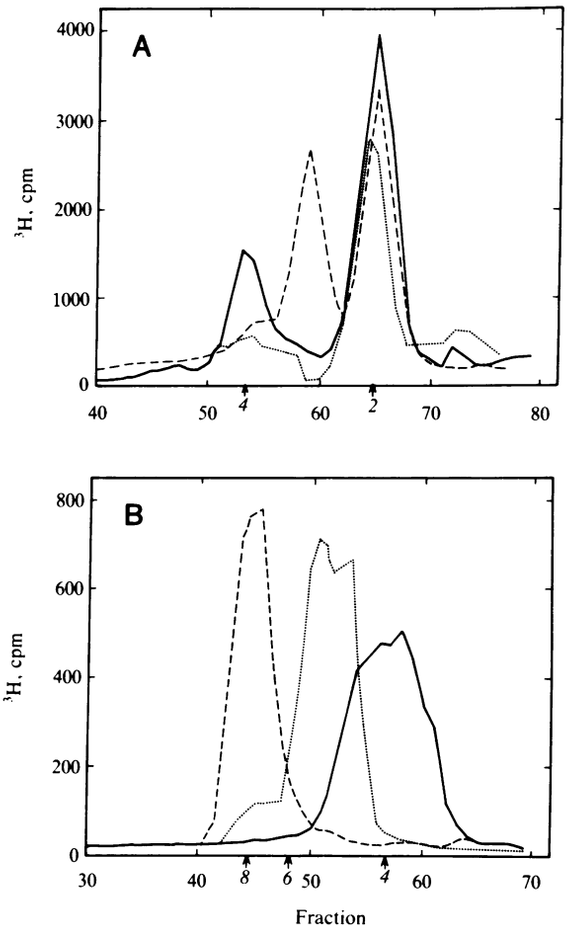

FIG. 1. Structural analysis of heparin fragments. Gel filtration of oligosaccharides on a polyacrylamide P4 column. Fractions of 0.6 ml were collected and evaluated by scintillation counting. Column calibration points corresponding to the elution volumes for oligosaccharides ranging from octasaccharide (*8*) to disaccharide (*2*) are indicated in the lower margins. (*A*) Nitrous acid cleavage of heparin fragments. Nitrous acid cleavage products were obtained from heptasaccharide (----) and hexasaccharide (····) prepared by enzymatic removal of monosaccharide units from the nonreducing end of the octasaccharide (—). To this end, the three oligosaccharides were treated with low pH nitrous acid, radiolabeled with NaB^3H_4, and gel filtered. (*B*) Endoglycosidase cleavage of active octasaccharide. Octasaccharide (-----) was affinity-fractionated with antithrombin and radiolabeled with either ^3H or with ^3H and ^{35}S. Subsequently, ^3H-labeled pentasaccharide (····) as well as ^3H- and ^{35}S-labeled tetrasaccharide (——) were produced by treatment of the respective octasaccharides with either platelet endoglycosidase or *Flavobacterium* heparinase. The various components were gel filtered.

Table 1. Binding of heparin fragments to antithrombin

Saccharide	Structure	K_d, M	$-\Delta G°$, kcal/mol
8	ID-GlcNAc-GlcUA-GlcNS - ID GlcNS - ID - AMN 6-OSO_3 3,6-OSO_3 2-OSO_3 6-OSO_3 2-OSO_3 6-OSO_3	1.5×10^{-7}	8.7
7	GlcNAc-GlcUA-GlcNS - ID - GlcNS - ID - AMN 6-OSO_3 3,6-OSO_3 2-OSO_3 6-OSO_3 2-OSO_3 6-OSO_3	2.5×10^{-7}	8.4
7*	GlcNAc-GlcUA-GlcNS - ID - GlcNS - ID AMN 3,6-OSO_3 2-OSO_3 6-OSO_3 2-OSO_3 6-OSO_3	$\approx 1 \times 10^{-4}$	≈5
6	GlcUA-GlcNS - ID - GlcNS - ID AMN 3,6-OSO_3 2-OSO_3 6-OSO_3 2-OSO_3 6-OSO_3	2.0×10^{-4}	4.7
5	GlcNS - ID - GlcNS - ID - AMN 3,6-OSO_3 2-OSO_3 6-OSO_3 2-OSO_3 6-OSO_3	2.0×10^{-4}	4.7
4	ID-GlcNAc-GlcUA-GlcNS - 6-OSO_3 3,6-OSO_3	$\approx 1 \times 10^{-3}$	≈4
4	ID† - GlcNS - ID - AMN 2-OSO_3 6-OSO_3 2-OSO_3 6-OSO_3	$\approx 1 \times 10^{-3}$	≈4

ID, iduronic acid; GlcUA, glucuronic acid; GlcNS, *N*-sulfated glucosamine; GlcNAc, *N*-acetyl glucosamine; AMN = anhydromannitol; $\Delta G°$ = standard-state free energy change based on 1 M concentration of reactants.
*Desulfated.
†Unsaturated.

can be assigned as indicated in Table 1. The affinities of these oligosaccharides for bovine and human antithrombin were determined by equilibrium dialysis. The data shown in Fig. 2 were used to calculate the values of K_d given in Table 1. It should also be noted that the minor structural variants present within the tetrasaccharide region of the octasaccharide do not significantly affect the avidity of the oligosaccharide for antithrombin, because no distortion of the theoretical binding isotherm is manifest. In addition, the virtually identical behavior of the bovine and human antithrombin with respect to the binding of octasaccharide and pentasaccharide provide evidence for a lack of species specificity in the mucopolysaccharide-binding site of the protease inhibitor. Furthermore, we have observed that when unlabeled octasaccharide is admixed with antithrombin in amounts just sufficient to saturate the protease inhibitor, labeled pentasaccharide is unable to bind to the antithrombin molecule (data not shown). This suggests that the octasaccharide and the pentasaccharide bind to the same site on the protease inhibitor.

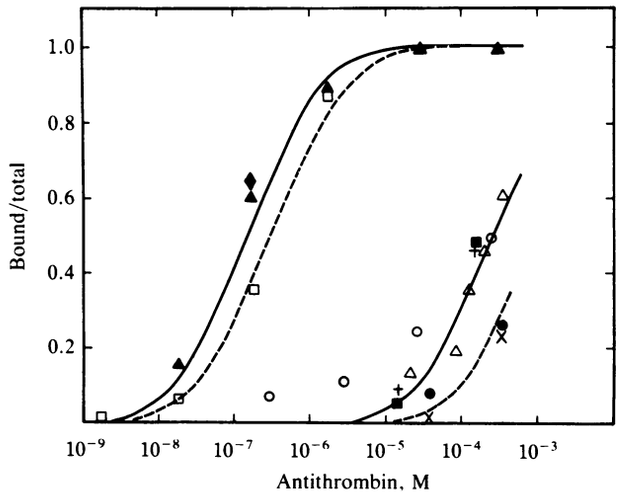

FIG. 2. Equilibrium dialysis of heparin fragments. Ratio of bound heparin to total heparin for different oligosaccharides as a function of antithrombin concentration were determined by equilibrium dialysis. The various lines represent a nonlinear least-squares computer fit of our data to a one-site binding model using dissociation constants indicated in Table 1. Data obtained using human antithrombin are represented as follows: ▲, octasaccharide; □, heptasaccharide; ■, hexasaccharide; +, pentasaccharide. Data obtained using bovine antithrombin are represented as follows: ◆, octasaccharide; ○, desulfated heptasaccharide; △, pentasaccharide; ●, nonreducing-end tetrasaccharide; ×, reducing-end tetrasaccharide.

The affinity-fractionated octasaccharide, radiolabeled with ^{35}S at *N*-sulfate groups, was treated with purified *Flavobacterium* heparinase. This enzyme is known to cleave certain sulfated glucosaminyl-sulfated iduronosyl bonds and thereby convert the nonreducing-end uronic acid into an unsaturated moiety. To this end, 60 units of *Flavobacterium* heparinase (17) were incubated for 3 hr with 100,000 cpm (≈10 μg) of octasaccharide. The environmental conditions were maintained at 1 mM calcium acetate/0.1 M sodium acetate, pH 7.2, and 30°C. The reaction mixture was subsequently analyzed by gel filtration on a polyacrylamide P4 column. Fig. 1*B* shows that the octasaccharide was completely degraded to tetrasaccharide fragments. These components were analyzed by HPLC/Particil. The chromatogram showed two major groups of tetrasaccharides that correspond to the nonreducing and reducing ends of the initial octasaccharide. Peak fractions containing the major nonreducing end tetrasaccharide were pooled, desalted by gel filtration, treated with nitrous acid at pH 1.5, radiolabeled at the anhydromannose reducing-end group with NaB^3H_4, and examined by HPLC/Particil. The data show that this radiolabeled heparin fragment is a tetrasaccharide that comigrates with the critical tetrasaccharide noted above and that the structure of the nonreducing-end tetrasaccharide has not been altered in the ^{35}S-labeling procedure (data not shown). The structure of the major nonreducing-end tetrasaccharide, obtained by digestion of the affinity-fractionated octasaccharide with *Flavobacterium* heparinase, is provided in Table 1. The affinity of this tetrasaccharide for bovine antithrombin was obtained by equilibrium dialysis (Fig. 2; Table 1). In additional experiments, we have observed that when unlabeled octasaccharide is admixed with antithrombin in amounts just sufficient to saturate the protease inhibitor, the labeled nonreducing-end tetrasaccharide is unable to bind to the antithrombin molecule (data not shown). This suggests that the octasaccharide and the nonreducing-end tetrasaccharide bind to the same site on the protease inhibitor.

The affinity-fractionated octasaccharide, radiolabeled at the reducing end with NaB^3H_4, was exposed to purified *Flavobacterium* heparinase and the resultant radiolabeled reducing-end tetrasaccharide fragment was separated as described above. The structure of this component is provided in Table 1. The affinity of this oligosaccharide for bovine an-

tithrombin was quantitated by equilibrium dialysis (Fig. 2; Table 1).

The affinity-fractionated octasaccharide, radiolabeled at the reducing end with NaB^3H_4, was also treated with α-iduronidase. To this end, 48 μg of α-iduronidase were incubated for 18 hr with 300,000 cpm (≈30 μg) of radiolabeled octasaccharide. The environmental conditions were maintained at 0.4 M NaCl/0.2 M sodium formate, pH 3.55, and 37°C. The resultant product was gel filtered on a polyacrylamide P4 column. This material was lyophilized and suspended in water. An aliquot of this oligosaccharide was degraded by nitrous acid at pH 1.5, radiolabeled with NaB^3H_4, and analyzed by gel filtration on a polyacrylamide P4 column. Fig. 1*A* shows that the resultant component consists predominantly of trisaccharide and disaccharide fragments. The data indicate that the α-iduronidase-treated oligosaccharide consists predominantly of a heptasaccharide (>70%) whose structure is provided in Table 1. The affinities of this heptasaccharide for bovine and human antithrombin were quantitated by equilibrium dialysis (Fig. 2; Table 1).

The heptasaccharide was subsequently treated with *N*-acetyl glucosamine sulfatase. To this end, 160 μg of *N*-acetyl glucosamine sulfatase was incubated for 18 hr with 200,000 cpm (20 μg) of heptasaccharide. The environmental conditions were maintained at 0.16 M sodium acetate (pH 5.5) and 37°C with 3 mM sodium azide added. The resultant product was gel filtered, lyophilized, and resuspended. To ascertain that the sulfate group had been removed from the *N*-acetyl glucosamine residue, an aliquot of the reaction product was degraded by nitrous acid at pH 1.5, radiolabeled with NaB^3H_4, freed of unincorporated ^3H counts, and subjected to gel filtration on a polyacrylamide P4 column. The re-sultant trisaccharide was harvested and compared on HPLC/Particil to a trisaccharide obtained from α-iduronidase-treated active tetrasaccharide (5) that had not been exposed to *N*-acetyl glucosamine sulfatase. The chromatogram showed that the trisaccharide obtained from the *N*-acetyl glucosamine sulfatase-treated heptasaccharide migrated close to the disulfated disaccharide region, whereas the control trisaccharide migrated close to the trisulfated tetrasaccharide region (data not shown). Based on these results, the predominant structure of the desulfated heptasaccharide is assigned as indicated in Table 1. The affinity of this fragment for bovine antithrombin was quantitated by equilibrium dialysis (Fig. 2; Table 1). It should be noted that the desulfated heptasaccharide was contaminated with residual octasaccharide (≈30%) as determined by gel filtration on a polyacrylamide P4 column. The binding data have been corrected to remove contributions from this contaminant.

The desulfated heptasaccharide was subsequently treated with *N*-acetyl glucosaminidase. To this end, 35 μg of *N*-acetyl glucosaminidase was incubated for 18 hr with 100,000 cpm (≈10 μg) of nonreducing-end desulfated heptasaccharide. The environmental conditions were maintained at 0.1 M sodium citrate (pH 4.2) and 37°C. The product was gel filtered, lyophilized, and resuspended. An aliquot of this material was degraded by nitrous acid at pH 1.5, radiolabeled with NaB^3H_4, freed of unincorporated ^3H counts, and analyzed by gel filtration on a polyacrylamide P4 column. Fig. 1*A* shows that the resulting fragments consist predominantly of disaccharides. These data indicate that *N*-acetyl glucosaminidase has converted the desulfated heptasaccharide to a hexasaccharide. The structure of the component is provided in Table 1. The affinities of these oligosaccharides for bovine and human antithrombin were quantitated by equilibrium dialysis (Fig. 2; Table 1).

DISCUSSION

In this communication, we examine the quantitative importance of various monosaccharide residues of an octasaccharide domain of heparin that are responsible for the binding of this oligosaccharide to antithrombin. To undertake this analysis, we have isolated a virtually homogeneous form of the octasaccharide that consists of the unique tetrasaccharide sequence noted above on the nonreducing end and a relatively common tetrasaccharide sequence: iduronosyl 2-sulfate → glucosamine 2,6-disulfate → iduronosyl 2-sulfate → anhydromannitol 6-sulfate, on the reducing end. The structure of this octasaccharide differs somewhat from that reported by Riesenfeld *et al.* (10) in that essentially no monosulfated disaccharides are present at the nonreducing end of the oligosaccharide.

To initiate the analysis, we have digested the octasaccharide with *Flavobacterium* heparinase, separated the resultant components by gel filtration as well as HPLC/Particil, and obtained the unique nonreducing-end tetrasaccharide with its reducing-end residue intact. By a similar approach, we have isolated the reducing-end tetrasaccharide, which now possesses an unsaturated uronic acid moiety at its nonreducing end. Direct measurements of the binding avidities of these two tetrasaccharides indicate that both of these components exhibit approximately equal strengths of interaction with respect to antithrombin. Thus, both halves of the octasaccharide contain groups that are important for interaction with the protease inhibitor. Furthermore, the sum of the binding energies of the two tetrasaccharides is within ≈8% of that of the octasaccharide. This latter result indicates that the relative spacial orientation of the two tetrasaccharides within the parent oligosaccharide is not of great importance with respect to the binding of octasaccharide to antithrombin.

To explore the contributions of the three monosaccharides on the nonreducing end of the unique tetrasaccharide sequence to the binding process, we have digested the octasaccharide with purified exoglycosidases, which remove monosaccharide residues or sulfate groups from the nonreducing end of the oligosaccharide. Alternatively, we have treated the octasaccharide with purified platelet endoglycosidase, which cleaves glucuronosyl-glucosamine bonds and, hence, removes the terminal three residues from the nonreducing end. These two approaches permit us to sequentially remove the iduronic acid, *N*-acetyl glucosamine 6-sulfate, and glucuronic acid residues from the nonreducing end of the octasaccharide. Direct measurements of the binding avidities of the reducing-end fragments, produced by enzymatic cleavage, indicate that the sulfate group of the *N*-acetyl glucosamine moiety is responsible for ≈45% of the binding energy of the octasaccharide. By contrast, the two nonsulfated uronic acid residues provide essentially no contribution to the binding energy of the oligosaccharide. It should be noted that Thunberg *et al.* (26) have removed the nonsulfated iduronic acid from the octasaccharide and have assessed the importance of this residue to binding of the oligosaccharide to antithrombin-Sepharose 4B. Their qualitative analysis indicated that this nonreducing-end iduronic acid residue is not essential for the interaction of the octasaccharide with the protease inhibitor.

The importance of the glucosamine residue of the unique tetrasaccharide that possesses the O^3-sulfate substituent to the interaction of octasaccharide with antithrombin was assessed by comparing the binding energies of the nonreducing-end trisaccharide to that of the nonreducing-end tetrasaccharide. The contribution of the nonreducing-end trisaccharide was calculated from the difference between the octasaccharide and pentasaccharide binding energies. Because the contribution of this tetrasaccharide (≈4 kcal/mol; 1 cal = 4.184 J) is not more than the apparent contribution of the trisaccharide (4.0 kcal/mol), it is unlikely that this glucosamine residue with all of its sulfate substituents can be responsible for a significant percentage of the binding energy

of the octasaccharide. Thus our data do not support the claims of Lindahl *et al.* (8) that the O^3-sulfate substituent within the unique tetrasaccharide sequence represents the critical structural element required for the binding of heparin to antithrombin. However, our analysis does not preclude the possibility that the O^3-sulfate group might play an important role in orienting the O^6-sulfate group within the nonreducing-end tetrasaccharide. Alternatively, the O^3-sulfate group might represent a biosynthetic marker that prevents sulfation of the uronic acid moieties within the unique tetrasaccharide sequence. As indicated in previous communications from our laboratory (4, 5), we believe that the two nonsulfated uronic acid residues are important elements of the binding region. The invariant nature of these residues suggests that the lack of sulfation of these moieties may be required to permit the *N*-acetyl glucosamine O^6-sulfate group to interact with a specific region on the antithrombin molecule.

Note Added in Proof. After the acceptance of this communication, a paper by Lindahl *et al.* (27) appeared that confirms in a qualitative manner the importance of the O^6-sulfate group within the octasaccharide.

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