Cleavage of the antithrombin III binding site in heparin by heparinases and its implication in the generation of low molecular weight heparin

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Heparin has been used as a clinical anticoagulant for more than 50 years, making it one of the most effective pharmacological agents known. Much of heparin's activity can be traced to its ability to bind antithrombin III (AT-III). Low molecular weight heparin (LMWH), derived from heparin by its controlled breakdown, maintains much of the antithrombotic activity of heparin without many of the serious side effects. The clinical significance of LMWH has highlighted the need to understand and develop chemical or enzymatic means to generate it. The primary enzymatic tools used for the production of LMWH are the heparinases from *Flavobacterium heparinum*, specifically heparinases I and II. Using pentasaccharide and hexasaccharide model compounds, we show that heparinases I and II, but not heparinase III, cleave the AT-III binding site, leaving only a partially intact site. Furthermore, we show herein that glucosamine 3-O sulfation at the reducing end of a glycosidic linkage imparts resistance to heparinase I, II, and III cleavage. Finally, we examine the biological and pharmacological consequences of a heparin oligosaccharide that contains only a partial AT-III binding site. We show that such an oligosaccharide lacks some of the functional attributes of heparin- and heparan sulfate-like glycosaminoglycans containing an intact AT-III site.

Heparin, a highly sulfated heparin- and heparan sulfate-like glycosaminoglycan (HLGAG) produced by mast cells, is a widely used clinical anticoagulant and is one of the first biopolymeric drugs and one of the few carbohydrate drugs. Heparin primarily elicits its effect through two mechanisms, both of which involve binding of antithrombin III (AT-III) to a specific pentasaccharide sequence, $H_{NAc/S,6S}GH_{NS,3S,6S}I_{2S}H_{NS,6S}$ (where 2S, 3S, and 6S indicate 2-O, 3-O, or 6-O sulfation, respectively; and NS and NAc indicate N-sulfation and N-acetylation of the glucosamine, respectively) contained within the polymer (1). First, AT-III binding to the pentasaccharide induces a conformational change in the protein that mediates its inhibition of factor Xa (FXa). Second, thrombin (factor IIa) also binds to heparin at a site proximate to the pentasaccharide AT-III binding site. Formation of a ternary complex between AT-III, thrombin, and heparin results in inactivation of thrombin. Unlike its anti-Xa activity that requires only the AT-III pentasaccharide binding site, heparin's anti-IIa activity is size-dependent, requiring at least 18 saccharide units for the efficient formation of an AT-III, thrombin, heparin ternary complex (2). Of specific note, 3-O sulfation of the glucosamine is a rare modification; accordingly only 30% of intact heparin chains contain an intact AT-III binding site (3).

Although heparin is highly efficacious in a variety of clinical situations, the side effects associated with heparin therapy are many and varied. Because of these limitations, for certain clinical indications, alternatives have been devised. One of the most promising alternatives, especially for venous thromboembolic complications, is low molecular weight heparin (LMWH) (4). LMWHs are derived from unfractionated heparin via controlled chemical or enzymatic breakdown of the heparin polymer (5). Ideally, controlled breakdown leaves intact the pentasaccharide binding site for AT-III while reducing the molecular weight of the polymer. As such, LMWHs possess high anti-Xa activity. However, LMWHs possess less anti-IIa activity than full-length heparin (4). Another consequence of the reduced molecular weight of LMWH as compared with unfractionated heparin is that LMWH therapy is associated with fewer side effects, especially heparin-induced thrombocytopenia (6, 7).

The three heparinases from *Flavobacterium heparinum* are enzymatic tools that have been used for the generation of LMWH (5,000–8,000 Da) and ultra-LMWH (≈3,000 Da). Heparinase I cleaves highly sulfated regions of HLGAGs at 2-O sulfated uronic acids, whereas heparinase II has a broader substrate specificity and cleaves glycosidic linkages containing both 2-O sulfated and nonsulfated uronic acids (8). Heparinase III, as opposed to heparinase I, cleaves primarily undersulfated regions of HLGAGs, namely, glycosidic linkages containing a nonsulfated uronic acid (8). Multiple investigations into the substrate specificity of the heparinases have increased their usefulness as tools to develop structure-function relationships for HLGAGs; a more detailed understanding is required to maximize their usefulness as generators of pharmacological LMWH.

As such, only preliminary data are available on the action of the heparinases toward oligosaccharide sequences containing intact AT-III sites and accordingly an unusual 3-O sulfated glucosamine. Tetrasaccharides containing 3-O sulfate have been shown to be uncleavable by any of the heparinases (9), suggesting that linkages with a 3-O sulfated glucosamine are resistant to cleavage. To begin to address this important issue, we recently undertook a series of studies to understand the enzymatic activity of heparinases I and II toward heparin oligosaccharides of variable sequence; one goal

Abbreviations: HLGAG, heparin- and heparan sulfate-like glycosaminoglycan; AT-III, antithrombin III; LMWH, low molecular weight heparin; FXa, factor Xa; AT-10, AT-III-fractionated decasaccharide; ΔU, a 4,5 unsaturated uronic acid; 2S, 3S, and 6S, 2-O, 3-O, or 6-O sulfation, respectively; NS and NAc, N-sulfation and N-acetylation of the glucosamine, respectively.

See commentary on page 10301.

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of these studies was to examine their ability to cleave the AT-III binding site (10, 11). A decasaccharide model compound (AT-10) was used in the studies and assumed a published sequence that contained an intact AT-III binding site, namely, $\Delta U_{2S}H_{NS,6S}IH_{NAc,6S}GH_{NS,3S,6S}I_{2S}H_{NS,6S}I_{2S}H_{NS,6S}$ (12), where ΔU is a 4,5 unsaturated uronic acid. In the accompanying paper in this issue of PNAS (13), we conclusively show through a variety of physical chemical techniques that the actual structure of AT-10 is $\Delta U_{2S}H_{NS,6S}I_{2S}H_{NS,6S}I_{2S}H_{NS,6S}IH_{NAc,6S}GH_{NS,3S,6S}$ and therefore does not contain an intact AT-III binding site. In light of the reinterpretation of the AT-10 structure, we sought to re-examine the action of heparinases I-III toward AT-III binding, 3-O sulfate containing oligosaccharides. Given that AT-10 (ultra-LMWH, molecular mass = 2769.3 Da) is derived from controlled heparinase I cleavage of heparin, we also sought to examine the functional consequences of an oligosaccharide that contains only a partial AT-III binding sequence, by using established bioanalytical techniques. Such an understanding of both heparinase action and functional consequences of a partially intact AT-III binding site is required for the efficient, optimal generation of LMWH for clinical use.

Materials and Methods

Materials. Pentasaccharides (Pentas) 1 and 2 were a generous gift of Robert Rosenberg, Massachusetts Institute of Technology. Hexa 1 was generated by using heparinase I digestion of heparin (8). Heparin was purchased from Celsus Laboratories (Cincinnati), and molar concentrations of stocks were calculated based on an average molecular mass of 13,000 Da. Enoxaparin was purchased from Avantis Pharmaceuticals (Chicago).

Digests. Heparinase I digests were completed as described (14, 15). Heparinase II or III reactions were completed in essentially the same way at room temperature in 10 μM ovalbumin, 1 μM dextran sulfate, and 10 mM ethylenediamine, pH 7.0. Short digestions were completed with 50 nM enzyme for 10 min whereas exhaustive digests were completed with 200 nM enzyme overnight. Mass spectra were collected by using parameters as outlined (13) and calibrated externally by using signals for protonated $(RG)_{19}R$ and its complex with a nitrous acid-derived hexasaccharide of the sequence $I_{2S}H_{NS,6S}$ $I_{2S}H_{NS,6S}$ $I_{2S}Man_{6S}$.

Equilibrium Fluorescence Titration Experiments. Titrations of human AT-III with either AT-10 decasaccharide or heparin were completed at 25°C by using a Fluorolog 2 machine (Spex Industries, Metuchen, NJ) (16, 17). Measurements were completed in 20 mM sodium phosphate, containing 0.1 mM EDTA and 0.1% PEG 8000, adjusted to either pH 7.4 or 6.0. With the pH 7.4 buffer, sodium chloride was added to a final concentration of 100 mM.

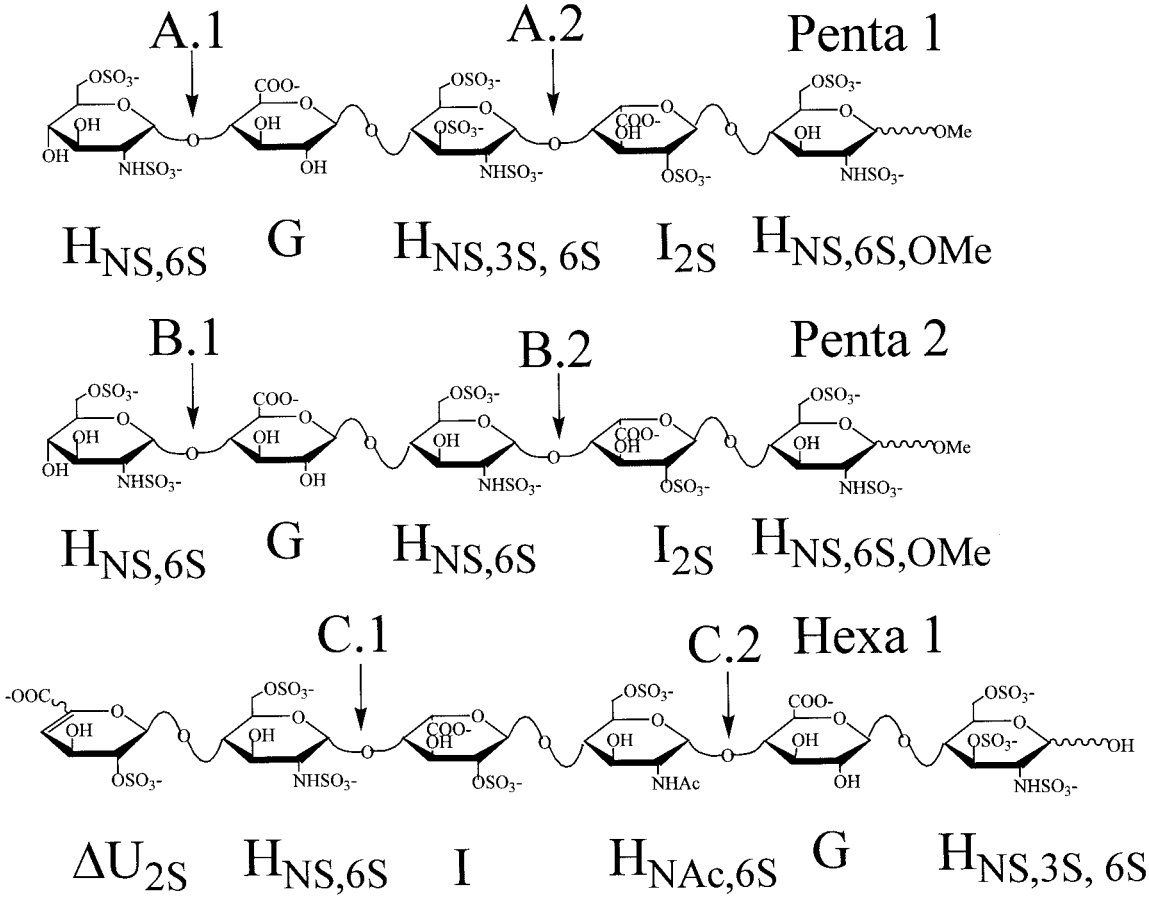

Fig. 1. Structures of the three oligosaccharide model compounds used in this study. (*A*) Pentasaccharide 1 (Penta 1) has the sequence $H_{NS,6S}GH_{NS,3S,6S}I_{2S}H_{NS,6S,OMe}$, contains a fully intact AT-III binding site, and has a calculated molecular mass of 1508.2. The two glycosidic linkages potentially susceptible to heparinase I, II, or III cleavage are labeled A.1 and A.2. (*B*) Pentasaccharide 2 (Penta 2), with the sequence $H_{NS,6S}GH_{NS,6S}I_{2S}H_{NS,6S,OMe}$ and a calculated molecular mass of 1428.1, is structurally identical to Penta 1, less a 3-O sulfate on the internal glucosamine, thus it does not contain a full AT-III site. As with Penta 1 the bonds potentially susceptible to heparinase cleavage are marked B.1 and B.2. (*C*) A heparinase-derived hexasaccharide (Hexa 1), with the sequence $\Delta U_{2S}H_{NS,6S}IH_{NAc,6S,}GH_{NS,3S,6S}$, also was used in this study. Hexa 1 (calculated molecular mass 1614.3) contains only a partially intact AT-III binding site; similar to AT-10 it is missing the reducing end $I_{2S}H_{NS,6S}$ disaccharide unit. As with Penta 1 and Penta 2, sites of potential cleavage are marked C.1 and C.2.

Table 1. Chemical structures and *m/z* values of the HLGAG oligosaccharides

Complex $(M + H)^+$	Saccharide (mass)	Chemical structure	Mass (calculated)
5842.0	1615.2	$\Delta U_{2S}H_{NS,6S}IH_{NAc,6S}GH_{NS,3S,6S}$ (Fig. 4*A*)	1614.3
5735.1	1508.3	$H_{NS,6S}GH_{NS,3S,6S}I_{2S}H_{NS,6S,OMe}$ (Fig. 2*C*)	1508.2
5655.1	1428.3	$H_{NS,6S}GH_{NS,6S}I_{2S}H_{NS,6S,OMe}$ (Fig. 3*C*)	1428.1
5316.3	1089.5	$\Delta UH_{NS,6S}I_{2S}H_{NS,6S,OMe}$ (Fig. 3*C*)	1088.9
5266.3, 5264.2	1036.5, 1036.1	$\Delta UH_{NAc,6S}GH_{NS,3S,6S}$ (Fig. 4*B* and *C*)	1036.9
5064.1	837.3	$H_{NS,6S}GH_{NS,6S}$ (Fig. 3*A*)	836.7
5143.4, 5144.0	916.7, 917.3	$H_{NS,6S}GH_{NS,3S,6S}$ (Fig. 2*A* and *B*)	916.8
4818.0, 4818.0, 4818.7, 4818.7	591.3, 591.3, 591.9, 592.0	$\Delta U_{2S}H_{NS,6S,OMe}$ (Figs. 2*A* and *B* and 3*A* and *B*)	591.5
4807.2, 4805.9	577.4, 577.8	$\Delta U_{2S}H_{NS,6S}$ (Fig. 4*B* and *C*)	577.5

Shown in column 1 is the *m/z* value of the protonated 1:1 complex of the saccharide and the basic peptide $(RG)_{19}R$. Column 2 shows the observed mass of the saccharide obtained by subtracting the mass of protonated basic peptide from the protonated 1:1 complex. The chemical structures of the saccharides for the corresponding peaks in the mass spectra are shown in column 3. Column 4 shows the theoretical masses calculated for the chemical structures. Note that the observed mass is within ± 1 Da of the calculated mass.

Fluorescence emission spectra were collected from 300–400 nm with a 280-nm excitation wavelength and a 5-s integration time. Briefly, the titration experiments were conducted as follows: sequential aliquots of either decasaccharide or heparin was added to a 1 μM solution of AT-III, the solution was allowed to come to equilibrium for 1 min, and an emission spectrum was collected. The fluorescence emission signal was adjusted to account for protein dilution.

Biological Measurements of Decasaccharide Activity. *In vitro* anticoagulant activity was determined as described (18, 19), according to the United States Pharmacopoeia. Thrombin and FXa generation inhibition assays were completed essentially as described. Briefly, either AT-10 decasaccharide, enoxaparin LMWH, or the synthetic AT-III binding pentasaccharide (Penta 1) used in this study was dissolved in sterile saline at the designated concentrations. To this sample was added an equal volume of fibrinogen-deficient plasma diluted 1:8 in 100 mM Tris·HCl, pH 8.5. In a separate sample, the same concentration of heparin oligosaccharide and actin was added in a 1:1 ratio to either Spectrozyme TH or FXa. In this manner, the intrinsic IIa and Xa generation was measured. In addition, to account for inhibition of thrombin and extrinisic generation of FXa, thromboplastin C was diluted 1:6 with either Spectrozyme TH or FXa. For all samples, the optical density was measured at 405 nm and results are expressed as a % inhibition compared with an unsupplemented saline control. For these assays, thrombin reagent (Fibrindex) was obtained from Ortho Diagnostics, and FXa was obtained from Enzyme Research Laboratories (South Bend, IN). Spectrozyme TH and FXa were obtained from American Diagnostica (Greenwich, CT).

Whole blood data also were used to determine the anticoagulant activity of AT-10. The assays, activated partial thromboplastin time and prothrombin time, was conducted in a manner similar to what has been previously reported (19). Activated partial thromboplastin time reagent was obtained from Organon Teknika–Cappel, and HepTest reagent was obtained from Haemachem (St. Louis, MO).

Results and Discussion

Enzymatic Action of the Heparinases Toward the AT-III Binding Site. Previously, we investigated the substrate specificity of heparinases I and II toward AT-10 (10, 11). However, these studies were carried out assuming a published structure (12). In light of the newly determined sequence of AT-10 (13), we re-examined the enzymatic action of heparinases I, II, and III toward oligosaccharides containing a 3-O sulfate that is critical for high affinity AT-III binding. For these studies, we used three oligosaccharides, two pentasaccharides (Penta 1 and Penta 2, Fig. 1), and a hexasaccharide (Hexa 1, Fig. 1). Of note is the fact that the pentasaccharides are synthetically derived whereas Hexa 1 is

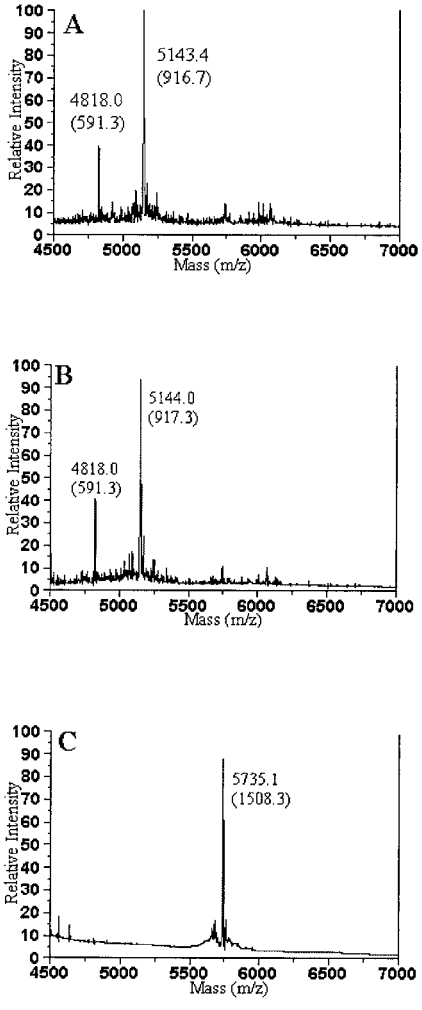

Fig. 2. Matrix-assisted laser desorption ionization mass spectra of (*A*) heparinase I, (*B*) heparinase II, and (*C*) heparinase III digestion products of Penta 1. Both heparinase I and II clip Penta 1 at the $G_{NS,3S,6S}\downarrow I_{2S}H_{NS,6S}$ linkage (site A.2) to yield a pentasulfated trisaccharide and a trisulfated disaccharide product. Penta 1 is not cleavable by heparinase III.

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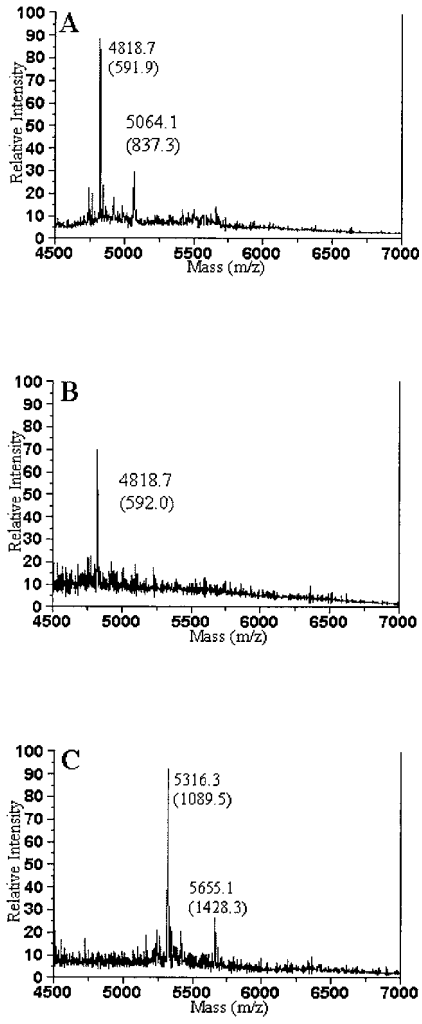

Fig. 3. Matrix-assisted laser desorption ionization mass spectra of (*A*) heparinase I, (*B*) heparinase II, and (*C*) heparinase III digestion products of Penta 2 complexed with $(RG)_{19}R$.

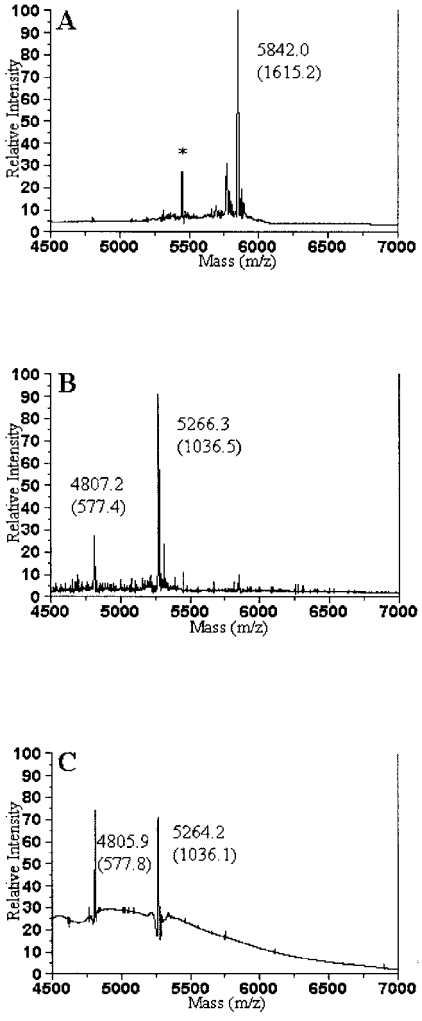

Fig. 4. Matrix-assisted laser desorption ionization mass spectra of (*A*) heparinase I, (*B*) heparinase II, and (*C*) heparinase III digestion products of Hexa 1 complexed with $(RG)_{19}R$. A minor contaminant in Hexal is marked with an ∗.

derived from treatment of heparin by heparinase I. As a result, unlike Penta 1 or Penta 2, Hexa 1 contains a $\Delta^{4,5}$ uronic acid at the nonreducing end. Furthermore, Penta 1 and Penta 2 differ from one another only by the presence (Penta 1) or absence (Penta 2) of a 3-O sulfate on the internal glucosamine residue (Fig. 1). The strategy used herein essentially involves treatment of each of the saccharides with heparinase I, II, or III, respectively, under exhaustive digestion conditions, followed by the identification of the resulting products by MS. The calculated mass of the saccharide substrates and products, their identity, and the observed mass is listed in Table 1.

In the case of Penta 1, only heparinase I and II, but not heparinase III, cleave the oligosaccharide into a pentasulfated trisaccharide of mass 916.7 and a trisulfated disaccharide of mass 591.3 (Fig. 2), indicative of cleavage at the I_{2S}-containing glycosidic linkage (linkage A.2 in Fig. 1). The data presented in Fig. 2 show that 3-O-containing linkages are resistant to heparinase I, II, or III cleavage. This resistance appears to be length-independent. Based on our previous understanding of the sequence of AT-10 (12), we reported that heparinase II could cleave a 3-O sulfate containing saccharide provided that it was of sufficient length (10). In light of the newly determined sequence for the AT-10 decasaccharide (13), as well as the data presented in Fig. 2, we reinterpret our previous findings and conclude that heparinase II does not cleave linkages with a reducing-end proximate 3-O sulfated glucosamine. In addition, resistance of this linkage to heparinase I, II, and III action is entirely caused by the presence of a 3-O sulfate as shown by heparinase I, II, and III treatment of Penta 2 (Fig. 3). In this case, all of the heparinases efficiently cleave the substrate. As with Penta 1, heparinase I cleaves at the I_{2S}-containing linkage yielding a trisaccharide of mass 837.3 and a disaccharide of mass 591.9 (cleavage at linkage B.2 in Fig. 1). Conversely, both heparinase II and III cleave at the now scissile unsulfated G-containing linkage (linkage B.1 in Fig. 1). Heparinase III cleaves only this linkage, giving a tetrasaccharide of mass 1089.5 and a monosaccharide (not observed). Heparinase II cleaves at linkage B.2 as well as B.1, reducing Penta 2 to a monosaccharide and two disaccharides, one of which is observed and has a mass of 592.0 (Fig. 3).

To explore further the substrate specificity of the heparinases toward the 3-O sulfated saccharides, we used Hexa 1, a 3-O sulfate-containing, heparinase I-derived hexasaccharide (Fig. 4). Hexa 1 also was chosen as a substrate for this study because it represents a nonreducing end truncation of AT-10 and contains the same $GH_{NS,3S,6S}$ moiety at the reducing end. We find that hexa 1 is susceptible to heparinase II and III cleavage but not heparinase I scission. Of note is the fact that cleavage of Hexa

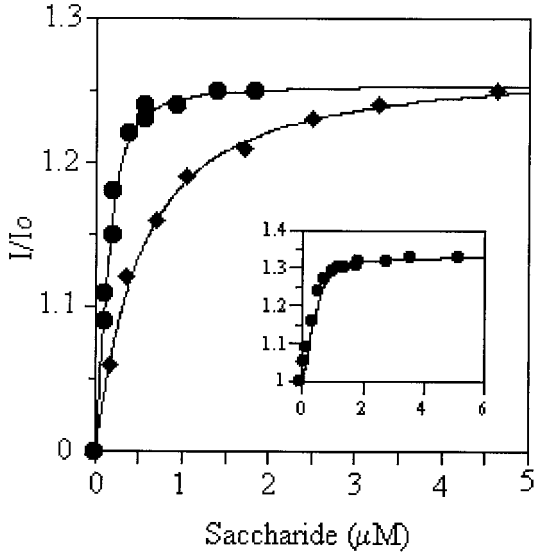

Fig. 5. Fluorescence titration of AT-III with either full-length heparin (●) or AT-10 (◆) at pH 6.0, I = 0.025. Data are plotted as the ratio of AT-III fluorescence upon the introduction of saccharide to the initial AT-III fluorescence (I/I_o) vs. concentration of added saccharide. The data were fitted by nonlinear regression and the K_D was determined. For heparin the measured K_D value was 10 nM, whereas for AT-10 this value was 800 nM. (*Inset*) The binding of heparin to AT-III at pH 7.4, I = 0.15. The measured K_D of 36 nM agrees favorably with other determinations of the affinity of heparin for AT-III.

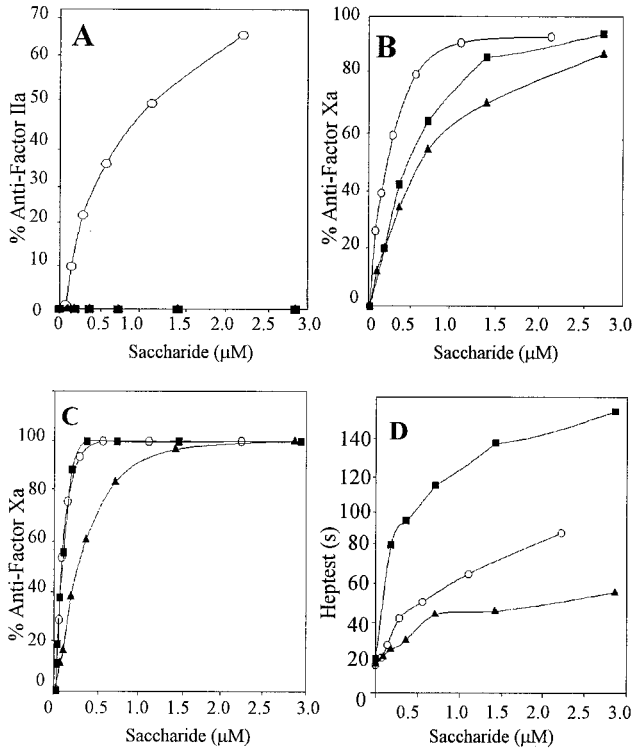

Fig. 6. Functional analysis of the AT-10 decasaccharide and comparison to the AT-III binding pentasaccharide. The *in vitro* anticoagulant activity of the AT-10 decasaccharide (▲) was compared with both the synthetic pentasaccharide (■) or enoxaparin (○), a LMWH generated through chemical cleavage of heparin. The activities of the three compounds was assessed by measuring either (*A*) anti-IIa activity, (*B*) anti-Xa activity, (*C*) anti-Xa activity using purified FXa, or (*D*) via HepTest. Also the activated partial thromboplastin time and the prothrombin time was measured wherein none of the compounds displayed significant activity, consistent with their high ratio of anti-Xa/anti-IIa activity (data not shown).

1 by either heparinase II or III does not occur at the G-containing linkage but rather at the I-containing linkage (cleavage at C.1 but not C.2 in Fig. 1). In the case of heparinase II cleavage, the products are a tetrasaccharide of mass 1036.5 and a disaccharide of mass 577.4. For heparinase III cleavage, the same products are observed, namely, a tetrasaccharide of mass 1036.1 and a disaccharide of mass 577.8. These results confirm our assessment that linkages with a reducing end proximate 3-O sulfate are protected from heparinase action, including heparinase II. With both heparinase II and III treatment of Hexa 1, a heparinase-resistant tetrasaccharide with the sequence $\Delta UH_{NAc,6S}GH_{NS,3S,6S}$ is formed. That this tetrasaccharide is resistant to further heparinase cleavage is consistent with previous observations (9). Also consistent with the known substrate specificity of heparinase I, Hexa 1, which contains no I_{2S} linkages, is not cleaved by this enzyme.

In light of these studies, it is now apparent that heparinases I and II can cleave the AT-III site at the $H_{NS,3S,6S}{\downarrow}I_{2S}H_{NS,6S}$ linkage, where the scissile linkage is designated with an arrow (A.2 of Penta 1 and B.2 of Penta 2, Fig. 1). Furthermore, with the newly assigned structure for AT-10, we find that linkages with a 3-O sulfate on the reducing end glucosamine, namely, $H_{NS,6S}{\downarrow}GH_{NS,3S,6S}$ are not cleavable by either heparinase II or III (A.1 of Penta 1 and B.1 of Penta 2, Fig. 1). In addition, we find that this inhibition is entirely the result of the unusual 3-O sulfate modification. Finally, taken together with our previous studies of the enzymatic action of heparinase I, the AT-10 structure reinforces the fact that heparinase I acts in an exolytic, processive manner on heparin/heparan oligosaccharides (11).

AT-III Binding to the AT-10 Decasaccharide. Our sequence analysis of AT-10 reported in the preceding paper (13) revealed that this decasaccharide does not contain an intact AT-III binding pentasaccharide sequence but rather contains only the nonreducing end trisaccharide unit. We sought to extend this sequence assignment and provide a functional context to this result by measuring the AT-III binding affinity of AT-10. At pH 7.4, I = 0.15, AT-10 has very little affinity for AT-III (Fig. 5). Conversely, under the same conditions, porcine intestinal mucosa heparin bound AT-III with an apparent K_D of 36 nM. To measure accurately an affinity of AT-10 for AT-III, the titration was completed instead at pH 6.0, conditions that are known to promote AT-III binding to saccharides. Under these conditions, AT-10 bound AT-III with an apparent K_D of 0.8 μM whereas the K_D for full-length heparin decreased to 10 nM. The measured K_D for AT-10 is comparable with similar saccharides with a truncated reducing end, with measured K_D values of 0.3–2 μM (17). Thus, the results of the titration experiments are consistent with AT-10 containing a partial AT-III pentasaccharide binding sequence (17). That AT-10 still possesses some affinity for AT-III is not surprising considering that the three saccharide units at the nonreducing end of the pentasaccharide sequence, namely, $H_{NAc,6S}GH_{NS,3S,6S}$, are primarily responsible for binding of the native state of AT-III, whereas the reducing end disaccharide unit, $I_{2S}H_{NS,6S}$, which is missing in AT-10, is important for binding the active, conformationally altered AT III. The measured K_D for AT-10 (0.8 μM at pH 6.0, I = 0.05) is ≈100 times higher than that of full-length heparin, confirming that AT-10 does not contain an intact AT-III pentasaccharide sequence. The decrease in AT-III affinity observed for AT-10 cannot be caused simply by a size issue because, in previous studies, the pentasaccharide alone has been shown to have an affinity similar to that of full-length heparin (17). Having measured the binding interaction between AT-III and the decasaccharide, we next sought to define the functional consequences of a HLGAG oligosaccharide that contains only a partial AT-III binding site.

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Biological Activity of AT-10. As might be expected for a oligosaccharide that does not contain an intact AT-III site, the biological activity of AT-10 is less than that of either enoxaparin (used here as an example of a LMWH) or the pentasaccharide, Penta 1 (Fig. 6). Consistent with the known mechanism of heparin-mediated inhibition of thrombin activity by AT-III, neither the decasaccharide nor the pentasaccharide have significant anti-IIa activity (Fig. 6*A*). In the case of the pentasaccharide this lack of activity is entirely the result of its size being insufficient to act as a template for the formation of an AT-III/IIa complex. For the decasaccharide, this size constraint is also a probable explanation (because rigorous biochemical studies have implicated oligosaccharides with at least 18 monosaccharide units being important for efficient complex formation) (2), although the lack of an intact AT-III binding site also may contribute to its reduced anti-IIa activity.

These results are confirmed and extended by examining the anti-Xa activity of the three by using FXa from serum (Fig. 6*B*) or purified FXa (Fig. 6*C*). As has been shown previously, inhibition of FXa by AT-III requires binding of the pentasaccharide motif only concomitant with a conformational change in AT-III. In the anti-Xa assay, both enoxaparin and the pentasaccharide have markedly higher activity than AT-10. The IC_{50} values of enoxaparin and the synthetic pentasaccharide are 66 nM and 39 nM, respectively, whereas that of AT-10 is 10-fold higher at 280 nM. These values are consistent with the lower affinity for AT-III of AT-10 as compared with heparin, which was determined in the AT-III fluorescence titration experiments (Fig. 5). That AT-10 possesses significant anti-Xa activity is not surprising because it can bind to AT-III albeit with lower affinity. The HepTest measurements (Fig. 6*D*) yield similar results, namely, enoxaparin and the pentasaccharide have significantly higher activity than the AT-10 decasaccharide. Taken together, the anti-IIa and anti-Xa activities of the AT-10 decasaccharide as compared with the pentasaccharide and enoxaparin agree well with the AT-III titration experiments as well as the known pharmacology of heparin's mechanism of inhibition of the coagulation cascade.

Summary. We show herein that heparinases I and II cleave the AT-III binding site, leaving behind the trisaccharide unit at the reducing end of the oligosaccharide. We also demonstrate that heparinase III does not cleave the AT-III site because of the presence of a 3-O sulfate on the internal glucosamine residue. Thus, the use of heparinase I or II for the generation of LMWHs requires extreme caution to ensure retaining intact AT-III sites in LMWH fragments. In fact, the results demonstrated herein show that heparinases I or II may be ideal agents for the neutralization of pharmacological doses of heparin (20–22).

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