Mechanisms Responsible for Catalysis of the Inhibition of Factor Xa or Thrombin by Antithrombin Using a Covalent Antithrombin-Heparin Complex*

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Covalent antithrombin-heparin (ATH) complexes, formed spontaneously between antithrombin (AT) and unfractionated standard heparin (H), have a potent ability to catalyze the inhibition of factor Xa (or thrombin) by added AT. Although ~30% of ATH molecules contain two AT-binding sites on their heparin chains, the secondary site does not solely account for the increased activity of ATH. We studied the possibility that all pentasaccharide AT-binding sequences in ATH may catalyze factor Xa inhibition. Chromatography of ATH on Sepharose-AT resulted in >80% binding of the load. Similar chromatographies of non-covalent AT + H mixtures lead to a lack of binding for AT and fractionation of H into unbound (separate from AT) or bound material. Gradient elution of ATH from Sepharose-AT gave 2 peaks, a peak containing higher affinity material that had greater anti-factor Xa catalytic activity (708 units/mg heparin) compared with the peak containing lower affinity material (112 units/mg). Sepharose-AT chromatography of the ATH component with short heparin chains (<12 monosaccharides) resulted in active unbound (40%) and bound fractions (190 and 560 units/mg, respectively). Factor Xa-ATH or thrombin-ATH inhibitor complexes gave chromatograms on Sepharose-AT with more unbound material compared with that of free ATH. Also, ATH did not bind to Sepharose-heparin, and the intrinsic fluorescence due to activation of AT in ATH by its heparin chain was reversed at higher [NaCl] than that required to dissociate non-covalent AT-H complexes. Thus, exogenous AT can compete with the AT moiety of AT for binding to the covalently linked heparin chain, leading to catalytic inhibition of factor Xa or thrombin. These data may suggest that access to pentasaccharide units in non-covalent ATH complexes by free AT may be facile.

Unfractionated standard heparin (H)† is a glycosaminoglycan (GAG) that catalyzes inhibition of the coagulant enzymes factor Xa and thrombin by the serine protease inhibitor (serpin) antithrombin (AT) (1–3). Reaction occurs via the allosteric activation of AT, due to H binding, followed by attack of the enzyme on the reactive center of the inhibitor (4). In the case of thrombin, binding to the heparin chain by the enzyme must also occur for efficient reaction to take place (3). After formation of thrombin-AT or factor Xa-AT inhibitor complexes, affinity of the AT moiety for the heparin chain decreases, leading to release of the catalyst for further reactions with AT and enzyme (5, 6). Although binding to thrombin is through non-selective interaction of negative charges on the GAG with the anion-binding exosite of the enzyme (7, 8), AT binding to H occurs through high affinity to a specific pentasaccharide sequence on the heparin chain (2, 9). Moreover, it has been shown that the rate-determining step for catalysis of thrombin (or factor Xa) inhibition involves the initial binding of AT and H (10).

Previously, we produced a covalent AT-heparin complex (ATH) to further study the mechanism of enzyme inhibition by H-activated AT (11, 12). Surprisingly, although serpin and GAG cannot dissociate, it was observed that ATH could catalyze the inactivation of thrombin (or factor Xa) by added AT (11). In fact, the specific catalytic activity of ATH was ~4-fold greater than that measured for reaction of factor Xa/thrombin + AT with starting H (11). Confirmation that inhibitory activity of ATH against either factor Xa or thrombin (in the presence of exogenous AT) was catalytic in nature became apparent from the fact that many-fold more molecules of enzyme were inactivated in the presence of excess added AT compared with that of ATH alone (11). Furthermore, plasma thrombin generation on fetal distal lung epithelial cells was inhibited more effectively in the presence of ATH than H due, in part, to the formation of thrombin-AT inhibitor complexes from plasma AT (13).

Investigations were carried out to determine components of the mechanisms involved in ATH catalysis of the AT + factor Xa (or thrombin) reactions. AT in the ATH complex has been shown to exist in the activated form due to direct interaction with a high affinity (pentasaccharide) sequence on the covalently attached heparin chain (11). Thus, one possible mode for ATH catalysis of factor Xa/thrombin inhibition would be via the activation of added AT molecules by the pentasaccharide site proximal to the AT component of ATH. However, because of the rapid velocity of the direct thrombin (factor Xa) + ATH reaction (second order rate constants for thrombin + ATH and variance; HMWH, high molecular weight H; LMWH, low molecular weight H; FACE, fluorophore-assisted carbohydrate electrophoresis.

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factor $X_a + \text{ATH}$ are $3.0 \times 10^9$ and $2.4 \times 10^8$ m$^{-1}$ min$^{-1}$, respectively (14); thrombin-$\text{ATH}$ (or factor $X_a$-$\text{ATH}$), inhibitor complexes might be generated before several cycles of the catalytic reaction could take place. Once factor $X_a$ or thrombin had formed a covalent bond with the AT portion of $\text{ATH}$, approach by free serpin and enzyme to the inhibited AT may be restricted due to steric reasons. Another explanation for the potent catalytic activity of $\text{ATH}$ is the possibility that during conjugate synthesis, AT may have selected for a small subpopulation of $\text{H}$ molecules that have more than one pentasaccharide (15). Thus, there are models, catalogue number $X$-$\text{ATH}$ preparations that contain multipentasaccharide GAGs. First, titration of $\text{ATH}$ by AT resulted in a 30–40% increase in intrinsic fluorescence, which is characteristic of AT activation by pentasaccharide binding (11, 14). Second, gel filtration of the non-covalent complexes formed between the heparin released from $\text{ATH}$ by protease treatment and excess free AT showed that $\approx 30\%$ of the heparin molecules were capable of binding $\approx 2$ AT molecules (14). However, assays of $\text{ATH}$ preparations have consistently shown that both anti-factor $X_a$ and anti-thrombin catalytic activities are $\approx 2$-fold greater than that of H with high affinity for AT (11). Thus, it would appear that AT molecules that have a single pentasaccharide ($\approx 70\%$) may also contribute to the observed catalytic potency.

We decided to study the possibility that all pentasaccharide units on $\text{ATH}$ heparin chains may be available to activate added AT molecules for factor $X_a$ (or thrombin) inhibition.

**EXPERIMENTAL PROCEDURES**

### Chemicals

All reagents were of analytical grade. Standard heparin (H) was from Sigma (grade I-A, sodium salt, 15-kDa average molecular mass, from porcine intestinal mucosa (Mississauga, Ontario, Canada)). Human antithrombin (AT) was from Bayer (Mississauga, Ontario, Canada). Nintyhydrin was from Fisher, and SnCl$_2$2H$_2$O and 2-methoxethanol were from Sigma. Stachrom heparin kits (containing the CBS 31.39 substrate for factor $X_a$) were obtained from Diagnostica Stago (Asnières, France) and anti-IIa kits were from American Diagnostica Inc. (Greenwich, CT). Protamine sulfate was obtained as the solid from Protamine Laboratories, Milano, Italy. The heparin values were converted from international units/ml to units/mg by dividing the heparin activity values by the milligram of AT/ml determined by amino acid analysis allowed for calculation of an extinction coefficient for AT in terms of AT concentration. Three different methods were used to evaluate the heparin content in $\text{ATH}$. Heparin mass concentration in stock $\text{ATH}$ solution was analyzed using the carbazole (19), Azure A (20), and Alcian blue (21) techniques. In each case, background measured in samples containing a similar concentration of purified AT was subtracted from values calculated for AT. Standards were prepared from solid commercial Sigma heparin. Moles of heparin in $\text{ATH}$ was derived by dividing the mass of heparin in $\text{ATH}$ samples by the $\text{ATH}$ heparin chain molecular weight. The number average molecular weight ($M_n$) of heparin released from $\text{ATH}$ by exhaustive protease treatment was determined by end group analysis of the $\alpha$-amino group of AT containing amide and short peptide linked to the heparin chains. In brief, $\text{ATH}$ was incubated with protease P-5147 and the heparin chains ($\text{H}$) isolated as described above. $\text{H}$ chains were dialyzed exhaustively against H$_2$O and freeze-dried. Analysis of hydrolyzed $\text{H}$ on the Beckman System 6300 High Performance Analyzer showed only a large free glucosamine peak and one unidentified peak (likely the l-lysyl-uronic acid linkage group reported previously (12)). To quantitate the $\text{H}$ amino acid end group, weighed out samples of $\text{H}$ (in 2 ml of H$_2$O) were incubated with ninhydrin reagent (0.2 ml of a solution prepared from 0.2 g of ninhydrin + 7.5 ml of 2-methoxethanol + 2.5 ml of 4 M sodium acetate, pH 5.5, + 10 mg of SnCl$_2$2H$_2$O with constant stirring and bubbling of a stream of commercial grade N$_2$ at 100 °C for 15 min. After cooling to 23 °C, the absorbance at 570 nm was measured. Absorbance resulting from any primary amino groups present on intrachain glucosamine residues was determined by analysis of similar amounts of freeze-dried samples of disaccharides recovered outside diazoisobutyrones containing reaction mixtures of $\text{ATH}$ + heparinase. Subtraction of the ninhydrin-glucosamine absorbance (a small proportion of the total) from the data gave results with absorbance exclusively due to reaction with the aldehyde-terminal amino acids. Results were compared with that of reactions with alanine standard to determine moles of terminal $\alpha$-amino group. Division of the weight of the sample by the moles of terminal amino groups gave the number average molecular weight per mol of $\text{H}$. Heparin chains released from $\text{ATH}$ by protease were also gel-filtered on a Sephadex G-200 column (Amersham Biosciences) to assess the polydispersity relative to commercial heparin. Finally, the number of moles of AT and heparin in $\text{ATH}$ stock solutions was used to calculate the heparin:AT mole ratio in $\text{ATH}$.

### Anti-Factor Xa and Anti-Factor IIa Assays

Anti-factor $X_a$ and anti-factor IIa activities were determined using the commercially available Stachrom heparin kit. Heparin standards (0.5, 0.4, and 0.8 anti-factor $X_a$ IU/m), controls, or saline (0.9%), diluted in a solution (0.05 M sodium acetate, pH 5.5, + 10 mg of SnCl$_2$2H$_2$O with constant stirring and bubbling of a stream of commercial grade N$_2$ at 100 °C for 15 min. After cooling to 23 °C, the absorbance at 570 nm was measured. Absorbance resulting from any primary amino groups present on intrachain glucosamine residues was determined by analysis of similar amounts of freeze-dried samples of disaccharides recovered outside diazoisobutyrones containing reaction mixtures of $\text{ATH}$ + heparinase. Subtraction of the ninhydrin-glucosamine absorbance (a small proportion of the total) from the data gave results with absorbance exclusively due to reaction with the aldehyde-terminal amino acids. Results were compared with that of reactions with alanine standard to determine moles of terminal $\alpha$-amino group. Division of the weight of the sample by the moles of terminal amino groups gave the number average molecular weight per mol of $\text{H}$. Heparin chains released from $\text{ATH}$ by protease were also gel-filtered on a Sephadex G-200 column (Amersham Biosciences) to assess the polydispersity relative to commercial heparin. Finally, the number of moles of AT and heparin in $\text{ATH}$ stock solutions was used to calculate the heparin:AT mole ratio in $\text{ATH}$.
heparin mass concentrations (mg/ml), as determined by protamine sulfate assay, were as described. 

**Protein Sulfate Assay**—The protamine sulfate assay is an aggregation assay used to determine the heparin mass concentration in a sample (22–24). Briefly, 0.2 ml of 1.0 mg/ml protamine sulfate solution in H2O was added to 0.5 ml of H standards, ATH standards, or unknown samples, followed by immediate vortexing. After 10 min at room temperature, 1.0 ml of 0.1 M arginine was added to the mixture and vortexed, followed immediately by the addition of 2.3 ml of 0.1 M Tris-HCl, pH 8.0, and further vortexing. Absorbances of H standards, ATH standards, or unknown samples were read at 470 nm within 1 h, and unknown samples were read from the appropriate H or ATH standard curve.

**Sephrose-AT Chromatography**—Lyophilized AT powder was reconstituted with 10 ml of sterile H2O from the kit. It was then dialyzed against coupling buffer before conjugation to CNBr-activated Sepharose beads. Conjugation of AT to the beads was done according to the manufacturer’s instructions and resulted in affinity matrix material containing 9.84 mg of AT/ml. Sepharose-AT columns (10 ml, pre-equilibrated with 0.15 M NaCl in 0.01 M phosphate, pH 7.3) buffer were loaded with either ATH (equivalent to 2 mg of AT and 0.6 mg of H), H, AT + H, H*, HMWATHF, LMWATHF, HMWH, or LMWH at similar loading levels (33.9 nmol of each species). After loading, the column was washed with 3 column volumes of 0.15 M NaCl buffer before elution of any bound material with a linear gradient (25 ml of 0.15 M NaCl buffer in the mixing chamber and 25 ml of 2 M NaCl buffer as limit solution). Finally the column was treated with 2 column volumes of 2 M NaCl buffer to ensure equilibration of the column with high salt. NaCl concentrations in the eluate were determined using a conductivity meter (EC Meter, Amber Science Inc., Eugene, OR). Fractions were analyzed for either protein or heparin by measuring absorbance (280 or 215 nm) or taking samples for assay with protamine (as described above), respectively. AT or heparin peaks were dialyzed against H2O, freeze-dried, and reconstituted in 0.15 M NaCl for further assays for activity (anti-factor Xa or anti-IIa, described above). Smaller columns of Sepha-

**Determination of Binding Affinities Using Intrinsic Fluorescence**—Aliquots of enzyme were mixed with 1 ml of 100 nM AT, 100 nM AT + 234 nM H, 100 nM ATH, or 100 nM LMWATHF in 0.02 M Tris-HCl, pH 7.4, to the cuvette, followed by titration with 5 M NaCl, 0.02 M Tris-HCl, pH 7.4, containing either 100 nM AT, 100 nM AT + 234 nM H, 100 nM ATH, or 100 nM LMWATHF, respectively. Thus, the NaCl concentration of AT, AT + H, ATH, or LMWATHF was increased from 0 to 2.25 M. Protein intrinsic fluorescence was measured (after each high salt solution addition (added in 10–100–µl increments) with an excitation at 280 nm and emission detected at 340 nm (with a 290 nm cut-off filter). Excitation and emission slit widths were 5 and 7 nm, respectively. The effect of NaCl on intrinsic fluorescence was deter-

**Statistical Analysis**—Data were compared for significant differences using either the Student’s t test (in the case of two groups) or by analysis of variance (ANOVA, for more than two groups). Upon finding a significant difference within several groups by ANOVA, testing between two groups within that set was carried out by t test. A p value of <0.05 was considered significant, and results were expressed as mean ± S.E.
Physicochemical Analysis of ATH—Stringent analyses of the protein and heparin content of ATH preparations were carried out to verify the heparin:AT mole ratio present in the conjugate. Aliquots of ATH stock solution were treated with HCl, and the hydrolysate was analyzed to determine amino acid content. Given the known sequence for human AT, the number of moles of acid-stable amino acids (alanine and arginine) recovered were used to calculate the molar concentration (in terms of AT) of the original ATH solution. Typical ATH stock solutions were 1.4 × 10⁻⁴ M in AT. Given a molecular weight for AT of 57,769 (calculated from the amino acid sequence and known carbohydrate content (17, 18)), absorbance readings at 280 nm for dilutions of ATH stock solutions gave an extinction coefficient of 0.641 for ATH concentrations of 1 mg of AT/ml. A value of 0.630 obtained for purified human AT was in agreement with that found previously (26). Three separate methods were used to determine the mass concentration of heparin in ATH solution. Background contribution due to AT in the ATH sample was assessed using AT solutions of similar concentration. Although AT control values were low in azure A and Alcian blue heparin assays, a significant value was obtained when the carbazole assay was applied (Table I). The relatively high signal given by AT controls in the carbazole assay was not surprising given that neutral sugars in the N-linked glycans of AT give H₂SO₄ dehydration products that condense with carbazole in the assay procedure (19, 27). Nevertheless, after correction for AT control values, heparin:AT mass concentration ratios were similar for all three assay methods (although precision was reduced for the carbazole procedure). Heparin:AT mole ratios for ATH were calculated from the heparin mass assays, given the number of moles of AT in the stock solution (as determined above) and a number average molecular weight for heparin chains in ATH of 16,900 ± 200 (average ± S.E.; amino acid end group analysis of chains released by protease). Results from the three heparin mass analysis procedures indicated that the heparin:AT mole ratio for ATH was close to 1:1 (Table I). Heparin mass concentration analyses of HMWATHF and LMWATHF using the Azure A method gave results that were proportional to the relative molecular weights of the conjugate heparin chains.

**Sepharose-AT Fractionation of ATH and H**—Chromatography of ATH on immobilized AT resulted in binding of >80% of the load (>70% as a high affinity fraction, Fig. 1A). In order to ensure that the Sepharose-AT column was not overloaded, chromatographies with different loading amounts were run. Similar chromatographies of H gave sizable peaks of unbound material (40% of the total recovery (Fig. 1B)). Sepharose-AT chromatographies of either ATH or H resulted in three distinct peaks as follows: peak 1 appeared in the wash fraction as unbound material, and peaks 2 and 3, which represent low and high affinity products, respectively, were eluted from the column by linear NaCl gradient (0.15 to 2 M). This three peak

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**TABLE I**

Comparative analyses for heparin content in covalent antithrombin-heparin complex

| Analytical method | ATH (µg H/nmol AT) | AT (µg H/nmol AT) | ATH corrected for background at AT (µg H/nmol AT) | HAT in ATH (mol/mol) |
|-------------------|-------------------|------------------|---------------------------------------------|---------------------|
| Carbazole         | 19.27 ± 1.32      | 1.86 ± 0.34      | 17.4 ± 1.3                                  | 1.03 ± 0.08         |
| Azure A           | 19.17 ± 0.19      | 0.17 ± 0.04      | 19.0 ± 0.2                                  | 1.12 ± 0.01         |
| Alcian blue       | 15.6 ± 0.6        | 0.0              | 15.6 ± 0.0                                  | 0.92 ± 0.04         |

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**RESULTS**

Chromatography of covalent ATH, unfractionated heparin, and non-covalent complexes of AT + H on Sepharose-AT. ATH (2 mg in terms of AT) (A), H (0.5 mg) (B), or AT (2 mg) + H (0.5 mg) (C) were chromatographed on 1 (inner diameter) × 12-cm (long) columns of Sepharose-AT (pre-equilibrated with 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3) with 1.3-ml fractions of effluent being collected. After loading, the column was washed for 30 fractions with 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3. Elution of bound material was with a linear gradient (25 ml of buffered 0.15 M NaCl in the mixing chamber and 25 ml of buffered 2 M NaCl as limit solution) followed by 2 column volumes of 2 M NaCl in buffer. Eluted material was detected by A280 (protein) or protamine sulfate assay (heparin (A270 × 4)). Apparent as either unbound, low affinity, or high affinity peaks were eluted from the column, as expected.

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**FIG. 1.** Chromatography of covalent ATH, unfractionated heparin, and non-covalent complexes of AT + H on Sepharose-AT. ATH (2 mg in terms of AT) (A), H (0.5 mg) (B), or AT (2 mg) + H (0.5 mg) (C) were chromatographed on 1 (inner diameter) × 12-cm (long) columns of Sepharose-AT (pre-equilibrated with 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3) with 1.3-ml fractions of effluent being collected. After loading, the column was washed for 30 fractions with 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3. Elution of bound material was with a linear gradient (25 ml of buffered 0.15 M NaCl in the mixing chamber and 25 ml of buffered 2 M NaCl as limit solution) followed by 2 column volumes of 2 M NaCl in buffer. Eluted material was detected by A280 (protein) or protamine sulfate assay (heparin (A270 × 4)). Apparent as either unbound, low affinity, or high affinity peaks were eluted from the column, as expected.
Catalytic Mechanisms of Covalent Antithrombin-Heparin

Covalent antithrombin-heparin complex (ATH), unfraccionated heparin (H), non-covalent mixture of antithrombin (AT) + H (1:1 molar ratio), or heparin from ATH (H') were fractionated on Sepharose-AT. The column was washed with buffer 0.15 M NaCl followed by elution with a linear NaCl gradient (0.15-2.0 M) and final high salt wash (2 M). Elution profiles (determined either by protein absorbance or protamine sulfate heparin mass assay) appeared as three peaks: unbound (peak 1), low affinity (peak 2), and high affinity (peak 3). Fractions comprising each peak were pooled, concentrated, and analyzed for heparin mass and activity (anti-factor Xa kit). Catalytic activities were determined as the ability to accelerate reaction of excess added AT with factor Xa (versus commercially available standard H). The units of activity were divided by the heparin mass to give specific activities (units/mg). Results are given as mean ± S.E. (n = 2). See “Experimental Procedures” for details.

### Table II

| Column load | Anti-factor Xa catalytic activity in units/mg (proportion of each peak as a percent of total eluate given in parentheses) |
|-------------|-----------------------------------------------------------------------------------------------------------------------------|
|             | Peak 1                                                                 | Peak 2                                                                 | Peak 3                                                                 |
| ATH         | 231 ± 7 (12)                                                            | 112 ± 47 (14)                                                          | 705 ± 50 (74)                                                          |
| H           | 0.4 ± 0.2 (41)                                                          | 8.4 ± 1.3 (17)                                                         | 463 ± 28 (43)                                                          |
| AT + H      | 5.0 ± 0.4 (37)                                                          | 7.1 ± 0.1 (17)                                                         | 447 ± 16 (46)                                                          |
| H'          | 1.2 ± 0.1 (7)                                                            | 5.6 ± 0.1 (10)                                                         | 660 ± 5 (83)                                                           |

Pattern (no affinity (unbound peak 1), low affinity (peak 2), and high affinity (peak 3)) was typical of separations of heparin-containing species on immobilized AT. Chromatography of non-covalent AT-H complexes on the AT column led to dissociation of protein and GAG, with free AT running slightly ahead of unbound H, followed by the usual low peak (peak 2) and high (peak 3) affinity gradient eluted peaks (Fig. 1C). The relative proportions and elution positions of peaks produced by Sepharose-AT fractionation of AT + H were essentially the same as those of H alone (compare Fig. 1, B and C) and in agreement with the 45–55% high affinity AT binding observed previously for this commercial H (14). Fractions in the peaks obtained from Sepharose-AT chromatographies were pooled and concentrated. Initial testing of the Sepharose-AT peaks showed that all ATH peaks had significant, direct non-catalytic activity against factor Xa, whereas peaks containing only H were completely inactive. Further assays were done to determine the ability to catalyze inhibition of factor Xa by added AT (catalytic anti-factor Xa assay). All assays for catalytic activity showed high sensitivity and reproducibility. As expected, although unbound and low affinity material from either H or AT + H chromatographies had very low catalytic anti-factor Xa activities (<10 units/mg heparin (Table II)), H with high affinity for AT had significant catalytic activity (463 or 447 units/mg for H or AT-H, respectively (Table II)). Alternatively, assays of unbound and low affinity material from Sepharose-AT chromatograms of ATH had moderate anti-factor Xa catalytic activities (231 and 112 units/mg heparin for peaks 1 and 2, respectively (Table II)), and high affinity ATH contained 1.53 times the activity of high affinity H (t test, p < 0.01). Thus, the vast majority of ATH with potent activity to catalyze the factor Xa + AT reaction was capable of strong AT binding, prior to complexation with a serine protease (i.e., factor Xa or thrombin). In order to verify that the heparin chains of ATH were responsible for the significant binding affinity to immobilized AT, ATH was treated with protease, and the heparin released (H') was purified on DEAE-Sepharose. The anti-factor Xa activity of H' was measured to be 644 units/mg. Gel filtration of H' showed that the ATH heparin chains had a much higher proportion of molecules with longer chain length than standard H (Fig. 2A). Chromatography of H' on Sepharose-AT resulted in 83% of the material having high affinity binding and potent (660 units/mg) anti-factor Xa activity (Fig. 2B and Table II). Therefore, the vast majority of heparin chains in ATH contained catalytically active, high affinity AT-binding sites.

### Sepharose-AT Chromatography of Different Molecular Weight Fractions of ATH and H—In order to further distinguish the sites within ATH heparin chains which were binding to immobilized AT, ATH was fractionated according to molecular weight by gel filtration on Sephadex G-200 under high ionic strength conditions. As a rule, ATH was gel-filtered under high salt conditions (2 M NaCl) to prevent possible binding of the AT moiety in one ATH molecule with the pentasaccharide on the heparin chain in another ATH molecule. However, no differences in size exclusion profiles (which would be indicative of complexes forming due to intermolecular ATH-ATH interactions) were observed under low ionic strength (0.15 M NaCl) conditions. SDS-PAGE analysis of the fractions from ATH material gel-filtered on Sephadex G-200 indicated that subpopulations of ATH molecules with discrete molecular weight ranges could be obtained across the peak (Fig. 3). ATH fractions with high molecular weight (first 2–9% of eluted material) or low molecular weight (last 2–9% of eluted material) were concentrated and designated as HMWATHF or LMWATHF, respectively (Fig. 4A). Because polydispersity of ATH results from variation in length of the heparin chains, HMWATHF and LMWATHF contained covalently linked heparin with greater and smaller numbers of saccharide units, respectively. Previous gel filtration analyses of the heparin chains from HMWATHF and LMWATHF (isolated after protease treatment of the conjugate) indicated that the heparin moieties had >83 and <10 saccharide units, respectively. To further confirm the molecular weight range of heparin in the LMWATHF preparation, protease-treated LMWATHF was gel-filtered on a calibrated Bio-Gel P-6 column (Fig. 5). The peak containing heparin chains was pooled and, after concentration, subjected to fluoro-phore-assisted carbohydrate electrophoresis (FACE) analysis for determination of the chain length. The molecular weight for heparin prepared from the LMWATHF was observed to range from 12 to 4 saccharide units (Fig. 6). Sepharose-AT chromatography of HMWATHF gave trace amounts (3% of recovery) of unbound material, followed by low and high affinity bound peaks (Fig. 4B) that had significant anti-factor Xa activities (210 and 762 units/mg heparin, respectively (Table III)). Thus, the ATH fraction with longer heparin chains tended to have slightly improved binding to exogenous AT with somewhat increased catalytic activities compared with the parent unfractonated preparation. The LMWATHF was further fractionated on Sepharose-AT into a significant amount of unbound material (~40% of recovery), a low affinity peak, and (relative to ATH and HMWATHF) a reduced amount of high affinity material (~30% of recovery (Fig. 4C)). Interestingly, although nearly half of the LMWATHF did not have affinity for exogenous AT, this unbound material possessed significant ability to catalyze reaction of factor Xa and AT (190 units/mg heparin (Table III)). In contrast to the HMWATHF, catalytic activity of the high affinity peak of the LMWATHF was considerably reduced, tending toward the level of that for H (ANOVA for specific activity of LMWATHF versus H, p > 0.05). As a control, H with similar chain lengths to that within HMWATHF and LMWATHF were prepared by gel filtration (Fig. 7A). Chromatography of high molecular weight H (HMWH) on the AT column resulted in <10% in the unbound fraction and an ≈1:3 ratio of low:high affinity peaks (Fig. 7B). Anti-factor Xa catalytic activities of unbound, low affinity, and high affinity HMWH peaks (0.5, 1.7, and 436 units/mg heparin, respectively (Table III)) were similar to the values for the corresponding H peaks (ANOVA, p > 0.05). In comparison, Sepharose-AT fractions of the HMWATHF had increased catalytic activities. The
majority of LMWH molecules (~75%) was unable to bind to AT and had no activity (Fig. 7C), whereas the small proportion of LMWH molecules with high AT affinity had significant activity (277 units/mg heparin (Table III)).

**Analysis of Anti-thrombin (Anti-IIa) Catalytic Activity**—All peaks obtained from chromatographies on Sepharose-AT were analyzed for the ability to catalyze reaction of added AT with thrombin (anti-IIa). The relative anti-IIa catalytic activities of unbound, low affinity, and high affinity peaks eluted from Sepharose-AT were directly proportional to AT-binding strength (Table IV). Furthermore, relative specific activities of peaks from ATH, H, and AT + H chromatographies were similar to those measured by the anti-factor Xa assays (Table II). Anti-IIa catalytic activities (Table IV) of the high affinity material (peak 3) of LMWATHF and particularly LMWH were greatly reduced compared with high affinity peaks of other species (in each case $p < 0.05$ (ANOVA)). This result would be expected given that thrombin requires longer chain heparin molecules in order to bridge both AT and the enzyme (3). The fact that the LMWATHF had any significant anti-IIa activity was interesting, given that the heparin chain length was not likely to bridge both AT and thrombin. Subtraction of the activity due to direct reaction of LMWATHF with thrombin (non-catalytic activity) gave a value of 106 units/mg for the high affinity material (peak 3).

**Sepharose-AT Chromatography of Factor Xa-ATH and Thrombin-ATH Inhibitor Complexes**—Covalent inhibitor complexes were formed by titration of ATH with either factor Xa or thrombin to ~100% equivalence, as shown by the detection of a small amount of remaining activity against chromogenic substrates. Treatment of the Xa- or thrombin-titrated ATH with heparinase, followed by SDS-PAGE, showed that ~95% of the AT (as ATH) had been converted to either factor Xa-AT or thrombin-AT bands. Factor Xa-ATH and thrombin-ATH complexes were used to test the effect of linkage to a serine protease on the affinity of ATH for the immobilized AT. Sepharose-AT chromatographic profiles for factor Xa-ATH and thrombin-ATH were compared with those for ATH fractionated on the same column (Fig. 8). An increase in the unbound fraction and a corresponding small decrease in bound material was noted for the inhibitor complexes relative to that for ATH.
(39, 28, and 12% as unbound material for factor Xa-ATH, thrombin-ATH, and free ATH chromatographies, respectively).

There was, however, no significant change in the position of elution for either low affinity or high affinity peaks due to reaction with factor Xa or thrombin. Analysis of peaks for anti-factor Xa activity showed that for both factor Xa-ATH and thrombin-ATH, unbound (peak 1) and low affinity (peak 2) peaks had significantly decreased activities (120 units/mg), whereas high affinity material (peak 3) had high activity (700–1000 units/mg). Anti-factor Xa assay of preformed factor Xa-ATH or thrombin-ATH complexes showed that activities were decreased relative to that for free ATH (20% for factor Xa-ATH and ~5% for thrombin-ATH). Furthermore, reaction of factor Xa or thrombin with ATH in the presence of added AT resulted in a 20–30% decreased formation of factor Xa-ATH or thrombin-ATH complexes.

Fluorescence Titrations of ATH and AT with NaCl—Intrinsic fluorescence of the protein in ATH or AT + saturating H (2.34-fold molar excess to AT) was measured at increasing NaCl concentrations, and the values were corrected for any changes in the intrinsic fluorescence of control AT that was titrated with NaCl under the same conditions (Fig. 9). For both ATH and AT + H, [NaCl] was inversely proportional to the AT intrinsic fluorescence induced by heparin pentasaccharide binding. However, significantly greater NaCl concentrations were required to reduce the intrinsic fluorescence intensity of ATH compared with that for AT + H (Fig. 9). Fifty % reversal of the heparin-induced intrinsic fluorescence in ATH and AT + H occurred at NaCl concentrations of 0.57 and 0.26 M, respectively. Similar fluorescence titrations of the LMWATHF gave complicated biphasic profiles with the low [NaCl] half of the curve showing very facile reversal of emission intensity,
of non-covalent mixtures of AT gave similar results. Fractionation was not a result of column capacity because application of the column gave 87% binding of the load (Fig. 10). Alternatively, application of AT to the heparin column gave 87% binding of the load (Fig. 10). Application of AT to the heparin column gave 87% binding of the load (Fig. 10).

Inhibition of factor Xa or thrombin by AT is potentiated by H due, in part, to binding of the serpin to a pentasaccharide sequence on the GAG which, in turn, allosterically activates the inhibitor (4). After reaction of factor Xa/thrombin with AT-H, the enzyme-serpin complex dissociates from H leaving the enzyme-serpin complex dissociated from H (Fig. 11). Evidence that the lack of affinity of AT in ATH for Sepharose-heparin, whereas AT in covalent ATH complexes could not. Evidence that the lack of affinity of AT in ATH for Sepharose-heparin, whereas AT in covalent ATH complexes could not.

**TABLE III**

| Column load | Anti-factor Xa catalytic activity in units/mg | Peak 1 | Peak 2 | Peak 3 |
|-------------|--------------------------------------------|--------|--------|--------|
|HMWATHF      | (3)                                        | 210 ± 23 (12) | 762 ± 49 (85) |
|LMWATHF      | 190 ± 53 (39)                             | 174 ± 0.2 (30) | 560 ± 33 (31) |
|HMWH         | 0.5 ± 0.0 (7)                             | 1.7 ± 0.0 (22) | 436 ± 50 (71) |
|LMW           | 0.6 ± 0.1 (75)                            | 1.7 ± 0.0 (7)  | 277 ± 10 (18) |

whereas the latter half of the curve was more coincident with that of ATH (Fig. 9). The proportion of species containing heparin with either low or high non-covalent affinity for AT (as indicated by the fluorescence data for the LMWATHF) was consistent with the Sepharose-AT chromatographic data in which ~40% of the LMWATHF was unbound (low affinity for exogenous AT) and had no catalytic activity (Fig. 4C and Table III).

**DISCUSSION**

Inhibition of factor Xa or thrombin by AT is potentiated by H due, in part, to binding of the serpin to a pentasaccharide sequence on the GAG which, in turn, allosterically activates the inhibitor (4). After reaction of factor Xa/thrombin with AT-H, the enzyme-serpin complex dissociates from H leaving the enzyme-serpin complex dissociated from H (Fig. 11). Evidence that the lack of affinity of AT in ATH for Sepharose-heparin, whereas AT in covalent ATH complexes could not. Evidence that the lack of affinity of AT in ATH for Sepharose-heparin, whereas AT in covalent ATH complexes could not.

**FIG. 7.** Preparation of HMWH and LMWH, followed by chromatography on Sepharose-AT. H (5 mg) was gel-filtered on a 2.6 (inner diameter) × 43-cm (long) column of Sephadex G-200 with 2 M NaCl as irrigant (A). Material in pooled fractions containing either HMWH (first 9% of peak) or LMWH (last 9% of peak) was separately chromatographed on Sepharose-AT (B and C, respectively). Chromatography (as in Fig. 1) gave unbound, low, and high affinity peaks 1–3, respectively.

Steps involved in the turnover of heparin during reaction of factor Xa and thrombin with permanently stabilized ATH. ATH has been shown to exhibit potent catalytic activity in the reaction of AT with factor Xa and thrombin (11). This finding was surprising given that covalently linked AT and heparin were unable to completely dissociate after formation of inhibitor complexes by direct reaction with factor Xa or thrombin (11, 12). One possible mechanism that might explain the catalytic activity of ATH was the presence of a second AT-binding pentasaccharide sequence on the covalently linked heparin chain that was separate from the one that activates the conjugate’s own AT moiety. Although studies showed that ~30–40% of ATH complexes contained 2 pentasaccharide units per molecule (14), specific catalytic activities of the conjugate were ~1.8–2-fold greater than that of the H fraction with high affinity for AT (11). Thus, in order to investigate further the basis for the catalytic properties of ATH, experiments were performed to probe the accessibility of the pentasaccharide site that interacts with the covalently linked AT. Deductions from results of interaction studies with ATH
TABLE IV

| Column load | Anti-Ⅰa activity in units/mg (proportion of each peak as a percent of total eluate given in parentheses) |
|-------------|------------------------------------------------------------------------------------------------|
|             | Peak 1 | Peak 2 | Peak 3 |
|ATH          | 223 ± 9.5 (12) | 146 ± 8.2 (14) | 636 ± 49 (74) |
|H            | 9.1 ± 0.5 (41) | 40.0 ± 5.8 (17) | 266 ± 34 (43) |
|AT + H       | 2.3 ± 0.0 (37) | 32.7 ± 1.6 (17) | 258 ± 9.5 (46) |
|HMWATHF      | 113 ± 2.4 (12) | 564 ± 37 (85) |
|LMWATHF      | 47.0 ± 4.3 (39) | 73.5 ± 10 (30) | 120 ± 5.4 (31) |
|HMWH         | 2.8 ± 0.1 (7) | 12.8 ± 0.1 (22) | 291 ± 33 (71) |
|LMWH         | 3.2 ± 0.1 (75) | 15.7 ± 0.3 (7) | 67 ± 0.1 (18) |

relied on precise determination of the structural components of the conjugate. Previously, we have analyzed the content of heparin in ATH by Alcian blue staining of SDS-PAGE gels of protease-treated ATH and compared the stain density with that of known amounts of standard H using laser densitometry (11). This methodology using Alcian blue staining for heparin quantitation has been validated previously by a number of investigators. We have analyzed a large molecular weight range (1000–30,000) of heparin isolated from heparin starting material (Sigma) or other commercial LMWHs and HMWHs, and we found no significant difference in stain bound per mg of heparin loaded. In fact, we have found previously that the intensity of Alcian blue stain bound per mg of GAG is the same for heparin, heparan sulfate, derman sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate, in agreement with the work of Bartold and Page (28). Many other reports support the validity of cationic staining (Alcian blue and toluidine blue) for quantitation of GAGs with varying molecular weights (29–32).

Small fraction samples were analyzed for heparin using a protamine sulfate turbidimetric assay. This protamine test for heparin was employed because of its very high sensitivity (<1 µg/ml could be detected) compared with other known methods. Also, data showing that similar protamine turbidimetric responses are given for a wide molecular weight range (300–25,000) of Sigma heparin and other heparins have been reported previously with this method (23).

Rigorous analyses of ATH for protein and heparin content gave further verification that the conjugate contained, on the average, one heparin chain per AT molecule. The mole concentration of ATH solutions in terms of AT (calculated from amino acid analyses of acid hydrolysates and the known amino acid sequence) was divided into the number of moles of ATH heparin (number average molecular weight = 16,900) determined from three different mass assay procedures. Analysis of all data resulted in the conclusion that the heparin:AT mole ratio was statistically consistent with that of a 1:1 complex. This outcome verified analyses carried out previously by different methods (Chan et al. (11) found the heparin:AT molar ratio in ATH to be 1.1). Given that ATH molecules contained one heparin chain per AT, the proportions of ATH molecules with different binding affinities could be easily compared by measuring the amount of AT (by absorbance) in each peak. Heparin mass analyses (although less sensitive) of pooled fractions with different affinities for Sepharose-AT confirmed this assumption.

Fractionation of ATH on immobilized AT resulted in >74% high affinity binding (Fig. 1A). Thus, because the vast majority of ATH molecules could form ATH-AT complexes but only a relative minority of the heparin chains in ATH contain two pentasaccharides (14), most of the ATH that possesses only one pentasaccharide was able to bind tightly to exogenous AT. Although the heparin component in ATH remains covalently attached to AT, added AT molecules are able to compete for binding to the pentasaccharide sequence that causes the AT moiety in ATH to be in an active conformation. Similar to covalent ATH, immobilized AT was able to compete for binding to H in ATH complexes, resulting in displacement of the AT (Fig. 1C). Heparin chains from ATH (H') were isolated after protease treatment of the conjugate. Chromatography of H' on Sepharose-AT showed that the vast majority of ATH heparin contained high affinity binding sites (Fig. 2), verifying that the GAG component of ATH has pentasaccharide sites that would be capable of binding to exogenous AT. Further analysis of peaks from the Sepharose-AT chromatographies revealed that binding affinity was directly proportional to the specific catalytic activity. For non-covalently linked heparin, only the material with high affinity binding (peak 3) likely contained heparin molecules with high specificity AT-binding sites (only high affinity peak 3 had significant anti-factor Xa activity (Table II)). In the case of ATH, because the pentasaccharide that interacts with the AT of ATH might be sterically hindered (due to the covalently linked AT), lower affinity material (peak 2) might retain significant catalytic activity that would be exhibited in the anti-factor Xa assays once factor Xa-ATH is formed. In fact, significant catalytic activities were observed in ATH fractions with decreased AT affinity as evidence of this (Tables II and III). ATH fractions with high AT affinity were ~1.53 times greater in anti-factor Xa activity than that of high affinity H material (Table II). Rosenberg et al. (15) have shown previously that the subfraction in commercial H that has two AT-binding sites per molecule has a greater specific activity than that for H with only 1 interaction site for AT (738 USP units/mg compared with 363 USP units/mg) due to greater pentasaccharide density along the chain. Comparison of our present results with those of Rosenberg et al. (15) indicates that the specific activities of high affinity ATH and H are in the range of that for 2 pentasaccharides and 1 pentasaccharide containing H chains, respectively. Closer inspection reveals, however, that the ratio of catalytic activity for 2 pentasaccharide heparins to that for 1 pentasaccharide heparin is significantly greater than the ratio of high affinity ATH activity to high affinity H activity (2.0 compared with 1.53). This would be expected, given that the AT-binding fraction of ATH contains significant amounts of 1 pentasaccharide heparin chains and the AT-binding fraction of H must contain some 2 pentasaccharide molecules.

An alternative hypothesis for the Sepharose-AT binding results was that covalently linked AT may be capable of intermolecular binding to the second (free) pentasaccharide in 2 pentasaccharide ATH molecules. Thus, in some cases, the immobilized AT might be simply dissociating ATH dimers. In an attempt to address this possibility, as well as to confirm the direct interaction of exogenous AT with the intramolecular pentasaccharide-binding site for the ATH, the ATH fraction containing heparin chains that were ≤12 monosaccharides in length (Fig. 6) were isolated. Because ATH of this size (representing ≤5% of ATH preparations) cannot contain 2 pentasaccharides, no excess (free) AT-binding sites are available. Sepharose-AT chromatograms of LMWATHF showed that ~50% bound to AT (Fig. 4C), which gave strong evidence for the direct competition of exogenous AT for the intramolecular pentasaccharide-binding site of the ATH. Furthermore, the specific catalytic activity of heparin chains in LMWATHF complexes that bound to AT (560 units/mg (peak 3, Table III)) was closer to that for high affinity H (463 units/mg (Table II)) than that for
Chromatography of inhibitor complexes of covalent ATH on Sepharose-AT. Covalent inhibitor complexes of ATH with either factor Xa (factor Xa-ATH) or thrombin (thrombin-ATH) were prepared by titration of ATH to equivalence with the appropriate enzyme. Resultant inhibitor complexes (0.25 mg in terms of AT) were loaded onto 1.5-ml columns of Sepharose-AT (pre-equilibrated with 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3). Unbound material (peak 1) was washed off with 40 ml of 0.15 M NaCl, followed by elution of low affinity (peak 2) and high affinity (peak 3) material with a linear gradient (25 ml of buffered 0.15 M NaCl in the mixing chamber and 25 ml of buffered 2 M NaCl as limit solution) and 30 ml of 2 M NaCl. Fractions (1.3 ml) were collected, and the percent of total eluate recovered from each chromatography was calculated.

Through a second pentasaccharide on the covalently linked heparin chain. Control experiments using low and high molecular weight fractions of H gave relatively similar results on Sepharose-AT to those for LMWATHF and HMWATHF. Whereas <30% of LMWH bound to immobilized AT (Fig. 7C), >70% of HMWH bound to the column (Fig. 7B).

Interestingly, the specific activity data given in terms of units/mg illustrate a fascinating property of HMWATHF (or HMWH) chains that have two pentasaccharides compared with LMWATHF (or LMWH) chains with one pentasaccharide. Logically, a heparin molecule that contains one pentasaccharide should have the same activity in units/mg as another heparin molecule that has two pentasaccharides but is twice the chain length. However, Rosenberg et al. (15) showed that although 20,000 molecular weight heparin with two pentasaccharide AT-binding sites had a specific activity of 738 units/mg, 7000 molecular weight heparin with one pentasaccharide had a specific activity of 363 units/mg. Thus, in terms of activity/heparin molecule, Rosenberg and co-worker’s (15) 2 pentasaccharide heparin was 14.8 units/nmol and the 1 pentasaccharide heparin was 2.54 units/nmol! Our results were consistent with this finding, in that high affinity fractions of the HMWATHF and HMWH both had higher units/mg than their low molecular weight counterparts (Table III). Rosenberg et al. (15) recognized this problem and proposed a cooperative mechanism for two pentasaccharide heparin chains, whereby the two AT-binding sites operated synergistically by giving a reduced off-rate. Previously, we have proposed the same rationale to explain why AT selects for enrichment of two pentasaccharide heparin chains during ATH formation (mean free distance of intramolecular diffusion between pentasaccharides in 2-pentasaccharide heparin molecule is less than that for intermolecular diffusion).

As expected, catalytic anti-IIa activity of high affinity fractions of ATH chromatographed on Sepharose-AT was several-fold higher than that for H or AT + H (Table IV). Previously, it has been shown that heparin chains of >18 saccharides in length are required to bridge both AT and thrombin during catalysis of thrombin inhibition (35). Thus, it was surprising that LMWATHF (with heparin chains ≤12 saccharides in length (Fig. 6)) possessed significant (albeit reduced) anti-IIa catalytic activity. We do not have a definitive explanation for this result. One possibility is that the heparin chains in LMWATHF, although short in length, have a higher negative charge density that may assist in greater electrostatic attraction to thrombin.

Further characteristics of the ATH catalytic mechanism

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**Fig. 8.** Chromatography of inhibitor complexes of covalent ATH on Sepharose-AT. Covalent inhibitor complexes of ATH with either factor Xa (factor Xa-ATH) or thrombin (thrombin-ATH) were prepared by titration of ATH to equivalence with the appropriate enzyme. Resultant inhibitor complexes (0.25 mg in terms of AT) were loaded onto 1.5-ml columns of Sepharose-AT (pre-equilibrated with 0.15 M NaCl in 0.01 M phosphate buffer, pH 7.3). Unbound material (peak 1) was washed off with 40 ml of 0.15 M NaCl, followed by elution of low affinity (peak 2) and high affinity (peak 3) material with a linear gradient (25 ml of buffered 0.15 M NaCl in the mixing chamber and 25 ml of buffered 2 M NaCl as limit solution) and 30 ml of 2 M NaCl. Fractions (1.3 ml) were collected, and the percent of total eluate recovered from each chromatography was calculated.

**Fig. 9.** Effect of NaCl concentration on the intrinsic fluorescence of covalent ATH complexes. One ml of buffered solutions (0.02 M Tris-HCl, pH 7.4) of ATH (100 nM), a low molecular weight fraction of ATH (LMWATHF, 100 nM), and non-covalent mixture of antithrombin (AT, 100 nM) + unfraccionated heparin (H, 234 nM) were titrated with buffered 5 M NaCl solutions of ATH, LMWATHF, or AT + H, respectively. Intrinsic fluorescence (excitation = 290 nm, emission = 340 nm) of the solutions at 25 °C was measured after each addition of titrant (μl). Percent of the total difference in fluorescence intensity between that in buffer and at final equilibrium (2.258 M NaCl) was calculated (% F.I.), adjusted for % change in fluorescence of AT alone (at each [NaCl]), and plotted against NaCl concentration.

The corresponding AT-binding peak of ATH (708 units/mg (Table II)), which is in agreement with the fact that most high affinity H molecules have only ~1 pentasaccharide. However, a significant proportion of the LMWATHF was unable to bind to Sepharose-AT. It is possible that the covalent linkage of heparin to AT in ATH may sometimes occur at more internal lysyl residues or the aldose linkage residue may be located at the start of the actual pentasaccharide sequence. Steric hindrance in the conjugates arising from either of these linkage situations might be too difficult for the Sepharose-AT to overcome. Chromatography of HMWATHF leads to almost complete binding of the load (Fig. 4B). Previously, it has been found that multienzyme block H tends to occur on long chain molecules (15, 34). Analyses of the specific activity of the HMWATHF high affinity peak gave results (762 units/mg (Table III)) suggesting that a high proportion of the conjugates contain 2 pentasaccharide chains. Thus, a significant amount of the interactions between the HMWATHF and immobilized-AT may have occurred through a second pentasaccharide on the covalently linked heparin chain. Control experiments using low and high molecular weight fractions of H gave relatively similar results on Sepharose-AT to those for LMWATHF and HMWATHF. Whereas <30% of LMWH bound to immobilized AT (Fig. 7C), >70% of HMWH bound to the column (Fig. 7B).

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Further characteristics of the ATH catalytic mechanism
were delineated. The effect of ATH reaction with factor Xa or thrombin on binding to Sepharose-AT was investigated in order to understand the capability of ATH-inhibitor complexes to catalyze further inhibition. Although >60% of either enzyme-ATH or ATH alone was bound by immobilized AT (Fig. 8), significantly more unbound material was recovered in the case of thrombin-ATH (28%) and, particularly, factor Xa-ATH (39%) inhibitor complexes compared with ATH (18%). Thus, complexation with factor Xa or thrombin may cause added steric hindrance of the ATH pentasaccharide toward exogenous AT. This hypothesis was confirmed by the fact that enzyme-ATH complexes (particularly factor Xa-ATH) had reduced anti-factor Xa activities compared with that of free ATH. Conversely, the presence of a vast excess of added AT inhibited the formation of enzyme-ATH inhibitor complexes, presumably by binding of the exogenous AT to the pentasaccharide site occupied by the AT in ATH. Increased physical obstruction by bound factor Xa or thrombin may be more critical in the case of molecules in LMWATHF that have smaller chain lengths for the initial electrostatic attraction to the immobilized AT (compare with Fig. 4, B and C). These latter results for ATH of varying molecular weights or in the form of inhibitor complexes led to studies of the relative affinity between the pentasaccharide and AT moieties. Direct determination of the binding of AT and heparin in ATH was accomplished by measuring the loss of intrinsic AT fluorescence when activating heparin is displaced (14, 36). Greater [NaCl] was required for 50% reversal of the heparin-induced intrinsic fluorescence in ATH (Fig. 9), which is consistent with the fact that because AT and heparin are covalently linked, complete dissociation is prohibited (regardless of the ionic strength, AT would always be in close proximity to the heparin-binding site). Fluorescence titrations of the LMWATHF with NaCl gave a complicated profile. The fluorescence of the LMWATHF decreased rapidly with small additions of NaCl, followed by a more gradual reduction in fluorescence (similar to ATH) at higher ionic strengths (Fig. 9). Thus, a portion of the molecules in the LMWATHF contain weakly interacting protein and GAG, whereas the remainder have AT and heparin which have strong intramolecular interactions. These data fit with the heterogeneity in affinity of different subfractions of LMWATHF for immobilized AT in that some heparin chains of the LMWATHF are more easily bound by exogenous AT, possibly due to decreased intramolecular association of AT and heparin. Binding of exogenous AT to molecules of LMWATHF that have strong intramolecular AT-pentasaccharide interactions may require longer GAG chains for initial intermolecular-electrostatic attractions. The molecules of the LMWATHF that did not bind to AT represented <3% of all AT molecules. Finally, probing with exogenous heparin showed that although the majority of AT bound to immobilized heparin, almost all AT passed freely through the Sepharose-heparin column (Fig. 10). Lack of ATH binding to immobilized heparin was probably due to strong negative charge repulsion between ATH GAG chains and heparin on the column. Because AT and heparin in ATH cannot dissociate, the heparin in ATH would likely be in too close proximity (on a charge basis) for the immobilized heparin to bind to the AT moiety of ATH. The likelihood that heparin was unable to access the AT in ATH because of electrostatic effects was further evidenced by the binding of Sepharose-heparin to AT in AT-H complexes via dissociation of the H (Fig. 10C). Furthermore, Sepharose-heparin chromatography of a mixture of AT + the heparin chains released from ATH by protease (H') resulted in binding of the AT and a lack of affinity for the H'. Finally, absence of ATH affinity for Sepharose-heparin was a further confirmation that intermolecular binding of ATH to other ATH molecules does not occur.

Originally, it was expected that the pentasaccharide bound by the AT in ATH might be hindered from other molecules because the AT in ATH is also covalently linked and cannot completely diffuse away from the AT-heparin chain (which can occur with AT that is only bound to a heparin molecule via the pentasaccharide (not covalently linked)). Although fluorescence data showed a resistance of the AT in ATH to be displaced from its pentasaccharide-binding site (presumably because the covalent linkage still keeps the AT tethered to the heparin), displacement from these non-covalent interactions by NaCl does occur (Fig. 9). Also, experiments with the heparin produced from protease-treated ATH (H') verified that ATH heparin chains, on their own, interact with immobilized AT and immobilized H in a similar way to that of standard H (pentasaccharide units of ATH heparin are the same as that in standard H).

Results from the study of ATH catalytic mechanisms have several implications. Because exogenous AT can bind to co-

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**Fig. 10. Chromatography of covalent ATH, unfractionated heparin, and non-covalent complexes of AT + H on Sepharose-heparin.** ATH (2 mg in terms of AT) (A), H (0.5 mg) (B), or AT (2 mg) + H (0.5 mg) (C) were chromatographed on 1 (inner diameter) × 12-cm (long) columns of Sepharose-heparin using elution conditions similar to those for Sepharose-AT given in Fig. 1. Eluted material was detected by A, B) (protein) or protamine sulfate assay (heparin (A, H)), and appeared as either unbound, low affinity or high affinity peaks 1–3, respectively. In the case of AT + H chromatographies, unbound (peak 1) AT (●) and H (◇) materials chromatographed separately.
valently linked AT-heparin, interchange of AT in AT-H complexes with free AT may occur through a displacement model. Alternatively, a mechanism can be envisaged in which free AT electrostatically attracts the GAG in AT-H, from the side opposite to that of the covalently linked AT, and causes a rotation of the heparin about its helical axis so that the pentasaccharide is now in the correct orientation for ion/hydrogen bonding to the exogenous AT. Also, because the off-rate of AT bound to the pentasaccharide is relatively rapid (37), the AT in ATH may frequently dissociate from its non-covalent interaction with the pentasaccharide on the covalently linked heparin chain so that exogenous AT may bind and be activated. Notwithstanding which model describes transition states involved in AT complexation with AT-heparin, interchange of AT in AT-H complexes with free AT may occur through a displacement model. Notwithstanding which model describes transition states involved in AT complexation with AT-heparin, interchange of AT in AT-H complexes with free AT may occur through a displacement model.

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