Recently, we developed a covalent antithrombin-heparin complex (ATH) as a possible treatment for respiratory distress syndrome. ATH reacted rapidly with thrombin and efficiently catalyzed the inhibition of either thrombin or factor Xa by exogenous antithrombin. In order to investigate mechanisms for the conjugate's unusual anticoagulant properties, changes in fluorescence due to covalent linkage or addition of exogenous antithrombin were studied in relation to reaction with thrombin derivatives or factor Xa. The emission spectrum of ATH was similar to that of antithrombin plus heparin mixtures. ATH quickly inhibited thrombin or factor Xa activities, as measured by a fluorogenic substrate. Fluorescein-labeled heparin was displaced from either thrombin or active site blocked thrombin by ATH, indicating that thrombin must bind to the conjugate's heparin moiety. Interaction of thrombin with ATH's heparin component was confirmed by a slow reaction rate of conjugate with a thrombin mutant that has weak heparin binding. Total intrinsic fluorescence increased when exogenous antithrombin was added to ATH, indicating that the catalytic mechanism may occur through a second inhibitor binding site. Thus, ATH reacts directly with thrombin through a bridge mechanism and probably catalyzes the reaction of thrombin with antithrombin by a second binding sequence on its heparin chain.

We have recently prepared a covalent conjugate of human antithrombin (AT) and standard heparin (SH) called ATH (1). The rationale for ATH synthesis was to construct an AT derivative that would have a sustained, increased antithrombotic activity and could be retained in the lung as a treatment for coagulation associated with respiratory distress syndrome. ATH was produced by incubation of AT plus SH, and the resultant complex was characterized by both an extremely fast reactivity with thrombin as well as an unexpected ability to catalyze the inhibition of thrombin by exogenous AT (1). Experiments, using a rabbit jugular vein thrombosis treatment model, have shown that ATH also has greater antithrombotic activity in vivo than that of AT plus SH mixtures. The superior antithrombotic activity of ATH was achieved without a significant increase in hemorrhagic side effects, compared with the bleeding observed with SH at similar plasma activity levels. In addition, instillation of ATH in the rabbit lung led to high anti-factor Xa (FXa) activities in lavage fluid taken up to 48 h after instillation with no significant activity appearing systemically (1). Inhibition of plasma thrombin generation on fetal distal lung epithelium by ATH was superior to that by AT plus SH (2). Thus, ATH has several of the properties required to potentially reduce the fibrin accretion that occurs during lung damage.

Given that the rate for direct (noncatalytic) reaction of thrombin with ATH was ~10 times faster than the reaction of thrombin with SH plus saturating amounts of AT (1), there was uncertainty whether direct thrombin inhibition by covalently linked AT and heparin followed the same mechanism as that of the noncovalent AT-SH complex. Covalent linkage of AT and heparin obviates the binding of inhibitor and catalyst, which is the rate-determining step in the reaction with thrombin (3). Further, due to the fact that the reactive site of the AT in ATH is permanently activated by bound heparin (1), it is possible that interaction of thrombin with the ATH heparin chain may not be critical for increased reaction velocity. However, the heparin moiety in ATH may have increased negative charge density compared with SH, since heparin's anticoagulant activity is increased with greater sulfonation (4). More negatively charged heparin chains would more readily attract thrombin, since thrombin binding to glycosaminoglycans is charge-dependent (5).

Catalytic activity of ATH for inhibition of thrombin by exogenous AT has been shown to be approximately 4 and 2 times greater than the corresponding activities for SH and heparin with high affinity for AT, respectively (1). Catalysis of thrombin inhibition by heparin is known to require the binding of AT to a specific heparin pentasaccharide sequence (6). After reaction with thrombin, the AT-thrombin inhibitor complex is released from the glycosaminoglycan chain so that the heparin molecule can bind another AT for thrombin inhibition. The exceptional catalytic activity of ATH was surprising due to the fact that the covalently linked AT cannot dissociate from the complex after reacting with thrombin. One possible explana-
Anticoagulant Mechanism of Antithrombin-Heparin

The second order rate constants for inhibition of thrombin by AFC-67 were both from Enzyme Research Laboratories Inc. (South Bend, IN). The genetic thrombin plus AT reaction (1).

In order to further develop ATH as a therapeutic agent, a clear understanding of its anticoagulant properties is required. Previously, Olson and Shore (7) have shown that there is an increase in the intrinsic fluorescence of AT when it binds to the pentasaccharide sites on heparin. In addition, the emission intensity of fluorescein-labeled heparin changes, dependent on whether it is bound to macromolecules such as thrombin. Therefore, we investigated the mechanisms of both the direct and catalytic anti-thrombin activities of ATH using differential fluorescence and molecular filtration techniques.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**All chemicals were of analytical grade. The AT used in all experiments was human AT from Bayer (Mississauga, Canada). Heparin was either grade I-A, sodium salt, standard heparin (200 anti-factor Xa units/mg, obtained from porcine intestinal mucosa), from Sigma (Mississauga, Canada) or 18,000 molecular weight heparin (sodium salt, 212 anti-factor Xa units/mg, from porcine intestinal mucosa) from Alexis Biochemicals (San Diego, CA). The average molecular weights (determined by gel filtration) of SH, heparin from Alexis Biochemicals, and heparin isolated after protease treatment of ATH were 15,000, 18,000, and 15,000, respectively. AT was prepared, as described previously (1), by incubation of AT and SH at 40 °C in pH 7.3 buffer, followed by purification on butyl-agarose and DEAE-Sepharose fast flow. The 18,000 molecular weight heparin was labeled with fluorescein by incubating 50 µl of 100 µg of fluorescein isothiocyanate (Sigma/mg of dimethyl sulfoxide with 10 µg of heparin dissolved in 300 µl of 1.0 M NaCl concentrate, pH 7.3, at 37 °C, with neutralization of excess reagent with 10 µl of 1 N HCl, pH 7.0, and gel filtration on a PD-10 (Amersham Pharmacia Biotech, Uppsala, Sweden) Sephadex G-25 column in H2O to separate the products. Thrombin and FXa were both from Enzyme Research Laboratories Inc. (South Bend, IN), while phenylalanyl-prolyl-arginyl chloromethyl ketone (Calbiochem). A thrombin mutant (R93) with substitution of alanine at Arg25, Arg27, and Arg101 (8), was a kind gift from Dr. Charles Esmon (Howard Hughes Medical Institute, Oklahoma City, OK). Substrate methyl-0-succinyl-isoueucyl-glutamyl-glycine-ariginyl-7-amino-3-trifluoromethyl-coumarin (AFC-67) was from Enzyme Systems Products (Dublin, CA). The catalytic anti-thrombin activities of ATH using differential fluorescence and molecular filtration techniques.

**Fluorescence Experiments—**All fluorometric determinations were performed using excitation at 280 nm and molecular fluorescence measurements were determined using excitation at 280 nm and emission at 505 nm, while excitation was at 492 nm and emission at 522 nm in experiments using fluorescein-labeled heparin. All slit widths were 5 nm.

**Determination of Rate Constants for Reaction of Thrombin or FXa with ATH—**The second order rate constants for inhibition of thrombin and FXa by ATH were determined. Thrombin (final concentration 2 nM) or FXa (final concentration 1 nM) was added, with rapid mixing (500–1000 rpm), to a fluorescence cuvette containing AFC-67 (final concentration 5 µM) and either ATH (final concentration 10 nM) or AT (final concentration 200 or 100 nM for thrombin or FXa, respectively) in 0.02 M Tris-HCl 0.15 M NaCl, pH 7.4, to make a total volume of 2000 µl. Fluorescence intensity due to the fluorophore released by substrate cleavage was monitored every 0.5 s. Fluorescence was plotted in order to determine the progressive enzyme inhibition. Curves were fitted to the data using the following equation: fluorescence intensity = k9 t + (c0 − c9 t)/(1 − e−k9 t)/k8, where c0 represents final steady-state concentration, c9 is the initial rate constant, k8 is the apparent rate constant for the change from c9 to c0, and t is time in minutes (10). Division of the apparent rate constant (k9) for enzyme inhibition by the inhibitor concentration gave the second order rate constant (k8) for the reaction. In some reactions, SH was included in the inhibitor solution, prior to enzyme addition, at a final concentration of 0.06–6000 µg/ml. Similar experiments were performed with R93 (final reaction concentration = 2 nM).

**Binding Studies—**The interactions between various AT and heparin derivatives were investigated in order to further characterize the mechanisms for direct thrombin inhibition by ATH. Previous workers have demonstrated that the intrinsic fluorescence of AT is increased when noncovalently bound to heparin (7). Thus, the effect of protamine sulfate or polybrene addition on the interaction between AT and heparin in ATH or SH mixtures (with or without the presence of FPR-thrombin) was determined by monitoring changes in intrinsic protein fluorescence (340 nm). Alternatively, fluorescein-labeled heparin (molecular weight 18,000) was mixed with either thrombin or FPR-thrombin, and the increase in emission from the fluorophore, due to decreased thrombin binding, was measured after addition of excess ATH or free heparin. Given that molecular weight 18,000 heparin was appropriate, that it was previously determined that heparin chains in ATH are slightly longer than those of SH (~18,000 versus 15,000 molecular weight (1)).

An examination of possible mechanisms for ATH's catalytic activities involved determination of the modes in which exogenous AT could bind to the conjugate. A mixture of 125I-AT plus excess ATH was gel-filtered on a Sephadex G-200 column (2.6-cm internal diameter × 46-cm height) in 0.15 M NaCl in order to determine if noncovalent 125I-AT-ATH complexes could form. To further evaluate AT binding to ATH, excess AT was added to ATH, and the resultant fluorescence was compared with the sum of emissions from similar quantities of AT and ATH to determine any net increase in fluorescence. Using the value for the increase in fluorescence of AT, when saturated with SH, the amount of AT bound to excess AT binding sites on AT could be calculated. A mixture of 125I-AT and 18,000 molecular weight heparin in ATH, the protein was removed from the AT glycaminoglycan chains. One mol of 125I-AT of 1.76 mg of heparin in 0.02 µmol phosphate 0.15 M NaCl, pH 7.3 (phosphate-buffered saline), plus 2 mg of P-5147 protease in 1.2 ml of 0.5 M Tris-HCl, pH 8.0, were heated in a sealed tube at 37 °C for 24 h, followed by the addition of 0.2 ml of 10 mg of P-5147/ml of 0.5 M Tris-HCl, pH 8.0, every 24 h up to a total of 10 ml of 0.5 M Tris-HCl, pH 8.0. This mixture was added to 125I-AT-ATH complex mixture applied to a DEAE-Sepharose Fast Flow column (15 ml of beads) equilibrated in the same buffer. After washing the column with 0.01 M Tris-HCl, 0.25 M NaCl, pH 8.0, the protein was eluted with 0.01 M Tris-HCl, 2.0 M NaCl, pH 8.0, and concentrated by pressure dialysis (under N2 versus 0.15 M NaCl. Heparin concentrate was gel-filtered on a 2.6-cm (diameter) × 46-cm (height) Sephadex G-200 column in 2 M NaCl, with heparin containing fractions being pooled and pressure-dialyzed against phosphate-buffered saline. The final heparin isolated from ATH was free of AT and contaminating protease, as judged by alcin blue (for heparin) and Coomasie Blue (for protein) staining after SDS-polyacrylamide gel electrophoresis (11). The capacity of the heparin from ATH to bind AT was compared with that for SH and 18,000 molecular weight heparin by measuring the increase in AT fluorescence (due to association with the heparin) when the protein was added to a known amount of glycosaminoglycan. In addition, either SH or heparin prepared from ATH was mixed with excess AT, followed by gel filtration on Sephadex G-200 in 0.15 M NaCl. AT present in the collected fractions was determined by absorbance measurements at 215 nm, and the proportion of AT bound to heparin (peaks higher in molecular weight than that of free AT) was calculated. Fluorescence of heparin combined with AT prior to gel filtration, the number of molecules of AT bound per heparin molecule could be estimated. Analysis of fractions (by SDS-polyacrylamide gel electrophoresis followed by alcin blue staining for glycosaminoglycan) from some of the Sephadex G-200 chromatographies was used to confirm recovery of heparin during gel filtration.
RESULTS

Effect of ATH Formation on AT Structure—Conformational changes in AT due to covalently bound heparin were assessed by comparing the emission properties of ATSH to ATH when each was excited at 280 nm. The intrinsic fluorescence of the AT component of ATH (nM fluorescence at 340 nm = 5.06) was elevated by 29% compared with native AT (nM fluorescence at 340 nm = 3.91). To determine if the difference in fluorescence intensities of ATH and AT was due to heparin binding, both AT-containing compounds were titrated with heparin. The addition of heparin to AT solution caused an increase in fluorescence (33%), which reached a maximum value slightly greater than that of ATH (Fig. 1). In contrast, the addition of heparin to AT had no significant effect on fluorescence intensity. Since changes in topology of proteins can be more clearly determined by analyzing a variety of unsaturated residues (which emit at different wavelengths), the spectra of both ATH and ATSH, with or without added heparin, were obtained. As observed in the single wavelength measurements, the intensity of the fluorescence scan of ATH was greater than the scan of AT (Fig. 2). No difference in emission from 310–360 nm was detected for ATSH compared with ATH plus added heparin. However, a dramatic increase in the fluorescence of AT was seen when combined with heparin. Interestingly, although the profiles of the emission spectra of ATH and AT plus heparin matched from $\lambda_{\text{max}}$ (340.5 nm) up to higher wavelengths, there was an increasing divergence from 325 to 310 nm (Fig. 2). Thus, even in the presence of heparin, the fluorescence intensity of AT was slightly decreased at the shorter wavelengths compared with ATH.

Reaction Rates of ATH or AT with Coagulant Enzymes—Rates of thrombin or FXa inhibition by either ATH or AT were determined. Results are shown in Table I. The second order rate constant for reaction of thrombin with ATH was 5000 and 4 times faster than for reaction with AT and AT plus heparin, respectively. The inclusion of exogenous heparin in reactions with thrombin plus ATH caused a significant decrease in the reaction velocity (Table I). In contrast, the second order rate constants for reaction of FXa with either ATH or AT plus heparin were comparable. Also, in the case of either ATH or AT, reactions with FXa were not inhibited by exogenous heparin at concentrations up to 40 $\mu$m (600 $\mu$g/ml, Table I). Inhibition of the FXa reaction with AT or ATSH was only observed with very concentrated heparin solutions (0.4 mM).

Mechanistic Studies of the Direct Inhibition of Thrombin by ATH—Since reaction of thrombin with ATH was significantly faster than the corresponding reaction of thrombin with AT + heparin ($3 \times 10^9 \pm 0.5 \times 10^9$ M$^{-1}$ min$^{-1}$ versus $7 \times 10^8 \pm 0.7 \times 10^8$ M$^{-1}$ min$^{-1}$; t test, $p < 0.01$), a number of experiments were performed to determine the relative importance of ATH's protein and glycosaminoglycan components. The addition of either protamine or polybrene (both of which bind to negatively charged heparin chains) to either ATH or AT plus heparin caused a reduction in the intrinsic fluorescence to levels that approached that of AT alone (Table II). These results indicated that a strong positively charged species could interfere with the noncovalent interactions between the heparin component in ATH and its endogenous AT moiety. Only the noncovalent binding of heparin and AT in ATH was affected, since native polyacrylamide gel electrophoresis of ATH plus protamine showed that ATH and the polycation migrated, slowly, as one large complex, without the release of AT (data not shown). Similarly, interaction between AT and heparin in either ATH or AT plus heparin mixtures bound to FPR-thrombin (active site-blocked thrombin) was decreased by polybrene, as shown by the characteristic drop in intrinsic fluorescence (Table II). The importance of binding of thrombin to the heparin constituent of ATH, during inhibition, was evaluated by competitive binding studies and using thrombin derivatives. Results are given in Table III. Both ATH and heparin were capable of displacing fluorescein-labeled heparin from active $\alpha$-thrombin. This was evident because the decrease in fluorescence due to binding of labeled heparin by thrombin was partly reversed.

![Fig. 1. Titration of covalent antithrombin-heparin complex or antithrombin with heparin](image1.png)

![Fig. 2. Fluorescence spectra of covalent antithrombin-heparin complex and antithrombin with or without added heparin](image2.png)
Enzymes (1–2 nM) were rapidly mixed with solutions containing inhibitors (≤10 nM) and the fluorogenic substrate APC-67 under pseudo-first-order conditions. Apparent first order rate constants for inhibition of enzyme substrate activity (calculated using the following equation: fluorescence intensity = v_f \cdot t + (v_i - v_f) \cdot (1 - e^{-kt})/k$ where $v_f$ represents the final steady-state rate, $v_i$ is the initial rate, $k$ is the apparent rate constant for the change from $v_i$ to $v_f$, and $t$ is the time in minutes) were divided by inhibitor concentration to give second order rate constants ($k_2$).

### TABLE I

| Exogenous [Heparin] | $k_2$ for IIa + ATH | $k_2$ for IIa + AT | $k_2$ for Xa + AT | $k_2$ for Xa + ATH |
|---------------------|---------------------|---------------------|---------------------|---------------------|
| µg/ml               | $\mu^{-1} min^{-1}$ | $\mu^{-1} min^{-1}$ | $\mu^{-1} min^{-1}$ | $\mu^{-1} min^{-1}$ |
| 0                   | 3.0 \times 10^5     | 6.0 \times 10^5     | 2.3 \times 10^6     | 9.9 \times 10^5     |
| 0.06                | ND                  | 5.5 \times 10^5     | ND                  | 7.0 \times 10^6     |
| 0.6                 | 2.8 \times 10^6     | 6.0 \times 10^5     | 2.4 \times 10^6     | 5.1 \times 10^7     |
| 6                   | 2.6 \times 10^5     | 7.3 \times 10^5     | 2.2 \times 10^6     | 1.8 \times 10^7     |
| 60                  | 4.8 \times 10^5     | 2.4 \times 10^5     | 2.3 \times 10^6     | 2.1 \times 10^7     |
| 600                 | 6.8 \times 10^5     | 3.1 \times 10^5     | 1.7 \times 10^6     | 2.1 \times 10^7     |
| 6000                | 6.0 \times 10^6     | ND                  | 2.5 \times 10^7     | ND                  |

\(^a\) IIa, thrombin. 
\(^b\) ND, not determined.

### TABLE II

**Effect of polybrene on the interaction of antithrombin and heparin in covalent antithrombin-heparin complex bound to active site-blocked thrombin**

The effect of polybrene (PB) on noncovalent binding of the AT and heparin components of ATH or mixtures of AT plus SH, in the presence of active site-blocked thrombin (FPR-IIa), was determined by measuring intrinsic fluorescence (excitation/emission = 280/340 nm; slit widths = 2 nm). Elevated fluorescence due to heparin binding by AT was decreased with polybrene, indicative that noncovalent AT-heparin interactions were reduced. Concentrations of ATH, AT, SH, and active site-blocked thrombin used in the experiments were 100, 100, 1000, and 100 nM, respectively. The [polybrene] is 125 µg/ml. Results with protamine sulfate (PS; 250 µg/ml) are also shown.

| Components in solution | Intrinsic fluorescence intensity |
|------------------------|---------------------------------|
| ATH                    | 295                             |
| ATH + FPR-IIa          | 795                             |
| ATH + FPR-IIa + PB     | 730                             |
| AT                     | 173                             |
| AT + SH                | 246                             |
| AT + SH + FPR-IIa      | 720                             |
| AT + SH + FPR-IIa + PB | 635                             |
| SH                     | 0                               |
| PS                     | 122                             |
| AT + PB                | 172                             |
| AT + SH + PB           | 173                             |
| AT + SH + PS           | 180                             |
| AT + SH + PS           | 173                             |

### TABLE III

**Interaction of covalent antithrombin-heparin complex with either thrombin or active site-blocked thrombin**

Displacement of fluorescein-labeled 18,000 molecular weight heparin (H) from either thrombin (IIa) or active site-blocked thrombin (FPR-IIa) by ATH or unlabeled (molecular weight 18,000) heparin (H) was evaluated by fluorescence measurements (excitation/emission = 492/522 nm). Increased fluorescence of labeled heparin indicated interference with binding to the thrombin species. The concentrations of labeled heparin, thrombin, active site-blocked thrombin, ATH, and heparin used in the experiments were 50, 100, 100, 623, and 1988 nM, respectively. Average values for $n = 3$ trials are shown.

| Components in solution | Fluorescence intensity |
|------------------------|------------------------|
| F-H (for IIa experiments) | 485                  |
| F-H + IIa              | 465                  |
| F-H + IIa + ATH        | 477                  |
| F-H + IIa + H          | 479                  |
| F-H (for FPR-IIa experiments) | 478          |
| F-H + FPR-IIa          | 444                  |
| F-H + FPR-IIa + ATH    | 464                  |
| F-H + FPR-IIa + H      | 467                  |

Up upon the addition of ATH or heparin (Table III). Removal of the fluorescein-heparin by ATH was due to competition for thrombin's heparin binding site and did not require reaction of thrombin with ATH's AT component, because a similar displacement of labeled heparin from FPR-thrombin could be achieved with ATH (Table III). Percentage reversals of the decrease in fluorescence of fluorescein-heparin, resulting from interaction with thrombin, by ATH (60 ± 3%) or heparin (71 ± 3%) were similar ($p = 0.16$, Student's t test). The reversal of fluorescein-heparin fluorescence reduction, due to FPR-thrombin binding, by ATH (59 ± 3%) compared with heparin (68 ± 2%) was also similar ($p = 0.15$). Further confirmation that interaction of thrombin with the ATH heparin moiety was important for inhibition was obtained from experiments with a thrombin derivative (R93) that had reduced binding to heparin due to a mutation at its anion binding exosite 2 (8). The second order rate constant for R93 reaction with ATH was calculated to be ~100 times slower than that for reaction with native α-thrombin (2.6 \times 10^7 M^{-1} min^{-1}), compared with 3.0 \times 10^9 M^{-1} min^{-1} (Table I) for native thrombin.

### DISCUSSION

SH has been used widely as an anticoagulant for treatment of thrombotic complications (12). Clinical application of heparin has reduced both mortality and morbidity of deep vein thrombosis and pulmonary embolism (13, 14). Additionally, heparin has been effective as a prophylactic for intravascular thromboembolic events (15).

Despite the successful treatment of a number of coagulopathies, heparin has several limitations. SH has a relatively short, dose-dependent, intravenous half-life (16). Although the plasma half-life of low molecular weight heparin is longer than SH (17), both SH and low molecular weight heparin have impaired activity against clot-bound thrombin (18). Furthermore, heparin’s effectiveness at anticoagulation within the vascular system cannot readily be extended to the treatment of extravascular thrombosis.

Prothrombotic activity occurring in the immature lung dur-
ing neonatal respiratory distress syndrome results in conversion of fibrinogen to fibrin and the formation of a hyaline membrane network within the intra-alveolar space (19, 20). Fibrin has been shown to cause impairment of surfactant function (21) and fibroblast proliferation, leading to bronchopulmonary dysplasia (22). Introduction of active heparin species into the lung of premature infants might prevent the procoagulant effects associated with neonatal respiratory distress syndrome. However, it has been demonstrated that heparin instilled into the airways of dogs was rapidly lost to the vascular compartment (23).

ATH has overcome many of the difficulties involved with heparin use. The plasma half-life of ATH (β-phase of 13 h (1)) was found to be longer than that for SH (0.32 h (1)) or subcutaneously administered low molecular weight heparin (peaking in plasma by 3 h and undetectable after 12 h (24)). Similarly, unlike heparin, activity from ATH instilled intratracheally into the lungs of rabbits could be recovered in lavage fluid 48 h after introduction, with no detectable conjugate in the circulation. Studies using a rabbit venous thrombosis treatment model have shown that ATH is superior (on an equimolar basis) to AT plus SH mixtures for reduction of clot size and fibrin accretion. Thus, ATH may be able to effectively inhibit clot-bound thrombin. However, in order to efficiently administer ATH in the treatment of thrombosis, ATH’s modes of action must be understood. In particular, the mechanisms involved in the rapid direct reaction of ATH with thrombin and the unexpected ability of ATH to catalyze thrombin inhibition by AT need to be further elucidated.

The functions of ATH’s components were investigated using differential fluorescence and molecular size fractionation techniques. Spectral analyses revealed that the AT in ATH was in

![FIG. 3. Binding of antithrombin to covalent antithrombin-heparin complex. Gel filtration of either 460 ng of 125I-AT plus 4 mg of unlabeled AT or 460 ng of 125I-AT plus ATH (equivalent to 4 mg in AT and 1 mg in heparin) were performed on a Sephadex G-200 column (2.6-cm diameter × 46-cm height) with 0.15 M NaCl as eluant. Loading volumes were 1 ml, and 3.72-ml fractions were collected. Elution of 125I-AT plus unlabeled carrier AT ( ) resulted in a peak at fraction 38, while a shift in radioactivity to a peak at fraction 17 occurred when 125I-AT was gel-filtered in the presence of ATH (○).](http://www.jbc.org/content/jbc/233/14/34734/F1.large.jpg)
a conformation which resembled that of AT activated by non-covalent heparin binding (Fig. 2). However, intrinsic fluorescence of ATH at lower wavelengths was slightly greater than that for noncovalent AT-SH complex; a result that may indicate a difference in the environment around tyrosyl residues in the conjugate (25, 26). Alternatively, interactions between the AT and heparin of ATH at nontryptophanyl residues may be different from the corresponding noncovalent binding of AT and SH, leading to modified allosteric effects at ATH tryptophan 49 (27). The reaction of thrombin with ATH was ~4 times faster than with AT plus SH (Table I), which is similar to results obtained before using a different technique (1). Also, as shown previously (1), thrombin reaction with ATH was inhibited by the addition of excess heparin. The reaction velocities of FXa plus ATH and FXa plus AT plus SH were similar, and exogenous heparin did not significantly affect the inhibition of FXa by ATH up to concentrations of ≤0.6 mg/ml (Table I). Thus, similar to reactions with AT, binding of ATH to thrombin may be competitively inhibited by binding of excess heparin to the enzyme (which does not occur with FXa), suggesting that interaction of thrombin with the ATH heparin chain might be important.

Studies of the mechanism for direct reaction of thrombin with ATH gave more evidence that inhibition may involve noncovalent interaction of the conjugate’s heparin chain to both the ATH protein component and thrombin. The addition of polybrene to ATH, with or without the presence of active site-blocked thrombin, caused a decrease in ATH’s intrinsic fluorescence. The magnitude of the polybrene-induced reduction of AT fluorescence correlates with the increase in fluorescence that occurs when heparin associates with AT (7). Thus, the AT and heparin moieties of ATH interact in a fashion similar to that of free AT and SH.

Further studies were undertaken to determine whether formation of the ATH complex obviated the template mechanism ascribed to reaction of thrombin with AT in the presence of heparin (thrombin and AT are co-localized by simultaneous binding of both enzyme and inhibitor to the heparin chain). The decreased emission from fluorescein-labeled heparin, resulting from binding to either active thrombin or FPR-thrombin, could be reversed by 60–70% with either AT or free heparin (Table III), which demonstrated that ATH could displace heparin from thrombin. Thus, reaction of AT with thrombin probably involves binding of thrombin to the ATH glycosaminoglycan chain. Interestingly, neither AT nor heparin alone could return the fluorescence of fluorescein-heparin to that observed prior to the thrombin (or FPR-thrombin) addition. A possible explanation is that some aromatic residues on thrombin may have a small quenching effect on the free fluorescein-heparin. The likelihood that binding of thrombin to the heparin component in ATH was important for inhibition was confirmed by a 100-fold slower second order rate constant for reaction of ATH with a thrombin mutant that has decreased affinity for heparin.

Investigations into the catalytic property of ATH gave further evidence that at least part of the catalysis is due to the presence of more than one AT binding site on some ATH heparin chains (1). Gel filtration of 125I-ATH plus excess AT showed that AT could form a complex with the conjugate (Fig. 3). A net increase in the intrinsic fluorescence of AT, when added to ATH, suggested that exogenous AT could bind to a pentasaccharide site on the ATH heparin component that was distinct from the region of noncovalent interaction with ATH’s endogenous AT (Fig. 4). Removal of the protein from ATH allowed direct examination of noncovalent binding of AT with ATH heparin. Fluorescence titrations by AT of ATH heparin, SH, or molecular weight 18,000 heparin illustrated that heparin isolated from ATH contained significantly more AT binding sites (Fig. 5). Combining known amounts of heparin from AT with excess AT, followed by gel filtration to separate bound and free protein, allowed for determination of the number of AT binding sites/heparin molecule (Fig. 6). It was calculated that the heparin obtained from ATH contained ~1.5 AT/glycosaminoglycan chain (Fig. 6). Also, it was clear that a portion of the ATH heparin molecules had at least two AT binding sites, because a peak was observed with a molecular weight equal to two AT plus one heparin (Fig. 6).

There are a number of reasons why the AT synthetic method may have resulted in molecules with heparin chains that have increased affinity for thrombin as well as AT. During the preparation, AT would rapidly bind to SH pentasaccharides (6), followed ultimately by the formation of irreversible Schiff bases and slow Amadori rearrangement (28). Thus, heparin molecules with pentasaccharide sequences proximal to the aldehyde terminus would more likely form adducts with AT. Rosenberg et al. (29) have shown that a small proportion (~1–3%) of SH molecules contain two or more high affinity AT binding sites. Statistically, these multipentasaccharide-bearing molecules would have a greater probability of binding AT. Furthermore, if an AT is bound to the site distal to the aldoe terminus on a two pentasaccharide-bearing heparin chain, intramolecular migration to the proximal AT binding sequence would occur more readily (mean free distance being smaller) than intermolecular diffusion. Therefore, although a minor subpopulation in SH, two or more pentasaccharide-containing heparin molecules may have been preferred for AT-heparin conjugation, which agrees with the suggested mechanism for AT catalytic activity. In addition, since movement by AT between two or more pentasaccharides on a heparin molecule would be assisted by a high negative charge density, thrombin binding to heparin in the resultant ATH complex would be enhanced (5).

The proposed mechanisms for the direct and catalytic inhibition of thrombin by ATH have a number of clinical implications. Strong binding of thrombin to ATH heparin could be important for the displacement of thrombin from fibrin monomer in clots. Moreover, the presence of a second pentasaccharide in ATH molecules would ensure the catalysis of thrombin by exogenous AT after ATH’s endogenous AT becomes linked to thrombin. However, further work is still required to assess the degree to which the pentasaccharide interacting with ATH’s endogenous AT may be involved in catalysis, especially after formation of ATH-thrombin complex. Nevertheless, the present work has demonstrated that binding of thrombin to the heparin component of ATH and activation of exogenous AT by multi-pentasaccharide ATH conjugates is an important mechanism related to ATH’s anticoagulant properties.

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Investigation of the Anticoagulant Mechanisms of a Covalent Antithrombin-Heparin Complex

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J. Biol. Chem. 1998, 273:34730-34736.
doi: 10.1074/jbc.273.52.34730

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