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Inhibition of Fibrin-Bound Thrombin by a Covalent Antithrombin-Heparin Complex[1]

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Numerous studies have shown that fibrin-bound thrombin (IIa) is protected from inhibition by antithrombin (AT) + heparin (H) due to the formation of a ternary fibrin·IIa·H complex. We investigated factors affecting the inhibition of fibrin·IIa by a covalent complex of AT and H (ATH). The rate of IIa reaction with ATH was decreased 2–3-fold by fibrin monomer as compared to 57-fold for AT + heparin with high AT affinity. Furthermore, although the reaction of AT + H with a IIa mutant with decreased H binding (RA-IIa) was inhibited 2–3-fold in the presence of fibrin, reaction rates of ATH + RA-IIa were not reduced by fibrin. The relative difference in the effect of fibrin on the ATH reaction with RA-IIa compared to that for reactions of AT + H with RA-IIa is consistent with the fact that, in the absence of fibrin, the rate of the ATH reaction with RA-IIa relative to IIa was much less reduced (8-fold) compared to the corresponding reactions of AT + H (decreased 306 fold). Similarly, the addition of excess H in the absence of fibrin gave only a small decrease in rate of ATH + IIa reaction. However, in the presence of fibrin, the addition of 40-fold excess H decreased the rate of ATH inhibition of IIa by 1 order of magnitude. Experiments with ATH containing low molecular weight heparin chains with low AT affinity showed that IIa inhibition requires ATH with long chains that activate the AT moiety. Finally, electrophoresis of fibrin ± (^{125}I-)IIa ± (^{125}I-)ATH on native and denaturing gels showed that ATH forms ATH-IIa complexes that remain bound to fibrin through the ATH component. Thus, ATH is a potent inhibitor of fibrin-bound IIa, likely due to the formation of fibrin·ATH-IIa as opposed to fibrin·IIa·H ternary complexes.

Key words: antithrombin-heparin, fibrin, heparin, rate, thrombin.

Heparin (H) is an anticoagulant glycosaminoglycan (GAG) that acts *in vivo* by accelerating the inhibition of thrombin (IIa) and factor Xa by antithrombin (AT) (*1–4*). Potentiation occurs through the formation of non-covalent AT·H complexes in which AT is bound to a high affinity pentasaccharide sequence on H (*5–7*). Binding to H causes a change in conformation of AT to a form that is activated towards reaction with its target serine proteases (*8, 9*). In the case of IIa, inhibition by AT·H also requires binding of the enzyme to the H chain in order to form a bridge between IIa and the inhibitor (*10*). Once the AT·H·IIa complex is formed, a covalent reaction occurs between the active site of IIa and the reactive centre loop of AT (*11, 12*). The affinity of H for AT in the AT-IIa inhibitor complex is decreased, which leads to the release of the H for catalysis of another AT + IIa reaction (*13, 14*).

The presence of fibrin has been shown to interfere with the inhibition of IIa by AT + H (*15, 16*). When IIa reacts with fibrinogen to form fibrin, the resultant polymerizing fibrin continues to bind the IIa and absorbs the enzyme into the developing clot (*17, 18*). Studies of the neutralization of fibrin- or clot-bound IIa have indicated that reactions with either AT + unfractionated heparin (UFH) or AT + low molecular weight heparin (LMWH) proceed at reduced rates (*19*), requiring 5- to 9-fold greater H concentrations for inhibition, equal to that with free IIa (*20*). Binding to fibrin occurs via the IIa anion binding exosite I (*18, 21*), which is involved in interactions of the enzyme with a number of substrates and inhibitors (*21, 22*). The addition of AT·UFH to fibrin-bound IIa results in the formation of a ternary complex between fibrin, IIa and UFH (*23*), which decreases IIa activity towards its substrates/inhibitors by changing the active site environment (*24*). Thus H, which has dissociated from AT·H, bridges binding sites on fibrin and the anion binding exosite II on IIa to assist in protection from inhibition (*25*). Moreover, it has been shown that UFH can even promote IIa binding to fibrin monomer and fibrin clots (*26*).

We have developed a covalent complex of AT and heparin (ATH) that has significant anticoagulant activities (*27*). ATH was shown to react rapidly with IIa (*27, 28*) to form a stable covalent ATH-IIa complex (*27*). Furthermore, ATH also possesses a potent ability to catalyze the inhibition of factor Xa or IIa by added AT (*27*). *In vitro* experiments

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showed that ATH is superior to AT + UFH as an inhibitor of plasma IIa generation on fetal distal lung epithelial cells (*29*). *In vivo*, ATH was found to have a prolonged intravenous half-life in rabbits compared to non-covalent mixtures of AT and UFH (*27, 30*). Additionally, ATH anticoagulant activity was retained in the rabbit lung air space for at least 2 days, without any systemic loss (*27*). Thus, there was evidence that ATH may be useful in the control of thrombin generation on alveolar epithelium in the pulmonary airspace, a factor that affects the progression of neonatal and adult respiratory distress syndromes (*31–35*). Further experiments were performed to determine the antithrombotic effects of ATH. Treatment of preformed clots in the rabbit jugular vein with ATH caused a reduction in clot size and fibrin accretion compared to increased clot size and accretion with the administration of equimolar amounts of non-covalent AT·UFH (*30*). Although the increased intravenous half-life of ATH allowed for a longer period of systemic anticoagulation during treatment, the fact that clot size was actually diminished suggested that ATH might be effective for inhibiting fibrin-associated IIa. Therefore, we decided to investigate possible mechanistic factors affecting the rate of the ATH reaction with fibrin-bound IIa.

EXPERIMENTAL

Materials—All reagents were of analytical grade. Human AT was from Bayer (Mississauga, ON, Canada) and UFH was from Sigma [grade I-A, Na salt, 15,000 average molecular weight, from porcine intestinal mucosa (Mississauga, ON, Canada)]. DEAE-Sepharose Fast Flow, Sephadex G-200 and cyanogen bromide-activated Sepharose were from Amersham Pharmacia Biotech (Uppsala, Sweden). Plasminogen-free human fibrinogen, human IIa and human factor Xa (Xa) were from Enzyme Research Laboratories (South Bend, IN, USA). The IIa chromogenic substrate *N*-*p*-tosyl-gly-pro-arg-*p*-nitroanilide (tGPR-pNA), hexadimethrine bromide (polybrene), gly-pro-arg-pro-amide (GPRP-NH_2), gelatin agarose and butyl agarose were from Sigma. S-2288 was from DiaPharma (West Chester, OH, USA). $Na^{125}I$ was from New England Nuclear (Mississauga, ON, Canada) and the IODO-BEADS® iodination reagent was from Pierce (Rockford, IL, USA). IIa with reduced binding affinity for H [designated RA-IIa, with exosite II mutations $arg^{93} \rightarrow$ ala, $arg^{97} \rightarrow$ ala, and $arg^{101} \rightarrow$ ala (*36*)] was a kind gift from Charles T. Esmon, Howard Hughes Medical Institute, Oklahoma City, OK, USA. Low molecular weight heparin (LMWH) was obtained from UFH by gel filtration on Sephadex G-200 to get a small molecular weight cut. The LMWH was recycled on the gel filtration column to obtain a fine fractionation with H of 1,000–4,000 Da molecular mass.

High Affinity and Low Affinity Heparin—Unless otherwise stated, all kinetics experiments were carried out using heparin (H) with high affinity for AT. H with high affinity for AT was prepared by Sepharose-AT chromatography of UFH. Sepharose-AT was prepared by reaction of AT with cyanogen bromide–activated Sepharose according to the manufacturer's instructions (approximately 2–5 mg AT per ml of packed gel). UFH (10 mg in 1 ml of 0.05 M Tris-HCl 0.15 M NaCl pH 7.4 buffer) was loaded onto a 50 ml column that was pre-equilibrated with buffer. Unbound, low affinity heparin was eluted with 2 column volumes of buffer. The unbound fraction containing low affinity heparin was exhaustively dialyzed versus H_2O, freeze-dried and stored dry at 23°C for future use. Bound high affinity H was eluted from the column with 90 ml of 2 M NaCl; 3 ml fractions were collected. H-containing fractions were identified [by Alcian blue staining (*27*)], pooled and treated with 3 volumes of ethanol. The precipitated material was dissolved in starting buffer and rechromatographed on the column. After repeating the chromatographic procedure 3 times, the final ethanol precipitate was redissolved in 0.15 M NaCl, and the H concentration in the resultant solution determined by a protamine sulfate turbidimetric assay (*13*). The high affinity H had an anti-Xa activity of 280 units/mg H and a mass average molecular mass of 15,000 Da, as determined by high-pressure liquid chromatographic gel filtration (*37*).

Preparation of Covalent Complexes—The synthesis of covalent antithrombin-heparin complex (ATH) has been described previously (*27, 38*). Briefly, AT (1.2 mg/ml) + UFH (66.7 mg/ml) was heated in 0.02 M phosphate, 0.15 M NaCl, pH 7.3, buffer at 40°C for 14 days, followed by the addition of 1 volume of 0.5 M $NaBH_3CN$ per 9 volumes of reaction mixture and a further heating at 37°C for 5 h. ATH was purified by a 2-step procedure utilizing butyl agarose hydrophobic chromatography to remove excess UFH, followed by DEAE Sepharose Fast Flow anion exchange chromatography to remove unreacted AT. The resultant ATH eluted from DEAE Sepharose Fast Flow was concentrated to 8.77 mg/ml AT (ATH protein extinction coefficient at 280 nm = 0.75), 2.23 mg/ml H (protamine sulfate turbidometric assay) using pressure dialysis versus 0.02 M phosphate, 0.15 M NaCl, pH 7.3. Concentrated ATH was stored at –80°C. ATH was also prepared by incubating AT with LMWH (molecular mass 1,000–4,000 Da) and purifying the product (ATLMWH) as described above. AT conjugated to low affinity heparin (ATLAH) was prepared in a similar manner. Also, ATH containing the fraction of LMWH with low affinity for Sepharose-AT (ATLMWLAH) was obtained by taking the low molecular weight fraction of ATLAH chromatographed on Sephadex G-200.

Preparation of Soluble Fibrin Monomer—Contaminating fibronectin was removed from the commercial fibrinogen by 2 incubations of 15 ml of 130 μM fibrinogen (molecular mass 340,000 Da) with 5 ml of gelatin agarose for 30 min, followed by centrifugation and collection of the fibrinogen-containing supernatant. The fibrinogen concentration was determined by absorbance at 280 nm using $\varepsilon^{1\%} = 15.1$ (*19*) (after correction for light scatter at 320 nm using the equation corrected $A_{280} = A_{280} - 1.7 \times A_{320}$ (*39*)). Soluble fibrin monomer was prepared by the following method. Purified fibrinogen (60–100 μM) was incubated with IIa (2 nM) at 37°C for 4–6 h, followed by centrifugation at 2,000 ×*g* for 5 min. The fibrin polymer pellet was placed in a dialysis bag (12,000–14,000 molecular weight cut-off), dialyzed versus H_2O (4°C) to remove fibrinopeptides A and B and then further dialyzed versus 0.02 M acetic acid until the fibrin dissolved (~8 h). The concentration of the soluble fibrin in solution was obtained by absorbance at 280 nm using a molecular weight of 340,000 and $\varepsilon^{1\%} = 14.0$ (*40*). Typically, 100 μM soluble fibrin was obtained and stored at –80°C. Soluble fibrin in 0.02 M acetic acid was neutralized with 40% v/v 1 M Tris-HCl pH 7.5 and polymerization was

blocked with 5 mM GPRP-NH_2 (*41*) to give soluble fibrin monomer for kinetics experiments.

Measurement of the Rate of Protease Inhibition—The rates of IIa, RA-IIa, and Xa reactions with inhibitors were measured discontinuously under pseudo first-order conditions (molar ratio of enzyme:inhibitor was ≤ 0.1). Aliquots of 20 nM IIa, 40–120 nM RA-IIa or 40 nM Xa (at 2-times final reaction concentration) were incubated in up to 8 separate wells of 96-well round-bottomed microtiter plates (Fisher, Nepean, ON, Canada) for 5 min at 23°C in 0.02 M Tris-HCl, 0.15 M NaCl, 0.6% polyethylene glycol 8000, pH 7.4 (TSP), containing 0.01 M GPRP-NH_2 ± H (0–10,000 nM) ± fibrin monomer (0–4,000 nM). An equal volume of either AT + H, ATH, ATLMWH, ATLAH, or ATLMWLAH (all at ≥ 10 times the enzyme concentration) was added to each well at time intervals ranging from 2–600 s. Termination of the reactions was carried out by the simultaneous addition to each well of 200 μl of a 10 mg polybrene/ml solution containing 222 μM tGPR-pNA substrate. Remaining enzyme activity was calculated from the rate of substrate hydrolysis determined by measuring the change in absorbance at 405 nm using a Spectra Max 340 Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The pseudo first-order rate constants for the inhibition reactions were calculated according to Eq 1.

$$V_t/V_o = e^{-k_1 t} \quad (1)$$

where: V_o = enzyme activity at time = 0 s, V_t = enzyme activity at time = t, and k_1 = the pseudo first-order rate constant. Apparent second order rate constants (k_2) were calculated by dividing k_1 values by the inhibitor concentrations. Due to the extremely rapid rates of enzyme inhibition at the concentrations of inhibitor and enzyme used, some reactions were carried out in the presence of S-2288, which acts as a competitive inhibitor (*42*). In cases where the competitive inhibitor (S-2288) was used, the pseudo first-order rate constant of inhibition was calculated according to Eq 2.

$$k_1 = (k_{app}) \times (1 + [S]/K_m) \quad (2)$$

where: k_{app} = the apparent pseudo first-order rate constant, [S] = the concentration of competitor and K_m = the Michaelis-Menton constant of enzyme for S-2288 (3 μM for thrombin, 2,000 μM for Xa). In a few experiments, pseudo first-order rate constants were calculated using equation 2 and compared to those calculated using equation 1 where direct measurement in the absence of S-2288 could be made using the same inhibitor concentrations. Equations 1 and 2 were confirmed to give equivalent values in this system. The effects of fibrin or H on the second-order rate constants for reaction of IIa, RA-IIa, or Xa with ATH were determined. Effects on rates for similar reactions with AT + H, ATLMWH, ATLAH, or ATLMWLAH were measured and comparisons made.

Binding Experiments—Detailed investigations of the complexes formed from combinations of ATH, fibrin, and/or IIa were carried out to determine the interactions that occur between components in the system. Buffer (0.02 M Tris·HCl, 0.15 M NaCl, 0.1% polyethylene glycol, pH 7.4) ± fibrin monomer (4000 nM final concentration combined with 5 mM (final) GPRP-NH_2) ± IIa (17 nM final) ± UFH (495 nM final concentration) ± inhibitor [either ATH or AT (17 nM final)] were mixed and incubated at 23°C for 10 min. In different experiments, ATH, AT, or IIa was spiked with ^{125}I-labeled ATH, AT, or IIa, respectively [proteins labeled using Na^{125}I and IODO-BEADS®, according to the manufacturer (Pierce)]. In order to determine if the H moiety in ^{125}I-ATH is involved in binding to fibrin monomer, UFH was added to a final concentration of 222 μM in some experiments. After incubation, the mixtures were combined with either 2-mercaptoethanol containing SDS sample buffer or 5% glycerol in buffer. Two-mercaptoethanol/SDS samples were heated at 100°C for 1 min and electrophoresed in SDS-PAGE gels according to the method of Laemmeli *et al* (*43*). Alternatively, samples in 5% glycerol only were electrophoresed in non-denaturing gels (no SDS). The gels were dried and autoradiography was performed. The degree of migration of radiolabeled species in the non-denaturing (native) gels indicates covalent and non-covalent associations that occur between the radiolabeled molecules and other non-radioactive components in the incubation mixtures. The degree of migration in the reducing SDS-PAGE gels verifies the formation of covalent complexes between radiolabeled molecules and non-radioactive molecules in the incubation mixtures.

Fluorescence Studies—Experiments were carried out to evaluate alterations in physical properties that occur during the interaction of ATH with fibrin-bound IIa. Fluorescence spectral determinations were used to assess any changes in environment (due to changes in conformation or binding) of tryptophanyl (and tyrosinyl) residues in the ATH/IIa/fibrin polypeptide chain(s). All fluorometric determinations were made, with rapid stirring in 1 × 1 cm quartz fluorescence cuvettes, in a cuvette chamber heated at 25°C, using a Perkin Elmer LS50B luminescence spectrometer. Experiments were performed in 0.02 M Tris·HCl, 0.15 M NaCl, pH 7.4, buffer. Intrinsic fluorescence measurements of the proteins were obtained at an excitation wavelength of 280 nm (with a 290 nm filter). The excitation slit width was 10 nm and the emission slit width was 7 nm. Mixtures of IIa (400 nM final concentration) ± ATH (400 nM final) ± fibrin monomer [1,000 nM final in the presence of GPRP-NH_2 (5 mM final)] in buffer were prepared and the fluorescence spectra obtained within 10 min. Differences in fluorescence spectral profiles were noted due to the addition of fibrin to IIa and/or ATH. Time course changes in peak fluorescence intensity (at 340 nm) were also recorded immediately after the addition of the various mixture components.

Statistics—Data were compared for significant differences using either 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 ± SEM with n ≥ 2.

RESULTS

Inhibition of IIa by ATH or AT + H in the Presence of Fibrin—ATH or non-covalent AT·H (AT bound to the fraction of UFH with high affinity to AT) was reacted for different times with IIa in the presence of varying concentrations of fibrin monomer (with added GPRP-NH_2 peptide to prevent polymerization) and the reaction rates were determined. Rate values (k_2) for the reactions of IIa with

either ATH or AT + H were plotted versus the concentration of added fibrin. The results are shown in Table I. Increasing fibrin concentration caused a decrease in the rate of IIa inhibition for both ATH and non-covalent AT + H mixtures. However, fibrin had a much smaller effect on the inhibition of IIa by ATH than on the inhibition of IIa by AT + H. Reaction rates for IIa inhibition by ATH decreased up to 3-fold in the presence of 4,000 nM fibrin, while rates of IIa reaction with AT·H were reduced by 57-fold when fibrin was present (Table I). Thus, fibrin monomer had 20 times as great an effect on inhibiting IIa reactions with AT·H compared to reactions with ATH. Additional experiments were carried out to determine the effect of fibrin on Xa inhibition by ATH. No decrease in the rate of Xa reaction with ATH was observed when fibrin monomer was added (Table II).

Inhibition of RA-IIa by ATH or AT + H in the Presence of Fibrin—In order to study the structural aspects of IIa that may be important for the differing effects of fibrin on reactions with ATH compared to AT + H, a IIa mutant (RA-IIa) with reduced binding to high affinity H was used. Direct reaction of RA-IIa with ATH was markedly slower than the corresponding reaction with IIa (Table I). However, the rate of RA-IIa inhibition by AT + H mixtures was decreased (relative to rates of IIa + AT + H reaction) to an even greater degree than that of the corresponding reactions with ATH. Thus, while ATH reacted at a 7.6-fold slower rate with RA-IIa compared to IIa, the reaction of AT + H with RA-IIa was more than 2 orders of magnitude slower than that with IIa (Table I). Increasing concentrations of H in the RA-IIa (10 nM) + AT (100 nM) + H (100 or 500 nM) reaction gave a several-fold increase in rate (6.68×10^5 and 2.0×10^6 M^{-1} min^{-1} for 100 and 500 nM H, respectively), indicating that RA-IIa still retained some H-binding ability (albeit greatly reduced). When fibrin was added to the system, the rate of RA-IIa inhibition by AT + H was only moderately decreased compared to reactions in the absence of fibrin (Fig. 1). Alternatively, there was no reduction in the reaction rate when fibrin was added to reactions of RA-IIa + ATH (Fig. 1 and Table I). In comparison, ATH was more reactive with the RA-IIa mutant than AT·H, regardless of whether fibrin was present.

Effect of Added H on the Inhibition of IIa by ATH with or without Fibrin—The importance of the H moiety of ATH for the inhibition of fibrin-bound IIa was investigated by carrying out IIa + ATH reactions in the presence or absence of added H and/or fibrin. The rate of IIa inhibition by ATH was decreased by only 10% when equimolar H was added (Fig. 2). The addition of a 40-fold molar excess of H caused a reduction of 1.3-fold in the reaction velocity. Alternatively, when 4,000 nM fibrin was present in reactions containing 10 nM IIa + 100 nM ATH, the addition of 4,000 nM H

TABLE I. **Effect of soluble fibrin monomer on the rate of inhibition of either α-thrombin (IIa) or thrombin with reduced heparin-binding affinity (RA-IIa) by either covalent antithrombin-heparin complex (ATH) or non-covalent mixture of antithrombin (AT) and heparin (H).** Pseudo first-order rate constants (k_1) were determined under pseudo-first order conditions using a discontinuous inhibition assay for remaining enzyme chromogenic activity. Apparent second order rate constants (k_2) were calculated by dividing k_1 values by the inhibitor concentrations. The effects of fibrin on the inhibition of IIa or RA-IIa by ATH or AT + H were assessed. Values are means ± SEM with $n \geq 2$. ND = not determined.

Enzyme	[Fibrin] (nM)	Reactions with ATH k_2 (M^{-1} min^{-1})	Reactions with AT + H k_2 (M^{-1} min^{-1})
IIa	0	$2.26 \pm 0.093 \times 10^9$	$3.02 \pm 0.051 \times 10^8$
	500	$2.19 \pm 0.063 \times 10^9$	$7.93 \pm 0.784 \times 10^6$
	1,000	$1.83 \pm 0.029 \times 10^9$	ND
	4,000	$1.01 \pm 0.127 \times 10^9$	$5.25 \pm 0.271 \times 10^6$
RA-IIa	0	$2.97 \pm 0.497 \times 10^8$	$9.85 \pm 2.71 \times 10^5$
	4,000	$5.50 \pm 0.970 \times 10^8$	$5.28 \pm 0.002 \times 10^5$

TABLE II. **Effect of fibrin on the inhibition of factor Xa (Xa) by covalent antithrombin-heparin complex (ATH).** Pseudo first-order rate constants (k_1) were determined under pseudo-first order conditions. Apparent second order rate constants (k_2) were calculated by dividing k_1 values by the inhibitor concentrations. The effect of fibrin on the inhibition of Xa by ATH was assessed. Values are means ± SEM with $n \geq 2$.

[Fibrin] (nM)	Relative rate for reaction of Xa with ATH k_2 (M^{-1} min^{-1})
0	$2.10 \pm 0.04 \times 10^5$
500	$2.54 \pm 0.25 \times 10^5$
4,000	$2.35 \pm 0.04 \times 10^5$

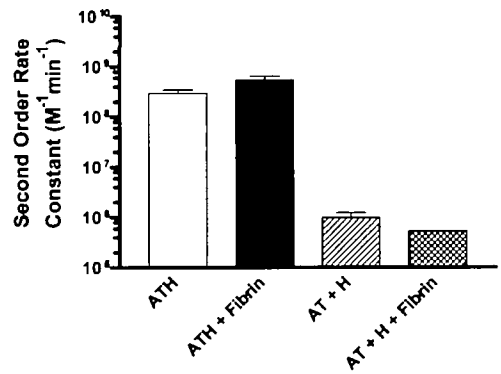

Fig. 1. **Effect of fibrin on the rate of inhibition of a thrombin variant (RA-IIa) by either covalent antithrombin-heparin complex (ATH) or a non-covalent mixture of antithrombin (AT) and heparin (H).** The effect of fibrin on the rates of inhibition of a thrombin variant (RA-IIa) with reduced H-binding affinity was studied. Inhibition of RA-IIa was either by ATH or a non-covalent mixture of AT + H. Apparent second-order rate constants were calculated as described in the text. Values are means ± SEM.

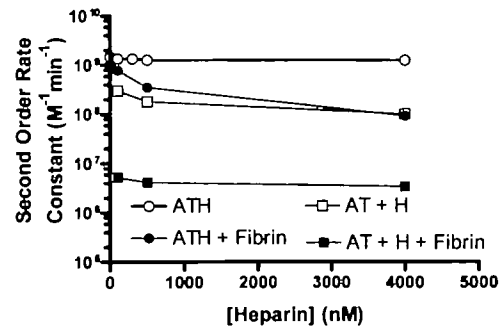

Fig. 2. **Effects of heparin (H) and fibrin on the rate of inhibition of thrombin (IIa) by either covalent antithrombin-heparin complex (ATH) or a non-covalent mixture of antithrombin (AT) and heparin (H).** The effect of varying H concentration on the rate of IIa inhibition by either ATH or AT was determined in the presence or absence of fibrin. Second-order rate constants were calculated as described in the text. Values are means ± SEM.

caused a 12-fold decrease in the rate of IIa inhibition compared to that in corresponding experiments without added H (Fig. 2). Thus, the addition of a significant molar excess of exogenous H decreased the rate of inhibition of IIa by ATH much more in the presence than in the absence of fibrin. In the case of the IIa reaction with AT, a large molar excess of H gave a significantly smaller rate reduction (Fig. 2).

Heparin Chain Length and Pentasaccharide Dependence of the ATH Reaction with IIa—The effect of H chain length and pentasaccharide content in ATH on the inhibition of IIa was studied. In comparison with ATH, the rates of the IIa reaction with ATH containing short H chains (ATLMWH) or ATH prepared using the fraction of UFH with low affinity for AT (ATLAH) were significantly lower (Fig. 3). In particular, the inhibition of IIa by ATLMWH was about 12-fold slower than the corresponding reaction with ATH. However, the inhibition of IIa by ATH containing a low molecular weight fraction of low affinity heparin (ATLMWLAH) was reduced by 2 orders of magnitude compared to native ATH (> 450-fold, Fig. 3). Therefore, both H chain length and AT affinity (pentasaccharide content) are critical for ATH reaction with IIa. In comparison, reaction rates of ATLMWH and ATLAH with Xa were also decreased compared to that with ATH (Fig. 4). As expected, the reaction velocity of ATLMWH with Xa was much less decreased compared to that of ATLMWH with IIa, given the fact that, unlike IIa, the inhibition of Xa with AT + H does not require the binding of the enzyme to the H chain (*44*)

Binding Experiments—Mixtures of (^{125}I-)ATH and fibrin monomer showed the formation of non-covalent complexes in non-denaturing gels (Fig. 5A). The formation of these non-covalent (^{125}I-)ATH·fibrin complexes was prevented by the presence of high H concentrations (222 μM). The non-covalent nature of the complexes between ATH and fibrin was verified by the fact that the radiolabeled ATH could be dissociated from the fibrin in SDS gels under reducing conditions (Fig. 5B). In contrast, mixtures of (^{125}I-)AT + UFH + fibrin monomer showed no binding of AT to fibrin or UFH [AT migrated as free AT on native gels (Fig. 5A)]. Control

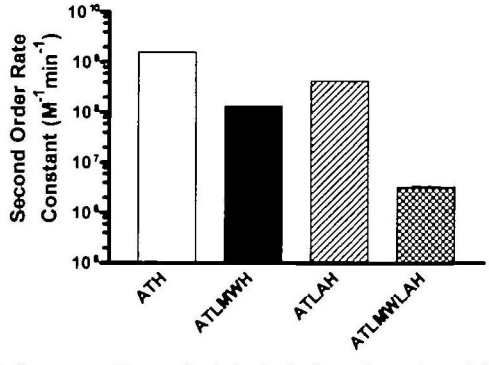

Fig. 3. **Influence of heparin (H) chain length and antithrombin (AT) binding site (pentasaccharide) content on the inhibition of thrombin (IIa) by covalent antithrombin-heparin complex.** Covalent antithrombin-heparin complex (ATH) was compared with covalent complexes of AT and a low molecular weight heparin fraction (ATLMWH), AT and a heparin fraction with low AT-affinity (ATLAH), or AT and a low molecular heparin fraction with low AT-affinity (ATLMWLAH). The second order rate constants of IIa inhibition by the various inhibitors were calculated as described in the text. Values are means ± SEM.

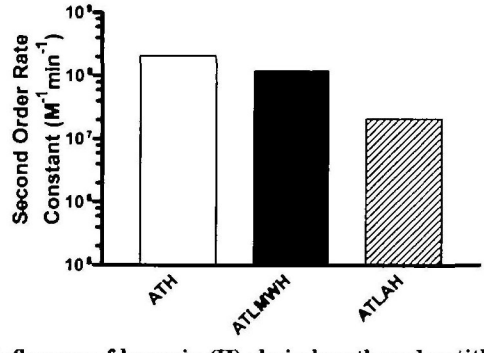

Fig. 4. **Influence of heparin (H) chain length and antithrombin (AT) binding site (pentasaccharide) content on the inhibition of factor Xa (Xa) by covalent antithrombin-heparin complex (ATH).** The second order rate constant for Xa inhibition by ATH was compared to that for the reaction of Xa with covalent complexes of AT and a low molecular weight heparin fraction (ATLMWH), or AT and a heparin fraction with low AT-affinity (ATLAH). Values are means ± SEM.

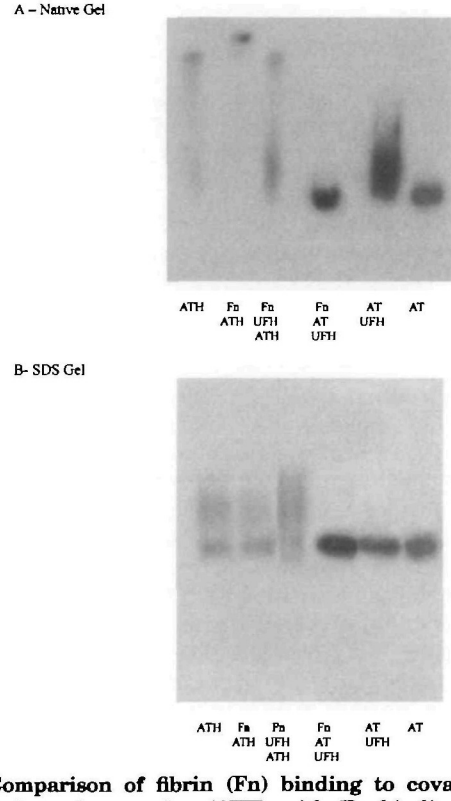

Fig. 5. **Comparison of fibrin (Fn) binding to covalent antithrombin-heparin complex (ATH) with Fn binding to antithrombin (AT) in non-covalent mixtures of AT and unfractionated heparin (UFH).** Solutions of ^{125}I-labeled covalent antithrombin-heparin complex (ATH) ± unfractionated heparin (UFH) or ^{125}I-labeled antithrombin (AT) ± UFH were prepared in the absence or presence of soluble fibrin monomer (Fn). Subsamples of the solutions were either mixed with 5% glycerol in buffer and electrophoresed on native gels under non-denaturing conditions (panel A) or heated for 1 min at 100°C with 2-mercaptoethanol/SDS and electrophoresed on SDS gels under denaturing conditions (panel B). Autoradiograms of the dried gels are shown.

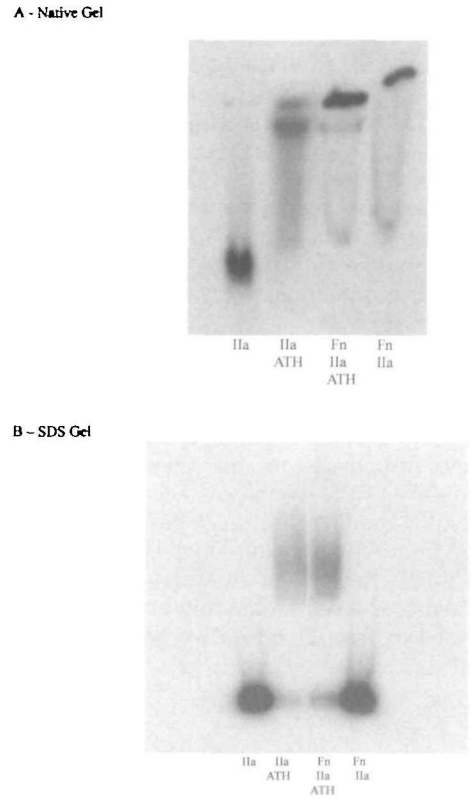

Fig. 6. **Binding of thrombin (IIa) to covalent antithrombin-heparin complex (ATH) in the presence of fibrin (Fn).** Solutions of ^{125}I-labeled thrombin (IIa) ± soluble fibrin monomer (Fn) ± covalent antithrombin-heparin complex (ATH) were prepared. Subsamples of the solutions were either mixed with 5% glycerol in buffer and electrophoresed on native gels under non-denaturing conditions (panel A) or heated for 1 min at 100°C with 2-mercaptoethanol/SDS and electrophoresed on SDS gels under denaturing conditions (panel B). Autoradiograms of the dried gels are shown.

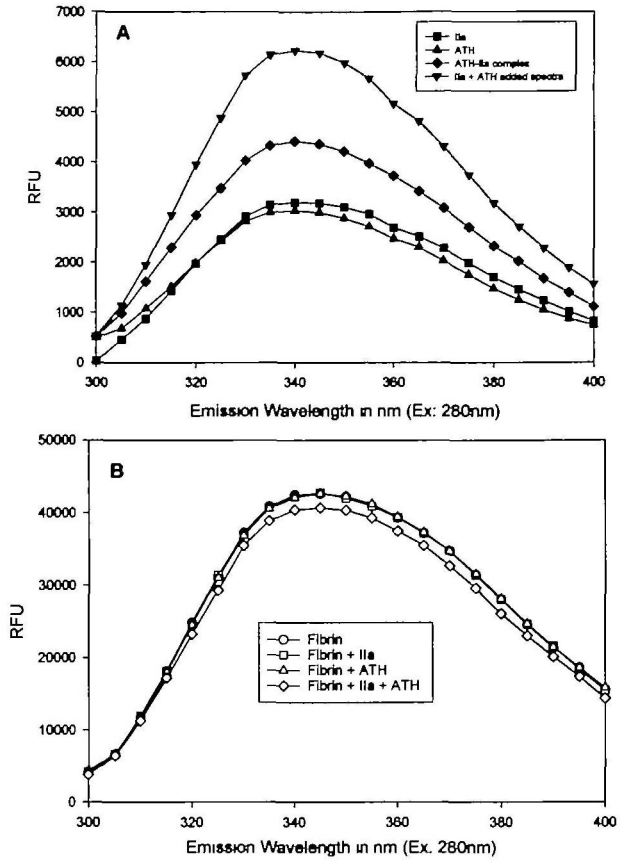

Fig. 7. **Spectral analysis of the intrinsic fluorescence of the reaction of thrombin (IIa) with covalent antithrombin-heparin complex (ATH) in the absence or presence of fibrin (Fn).** Solutions of 400 nM thrombin (IIa), 400 nM covalent antithrombin-heparin complex (ATH), and 400 nM ATH-IIa complex (IIa reacted with equimolar ATH) were prepared and spectral scans carried out (panel A). The mathematical sum of spectra for IIa and ATH (added spectra) is shown for comparison. Similar solutions of 1000 nM soluble fibrin monomer ± 400 nM IIa ± 400 nM ATH were prepared and scanned (panel B). Fluorescence spectral scans were performed from 300 to 400 nm with excitation at 280 nm (290 nm filter). Relative fluorescence units (RFU) from the spectrometer are plotted versus emission wavelength.

experiments with (^{125}I-)AT + UFH resulted in the expected non-covalent complexes of AT·H (Fig. 5). The interaction of ATH with IIa ± fibrin was examined. Using ^{125}I-IIa showed that IIa formed a complex with fibrin monomer [^{125}I-IIa had a reduced rate of migration in non-denaturing gels in the presence of fibrin compared to that of ^{125}I-IIa alone (Fig. 6A)] that was non-covalent [dissociated in SDS PAGE to migrate at the same position as ^{125}I-IIa alone (Fig. 6B)]. In the absence of fibrin, ATH formed a 1:1 complex with (^{125}I-)IIa (Fig. 6A) that was verified to be covalent under the dissociating conditions of an SDS-PAGE gel (Fig. 6B). However, the combination of ATH with (^{125}I-)IIa in the presence of fibrin monomer gave a ^{125}I-IIa band that migrated at an intermediate position between that of ^{125}I-IIa + ATH and ^{125}I-IIa + fibrin in non-denaturing gels (Fig. 6A). ^{125}I-IIa was determined to exist as a covalent complex with ATH when mixed fibrin followed by ATH, since the radiolabel migrated as a high molecular weight smear on denaturing SDS-PAGE [similar to incubations of ATH + (^{125}I-)IIa in the absence of fibrin (Fig. 6B)]. Therefore, although (^{125}I-)IIa reacted to form inhibitor complexes with ATH in the presence of fibrin, covalent ^{125}I-IIa-ATH continued to interact with fibrin under physiological conditions. The addition of exogenous H in experiments with ^{125}I-IIa + ATH + fibrin decreased the yield of fibrin-bound ^{125}I-IIa-ATH. Varying the order of ATH and ^{125}I-IIa addition to the fibrin had no significant effect on the outcome of the experiments.

Fluorescence Studies—A mixture of equimolar amounts of IIa and ATH gave a combined intrinsic fluorescence at 340 nm that dropped dramatically over time, indicative of changes in the environment of tryptophanyl groups on IIa and/or ATH occurring during covalent IIa-ATH inhibitor complex formation. This rapid fluorescence decrease occurring when covalent IIa-ATH complexes were forming was observed in the presence or absence of fibrin. Spectral emission scans of various combinations of ATH, IIa and fibrin were obtained. In the absence of fibrin, ATH + IIa mixtures gave a fluorescence profile, at final equilibrium, that was significantly less than the sum of the individual fluorescence spectra for the ATH and IIa before mixing (Fig. 7A). Even more striking was the observation that the addition of fibrin (1,000 nM) to ATH (400 nM) + IIa (400 nM) resulted in an emission peak that was not increased com-

pared to that of fibrin + IIa or fibrin + ATH (Fig. 7B). Thus, ternary complexes of fibrin·IIa·ATH have a significantly altered environment for fluorescent residues in the polypeptide chains compared to fibrin·IIa or fibrin·ATH complexes.

DISCUSSION

Treatment of patients with thrombosis requires the control of IIa generation and the subsequent action of thrombin on fibrinogen to form fibrin clots. IIa inhibition is critical for reducing of prothrombotic complications since active IIa promotes its own formation by feedback activation of coagulation factors V, VIII, and XI (*45–47*). Both UFH and LMWH have been successfully used to ameliorate IIa-induced coagulation *in vivo*. The inhibition of IIa by H relies on the activation of the plasma inhibitor AT (*9*), followed by binding of the AT·H complex to IIa through the H moiety (*10*). The reaction of AT and IIa in the AT·H·IIa complex results in an inactive covalent IIa-AT product (*11, 12*) that dissociates from the GAG chain (*13, 14*). Thus, the H molecule is free to catalyze another AT + IIa reaction.

After fibrin has been generated, IIa can remain bound to fibrin through the anion-binding exosite I of the enzyme (*18, 21*). Furthermore, fibrin-bound IIa has been shown to retain procoagulant enzymatic activity (*48–50*). However, fibrin-bound IIa is resistant to inhibition by either AT·UFH or AT·LMWH complexes (*19, 20*). The mechanism for the protection of IIa inhibition by fibrin involves formation of a fibrin·IIa·H complex (*23, 24*), and the binding of free H (dissociated from AT) to fibrin can actually recruit IIa to the fibrin surface (*26*). In this trapped form, with fibrin bound to IIa through anion-binding exosite I and H bound through anion-binding exosite II (*25*), the enzyme is unable to interact with approaching AT·UFH or AT·LMWH. Thus, the bridging of IIa and fibrin by free H is a critical step in the prevention of successful neutralization of fibrin-bound IIa and clot extension.

To address the limitations of H inhibition of fibrin-bound IIa, we have developed an active, covalent complex of AT and H (*27*). We showed that ATH reacts rapidly with IIa and Xa *in vitro* (*27, 28*). Experiments *in vivo* indicated that ATH might be capable of facile reaction with fibrin-bound IIa, since intravenous administration of low dose ATH caused a reduction in the size of venous thrombi compared to an increase in clot size with treatments with the same molar concentrations of non-covalent AT + UFH. Given the antithrombotic potency of ATH and the fact that the GAG chain of ATH does not dissociate to form a ternary fibrin·IIa·H complex, we decided to study the effects of fibrin on the inhibition of IIa by ATH *in vitro*.

Reactions were carried out with IIa + inhibitors, with or without varying concentrations of fibrin monomer, and the rate of IIa neutralization was determined (second order rate constant, k_2). Although the inhibition of IIa by ATH was impaired by fibrin monomer, the inhibition by fibrin was only 4% of that observed for IIa + AT·H (Table I). This result is consistent with preliminary studies on the effect of fibrin on IIa inhibition by ATH done at our institution (*51*). Also, this finding is in agreement with the suggestion that free H (not sterically hindered by covalently-linked AT) is required to form a fibrin·IIa·H complex that prevents the approach of AT-bound H. In the case of Xa inhibition by AT·H, entrapment of the enzyme in a fibrin·Xa·H complex is unlikely since the binding of Xa to H is relatively weak (*52*). However, a Xa binding site on fibrin has been identified [distinct from that for IIa (*53*)] and experiments have shown that clot-associated Xa is resistant to inhibition by AT + H (*54*). Indeed, previous work at our institution showed that fibrin reduces the rate of Xa inhibition by AT·H to a moderate degree (*51*). In comparison, the rate of Xa inhibition by ATH was not reduced by 4,000 nM fibrin (Table II). Thus, similar to IIa, the binding of Xa to fibrin has a low impact on reaction with ATH.

It is possible that AT·H was unable to react with the IIa in fibrin·IIa·H complexes due an inability of the approaching H moiety to bind to exosite II of IIa. Therefore, unlike the H in AT·H (which dissociates to form a complex with IIa·fibrin) the GAG portion of ATH may assist in the reaction with fibrin-bound IIa by bridging the AT to anion-binding exosite II of IIa. A mutant with reduced H-affinity (RA-IIa) was used to test the importance of H binding to fibrin-associated IIa. While inhibition rate of RA-IIa by AT + H was 0.3% that with IIa, the reaction rate of RA-IIa + ATH was decreased by only 7.6-fold compared with IIa + ATH (Table I). This suggestes that because the AT in ATH is always activated, reactions with IIa are more rapid (rate-determining step of AT·H formation eliminated), which partially moderates any problems with the bridging of IIa and AT by the heparin moiety. Further experiments showed that the rate of RA-IIa reaction with AT + H was decreased 2-fold when fibrin was present, compared to a mild increase in rate of inhibition of RA-IIa by ATH with fibrin (Fig. 1 and Table I). Since the binding of H to IIa is charge-dependent, we challenged the reaction of IIa with ATH (±fibrin) by the addition of exogenous H. The addition of up to a 40-fold molar excess of H caused a modest (~20%) decrease in the rate of the reaction of IIa with ATH (Fig. 2). Alternatively, when fibrin was present, the presence of a 40-fold molar excess of H in the ATH + IIa reaction mixture led to a 12-fold rate reduction (Fig. 2). The fact that H competitively inhibited the ATH + IIa reaction to a greater degree when fibrin was present suggests that the H moiety in ATH might be involved in interactions in addition to those that occur when fibrin is absent. In contrast, similar reactions of IIa + AT in the presence of a 40-fold excess of H in 4,000 nM fibrin reduced the rate by ~40% compared to experiments with equimolar added H (Fig. 2). Structural characteristics of the ATH reaction with IIa were considered. Studies of ATLMWH showed a marked decrease in the rate for inhibition of IIa compared to that for ATH + IIa (Fig. 3), consistent with the fact that IIa inhibition by AT + H requires IIa to bind to the H chain (*10*). The velocity of the IIa reaction with ATLAH was significantly less than with ATH (Fig. 3). Thus, the activation of the AT in ATH by the H pentasaccharide is important for IIa inhibition and simple covalent linkage of AT and H is insufficient for rapid reaction. ATLMWLAH had a vastly reduced rate of reaction with IIa, confirming the importance of both bridging to IIa and the activation of AT in the mechanism of IIa inhibition by ATH (Fig. 3). Similar studies were carried out with ATH derivatives and Xa. Compared with ATH, reactions of ATLMWH with Xa (Fig. 4) showed a much smaller decrease in the reaction velocity than reactions of ATLMWH with IIa (6.7-fold smaller reduction in the inhibition rate of Xa than IIa). This is not surprising given that the inhibi-

tion of IIa by AT·H requires binding of the enzyme to the GAG while the inhibition of Xa does not (*44*). However, the rate of the reaction of Xa with ATLAH was significantly less than that with ATH (Fig. 4). As expected, activation of the AT in ATH by the H pentasaccharide is also important for Xa inhibition.

The molecular interactions of ATH with IIa + fibrin were assessed. Direct binding experiments showed that, unlike the AT in AT·H, ATH binds strongly to fibrin monomer in buffer (Fig. 5). Previous reports have shown that since the H in AT·H has sufficient affinity for fibrin, AT in the complex can dissociate, allowing for fibrin·H formation (*26, 55*). Conversely, since AT is covalently linked to H in ATH, binding of the H moiety in the conjugate to fibrin would necessitate AT-fibrin localization. The importance of heparin chains of ATH in fibrin·ATH was evident from the fact that high H concentrations prevented complex formation (Fig. 5). Further experiments with ^{125}I-IIa verified that ATH reacts rapidly with fibrin-bound IIa to form IIa-ATH inhibitor complexes that remain bound to the fibrin (Fig. 6). Retention of IIa-ATH by fibrin is likely through the GAG chain of ATH, since the addition of large quantities of exogenous H inhibited the reaction of ATH with IIa·fibrin (Fig. 2). ATH binding to fibrin may assist in localizing the inhibitor with fibrin-bound RA-IIa, partially compensating for the fact that RA-IIa lacks H-binding affinity. Fluorescence measurements gave further details of the characteristics of the interactions between fibrin, IIa and ATH. The addition of either ATH or IIa to fibrin (Fig. 7) gave essentially no net increase in RFU over that for fibrin alone. This outcome may have arisen either by fluorescence transfer from the fluorescent residues on fibrin to those on IIa/ATH or structural changes induced by binding. However, the decrease in RFU with IIa and ATH binding to fibrin, compared to that for either IIa + fibrin or ATH + fibrin, implies that significant conformational/environmental changes occur during the reaction of ATH and IIa on fibrin that were not observed with fibrin bound to either ATH or IIa alone (Fig. 7). A model for the inhibition by ATH of IIa bound to fibrin can be constructed that incorporates all of the observations to date. ATH reacts with fibrin-bound IIa to form a covalent ATH-IIa inhibitor complex that remains bound to the fibrin (Fig. 8A). However, non-covalent AT·H interacts with fibrin-bound IIa to form a ternary fibrin·IIa·H complex and dissociated free AT (Fig. 8B).

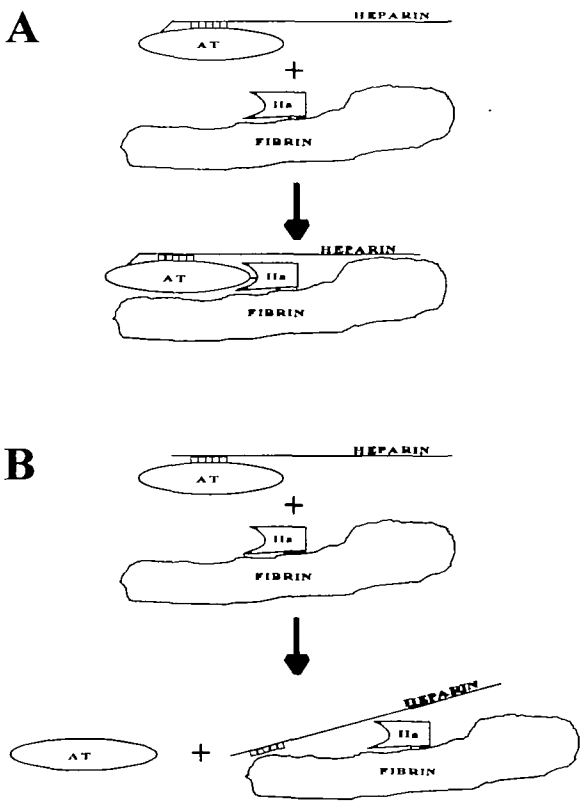

Fig. 8. **Model of the inhibition of fibrin-bound thrombin (IIa) by covalent antithrombin-heparin complex (ATH).** Panel A shows a proposed reaction mechanism for the inhibition of thrombin (IIa) on fibrin by covalent antithrombin-heparin complex (ATH). For comparison, panel B shows the formation of ternary fibrin·IIa·heparin complex + free antithrombin (AT) when non-covalent AT·heparin approaches fibrin-bound IIa.

In summary, covalent ATH can inhibit fibrin-bound thrombin at a rate much faster than that of non-covalent AT·H complexes. Although binding to fluid phase IIa by the GAG chain on ATH appears to be important, the activated AT moiety in the conjugate reacts with a IIa mutant with has reduced H-affinity to a similar degree whether fibrin is present or not. Thus, it seems likely that ATH can access the H-binding site on IIa that is fibrin-bound and react rapidly due to the permanently activated covalently-linked AT. The importance of AT activation by the pentasaccharide on the GAG component of ATH was verified by the reduced activity of AT complexed to low affinity H. Direct binding experiments showed that ATH interacts with fibrin and the final ATH-IIa inhibitor complex formed remains fibrin-bound (likely through the H moiety). Although binding regions for H on fibrin have been identified (*56*), detailed mapping of the binding site has not been reported. Future experiments are being planned to determine the structural locations on fibrin responsible for ATH interactions.

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