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Analysis of Inhibition Rate Enhancement by Covalent Linkage of Antithrombin to Heparin as a Potential Predictor of Reaction Mechanism

Sanjay Patel[1], Leslie R. Berry[1,2] and Anthony K.C. Chan[1,2,3,*]

[1]Henderson Research Centre, 711 Concession Street, Hamilton, Canada L8V 1C3; [2]Department of Pediatrics, McMaster University, Hamilton, Canada L8S 4L8; and [3]Hospital for Sick Children, University of Toronto, Toronto, Canada M5G 1X8

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Antithrombin (AT) inhibition of coagulation enzymes is catalyzed by unfractionated heparin (UFH) and other heparinoids. Reaction proceeds either *via* conformational activation of the inhibitor or template-mediated binding of both inhibitor and protease. We investigated if the relative inhibition rates of AT + UFH and covalent AT-heparin conjugate (ATH) with coagulation factors might be indicative of the mechanism involved. Rates were determined by discontinuous assay and mechanisms were probed by a variety of binding studies with UFH or ATH heparin chains. Rates were increased more than 2-fold with ATH over AT + UFH in reactions with thrombin, factor (F) VIIa + tissue factor + Ca^{2+} + lipid, FIXa and FXIa, but not with FXa or FXIIa. In comparison, UFH or ATH heparin binding (evidence of a template mechanism) was only observed with thrombin, tissue factor, FIXa and FXIa. Thus, inhibition rate enhancement by conjugation of AT with heparin were predictive of inhibitor·enzyme template bridging by heparin. Rationales behind this novel concept are discussed.

Key words: anticoagulant, antithrombin, coagulation factor, heparin, mechanism.

Abbreviations: TF, tissue factor; F, factor; AT, antithrombin; UFH, unfractionated heparin; ATH, covalent antithrombin-heparin; hiATH, heat-inactivated ATH; PC/PS, phosphatidyl choline/phosphatidyl serine.

The coagulation cascade is a series of proenzymes in plasma that are activated in turn to generate thrombin, which converts fibrinogen into a polymerizing fibrin clot (*1, 2*). *In vivo*, primary activation of the clotting system after vascular damage occurs via factor (F) VIIa complexed to tissue factor (TF) (*3, 4*). However, once formed, thrombin can feedback to augment its formation by activating FXI, FVIII, and FV (*5–8*). This secondary involvement of the intrinsic pathway gives rise to the vast increase in thrombin generation rate or burst in coagulation, which is sustained by FXIa (*9*). FXI activation can also be achieved by surface-contact activated FXII (*10*), although the relative importance of this factor in coagulation is under debate (*11*).

During normal hemostasis, enzymes within the cascade function to prevent hemorrhage and contribute to vascular repair (*12*). Overt procoagulant activity can result in thrombosis (*13, 14*) while defects in thrombin generation lead to excessive bleeding (*15–17*). As a complement, coagulation inhibitors limit and control the process. Plasma antithrombin (AT) is a major inhibitor that is variously able to neutralize FXIIa (*18*), FXIa (*19*), FIXa (*20*), FVIIa (*21*), FXa (*22*), and thrombin (*23*). Clinically, inhibition of coagulation factors by AT is significantly accelerated by the use of unfractionated heparin (UFH) (*24*). UFH can exert its effect via conformational activation by binding to AT and allosterically converting the serpin into a structural form that is much more reactive towards the protease (*25*). Alternatively, UFH may act as a template through binding to both inhibitor and enzyme, thus localizing the molecules for reaction (*26*). In this mechanism, conformational activation of AT by UFH occurs but additional reaction rate enhancement is gained by simultaneous binding of UFH to the enzyme, thus assisting approach of the coagulation factor towards the activated inhibitor (*26*). Which mechanism predominates, depends on the ability of UFH to form useful complexes with the enzyme. Presently, numerous investigations are being carried out with the different coagulation factors to confirm whether only conformational activation occurs during inhibition by AT·UFH or if template-mediated inactivation is involved.

Over the last decade, we have employed a covalent complex of AT with heparin (ATH) to study anticoagulant reactions *in vitro* and *in vivo* (*27, 28*). Covalent linkage of AT and heparin results in more potent anticoagulant activity (*29*), an increased intravenous half-life (*27*), effective inhibition of fibrin-bound thrombin (*30*), and an increased antithrombotic/hemorrhagic ratio (*31*). In-depth studies revealed that while ATH neutralization of thrombin was approximately 4 times more rapid than UFH + saturating AT, no significant difference in rate was observed for reactions of FXa + ATH compared to FXa + AT·UFH (*32*). Previously, studies in purified buffer

*To whom correspondence should be addressed. Tel: (905) 527-2299 Ext: 43559, Fax: (905) 575-2646,
E-mail: achan@thrombosis.hhscr.org

systems have shown that while thrombin neutralization by AT requires heparin bridging for maximal inhibition rates (*26, 33*), UFH accelerates FXa reaction with AT only through conformational activation of the inhibitor (when Ca^{2+} is absent) (*33, 34*). Thus, a correspondence was observed between enhancement of the protease inhibition rate by covalent linkage of AT to heparin and a template mechanism. Conversely, no increase in rate for ATH over AT + UFH occurred in reaction with FXa under conditions where only a conformational mechanism is known to operate. It seemed appropriate to inquire if the reaction rate of ATH with coagulation proteases, relative to that for AT + UFH, might be an indicator of whether a template (ATH has an increased rate) or a conformational activation (reaction rate for ATH and AT + UFH are similar) mechanism is functioning. If this axiom holds, then further understanding might be gained from study of other ATH structural characteristics. To test this hypothesis, we have investigated the kinetics and heparin binding of coagulation factors with ATH and AT + UFH to correlate relative reaction rates with the potential heparin-mediated mechanisms.

MATERIALS AND METHODS

Chemicals—All reagents were of analytical grade. Hexamethrine bromide (Polybrene) was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). Prionex 10% was purchased from Centrechem, Inc. (Norwalk, CT, USA). Magnesium nitrate and ammonium molybdate were from Sigma (St. Louis, MO, USA). Heparinase was purchased from ICN Biomedicals (Aurora, OH, USA). Heparin-Agarose was from Sigma. CNBr-activated Sepharose 4B beads were purchased from Amersham Biosciences (Uppsala, Sweden). Unfractionated sodium heparin (UFH) was obtained from Sigma. High affinity heparin was prepared from UFH using a previously published method (*30*). S-2222 substrate for FXa (*N*-benzoyl-isoleucyl-glutamyl-glycyl-arginyl-*p*-nitroanilide·hydrochloride and its methyl ester), S-2238 substrate for thrombin (H-D-phenylalanyl-pipecolyl-arginyl-*p*-nitroanilide·dihydrochloride), and S-2366 substrate for FXIa (L-Pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroanilide·hydrochloride) were obtained from Diapharma (West Chester, OH, USA). Pefachrome 5979 substrate for FVIIa (CH_3SO_2-(D)-3-cyclohexylalanyl-2-aminobutyryl-arginyl-p-nitroanilide·acetate), Pefachrome 3107 substrate for FIXa (H-D-leucyl-phenylglycyl-arginyl-p-nitroanilide·diacetate), and Pefachrome 5963 substrate for FXIIa (H-D-hexahydrotyrosinyl-glycyl-arginyl-p-nitroanilide·diacetate) were from Centerchem Inc. (Stamford, CT, USA). Human antithrombin (AT) was from Affinity Biologicals (Ancaster, ON, Canada). Human factors: thrombin, VIIa, IXa, Xa, XIa, and XIIa were supplied by Enzyme Research Laboratories (South Bend, IN, USA). Recombinant human tissue-factor (non-lipidated) (TF) was obtained from American Diagnostica (Stamford, CT, USA). ATH production followed the previously described method (*27*).

ATH Preparation—Production of ATH has been described previously (*27*). Briefly, AT (1052 mg) and UFH (64 g) were separately dialyzed against 2 M NaCl followed by PBS, then mixed together in a total volume of approximately 900 ml and incubated for 14 d in a water bath set at 40°C. Subsequently, 0.5 M $NaBH_3CN$ was added to the mixture (final concentration of 0.1M) and incubation was continued for another 5 h at 37°C. ATH was purified from the reaction mixture by a two-step procedure involving hydrophobic chromatography (Butyl-Sepharose; Amersham Biosciences, Baie d'Urfé, QU, Canada) followed by anion exchange chromatography (DEAE-Sepharose; Amersham Biosciences) (*27, 35*).

Preparation of Heat-Inactivated ATH (hiATH)—To test a coagulation protein's capacity to bind ATH's heparin chain, synthesis of an ATH molecule with no inhibitory activity (no active AT component) was used. Thermally inactivated AT is unable to react with thrombin due to an insertion of the reactive centre loop into the five-stranded A β-sheet (*36*). To prepare heat-inactivated ATH (hiATH), 600 μl of 1 mg/ml native ATH was dialyzed against 0.25 M sodium citrate 10 mM Tris-HCl pH 7.4 at room temperature. Following dialysis, the recovered ATH was heated at 80°C for 8 h in a water bath and then cooled at room temperature. The resultant material (hiATH) was dialyzed twice against 500 ml of 0.02 M Tris-HCl 0.15 M NaCl pH 7.4 at room temperature for 24 h (12 h for each 500 ml). The final concentration of the hiATH was calculated by dividing the sample absorbance at 280 nm by 0.75 (extinction coefficient for 1 mg/ml of human AT). The inability of hiATH preparations to react with the coagulation proteases was confirmed by inhibition tests with thrombin.

Determination of Second Order Rate Constants (k_2 Values)—Second-order rate constants for the inhibition of thrombin, FXa, FVIIa + TF + phosphatidyl choline/phosphatidyl serine (PC/PS), FIXa, FXIa, and FXIIa, by ATH and AT + UFH were measured under pseudo-first order conditions. A minimum AT:enzyme molar ratio of 10:1 was maintained for each reaction. The AT concentration ranged from 10 to 600 nM and enzyme concentrations ranged from 1 to 60 nM. Accordingly, UFH (MW = 15,000) was tested at concentrations ranging from 1 to 20,000 nM. AT, ATH, enzyme, and UFH were all diluted in 0.02 M Tris-HCl 0.15 M NaCl 0.6% polyethylene glycol 8,000 pH 7.4 (TSP) buffer. In the case of FVIIa, TF (37.5–50 nM) and PC/PS vesicles (3:1 mole ratio; 75–100 μM in total phosphorus) where included and reactions carried out in 5 mM $CaCl_2$. For FIXa inhibition experiments, a TSP + 0.5% Prionex buffer was used for all dilutions. The reaction protocol was as follows. At room temperature (23°C), 10 μl aliquots of enzyme were incubated in six separate wells of a 96-well flat-bottom microtiter plate (Fisher, Nepean, ON, Canada) for 5 min. To well number 6, 10 μl of TSP was added. At time intervals ranging from 3 to 820 s, a 10 μl volume of either AT + UFH or ATH was added to each well (wells 1–5). After incubation, the inhibition reactions were terminated by the simultaneous addition of 80 μl of 1.25 mg polybrene/ml TSP solution containing 0.5–1.3 mM of the appropriate chromogenic substrate to each well. After terminating the reaction, the residual enzyme activity for each well was determined by measuring the absorbance at 405 nm for a specific time period (ranging from 5 to 20 min) using a SpectraMax

Plus 384 plate reader. A graph of ln (V_t/V_o) versus inhibition time, where V_o represents initial enzyme activity (well 6 = no inhibitor added) and V_t represents the enzyme activity at time t, was plotted (*37*). The pseudo- first order rate constant was then calculated from the slope of the graph ($k_1 = -$slope) (*37*). Determination of the second order rate constant (k_2) was calculated by dividing the k_1 value by the inhibitor concentration ($k_2 = k_1/[AT]$) (*37*). The average of at least five experiments was used to calculate the final second order rate constant for inhibition of each enzyme. Maximal rates for reactions of AT + UFH were determined by testing varying concentrations of heparin over a range of more than three orders of magnitude.

Analysis of Varying Heparin Concentration versus Inhibition Rate—In experiments on the inhibition of factors by AT + UFH, plots of k_2 versus heparin concentration were constructed in order to assess trends in the data which may be consistent with potential mechanisms involved. In theory, if k_2 values rose to a maximum, followed by reduced rates with further increases in [UFH], a template-mediated inhibition mechanism may be indicated (excess UFH yields inhibitor·UFH and enzyme·UFH complexes that cannot bridge). Alternatively, a rise in measured reaction rate to a maximum plateau, as [UFH] increased, would be suggestive of a conformational AT activation mechanism (at saturation, only inhibitor·UFH forms). Thus, the curve profile might give an initial indication of tendencies towards a particular mechanism that could be further confirmed by direct experimentation. Curve shapes in plots of k_2 versus heparin concentration were assessed by fitting the data to equations representing either a template model (rise to peak; five-parameter double rectangular hyperbola) or a conformational activation model (asymptotic plateau; three-parameter exponential rise to max). R^2 values for the peak and plateau models were used as relative indications for goodness of fit. Additionally, a statistical comparison (Student's t-test) between data for the highest k_2 value observed and the k_2 value at the highest heparin concentration tested, was conducted as further evidence of either a peak (k_2 values different) or a plateau (k_2 values similar) curve shape.

Binding of Coagulation Enzymes to Immobilized Heparin—Heparin-binding studies were carried out to gather evidence of support for either a template-mediated (significant heparin affinity) or a conformational activation (low heparin affinity) mechanism of enzyme inhibition. Thus, interaction of the various coagulation proteins with Heparin-Agarose was tested. Using a microfuge tube, protein (500 nM) dissolved in 400 µl of TSP (supplemented with 5 mM Ca^{2+} in the case of FVIIa and 5 mM Ca^{2+} + 75 µM PC/PS in the case of TF) was incubated with a 200 µl volume of pre-equilibrated, packed Heparin-Agarose beads at 23°C using gentle mixing. Following a 10-min period, the bead solution was centrifuged and the supernatant was collected. Successive incubations were carried out in TSP (supplemented with Ca^{2+} for FVIIa and TF) adjusted to contain increasing concentrations of NaCl (0.4, 0.6, 1.0, and 2.0 M), followed by further collection of supernatants. Absorbance measurements of each supernatant were made at 215 nm to detect the presence of protein in order to determine the effect of ionic strength on binding.

HiATH Binding to Coagulation Enzymes Run on Native PAGE—Similar to heparin-binding studies, an attempt to establish the ability of enzymes to interact with heparin chains in ATH was done by testing affinity of enzymes towards a form of ATH (hiATH) in which the AT moiety is in a conformation that cannot react with the protease (*38*). Coagulation enzymes were mixed with hiATH, followed by native PAGE to identify those factors with the capacity to bind the heparin chains. In these experiments, 4.47×10^{-11} moles of enzyme were incubated either in the absence or presence of 5.6×10^{-11} moles of active or heat-inactivated conjugate dissolved in TBS. After a short incubation, samples were mixed with an equal volume of trace bromothymol blue tracking dye in 10% glycerol and loaded onto the gel (7.5% polyacrylamide separating gel and 4% polyacrylamide stacking gel). Electrophoresis was at a constant voltage of 200 V in 0.02 M Tris 0.2 M glycine adjusted to pH 8. Neither bromothymol blue nor glycerol were shown to interfere with binding in control gels run for short periods. Due to its size, electrophoresis of FXIIa was performed in a 4% separating gel (no stacking gel). Due to its elevated pI (8.9–9.1) (*39, 40*), FXIa was not tested for hiATH binding using PAGE. Following electrophoresis, proteins in the gels were stained with Coomasie Brilliant Blue R250 (Sigma) and dried using a BioRad gel dryer.

HiATH Binding to Immobilized Coagulation Factors—Although interaction with solution phase hiATH was preferred, PAGE analysis of hiATH binding to several coagulation factors was problematic. Thus, further affinity testing was carried out by interaction of hiATH with coagulation factors immobilized onto beads. Coagulation factor-Sepharose beads were prepared using CNBr-activated Sepharose according to the manufacturer. One hundred microliters of 1,000 nM protein was incubated with 200 µl of 0.1 M $NaHCO_3$ 0.5 M NaCl pH 8.3 (coupling buffer) in an Eppendorf tube. CNBr-activated Sepharose 4B beads were then prepared by weighing out 0.5 g of powder in 1 mM HCl. Once swelled, 3.07 mg of wet packed beads were: taken, mixed with coupling buffer for 2 h, periodically shaken, and stored overnight at 4°C. The protein + bead reaction solution was microfuged at 12,000 rpm and the supernatant removed. Excess ligand was separated from the beads by four washings with 0.5 ml coupling buffer. In addition to removing excess ligand, remaining active groups on the Sepharose beads were blocked by mixing with 0.5 ml 0.1 M Tris-HCl buffer, pH 8.0 for 2 h. With excess ligand removed and active groups blocked, the beads were subjected to three cycles of washes with alternating pH to further hydrolyze any remaining reactive groups. A cycle was composed of an initial washing with 0.1 M acetate-0.5 M NaCl, pH 4.0 and a secondary wash with 0.1 M Tris-HCl-0.5 M NaCl, pH 8. Following removal of the last wash, the final coagulation factor-Sepharose bead mixture was resuspended in TBS and stored at 4°C.

For immobilized coagulation enzymes, the concentration of enzyme bound to Sepharose beads was measured by substrate cleavage to quantify the minimum amount

of active enzyme available for hiATH binding. In the protocol, protein-Sepharose beads were washed with 200 µl of a 0.02 M Tris-HCl-2 M NaCl, pH 7.4 to remove any potentially bound materials, followed by washes with three volumes of 200 µl TBS buffer. Next, 200 µl of 0.5 mM of substrate solution in TBS was mixed with the protein-Sepharose beads for 60 s. After the incubation period, the substrate bead solution was microfuged and 180 µl of the supernatant was removed into a 96-well microplate well for an endpoint absorbance reading at 405 nm. Comparison of this milliOD/min reading to that of enzyme standards allowed for an estimate of immobilized enzyme concentration. This result confirmed the theoretical capacity of conjugated ligand for binding. Since there was no enzyme activity associated with the tissue factor ligand, it was assumed that most of the protein was linked to the beads since no absorbance at 280 nm remained in the conjugation supernatant.

For binding experiments, a sample of hiATH (7.42 µl of 13.47 µM hiATH diluted in 42.58 µl TBS) was mixed with the protein-Sepharose (total prepared as described above) and periodically shaken for 10 min. Following this incubation period, the solution was microfuged and the supernatant collected. TBS containing increasing NaCl concentrations (0.4, 0.6, 1.0, and 2.0 M) was successively incubated with the bead conjugate mixture, with collection of supernatant in each case. Absorbance readings of each supernatant at 215 nm were performed to determine binding and elution of hiATH from the coagulation factor-Sepharose beads.

Statistics—Data were expressed as mean ± SEM ($n \geq 5$). Tests of multiple groups were done by ANOVA. In the case of comparison between two groups, a two-tailed student's *t*-test was used. Values of $p < 0.05$ were considered significant.

RESULTS

Determination of Maximal Second Order Rate Constants for AT + UFH—Analysis of the k_2 values for inhibition of coagulation factors at varying UFH concentrations was used to determine peak rates of reaction. Results with vitamin K-dependent factors are shown in Fig. 1. The plot of heparin concentration versus second order rate constants for thrombin inhibition by AT+UFH reveals an ascending and descending curve (Fig. 1A), typical of a template-mediated inactivation mechanism (*41*). Curve analyses confirmed the peak nature of the profile, whereby a low R^2 value for a plateau curve fit was obtained (0.006) and the maximum k_2 value observed was statistically different ($p = 0.0074$) from the k_2 value measured at the highest heparin concentration tested. The maximum k_2 for AT + UFH reaction with thrombin was measured to be $7.99 \pm 0.55 \times 10^8\,M^{-1}\,min^{-1}$. Inhibition of FXa by AT with varying [UFH] yielded rate values that plateaued at high UFH concentrations (Fig. 1B). Maximum k_2 for FXa + AT + UFH was $1.59 \pm 0.19 \times 10^8\,M^{-1}\,min^{-1}$. Further analyses confirmed the rise to plateau consistent with conformational activation since values for the maximum k_2 and the k_2 at highest heparin concentration were similar ($p = 0.4213$). Reactions between FVIIa/TF + AT with varying [UFH] yielded rate constants ascending to a peak value (Fig. 1C). The maximal k_2 value obtained was $3.67 \pm 0.11 \times 10^5\,M^{-1}\,min^{-1}$. Finally, the plot of k_2 versus UFH in reactions of FIXa + AT + UFH shows an ascending and descending profile (Fig. 1D). Comparison of the peak reaction rate ($5.89 \pm 0.62 \times 10^6\,M^{-1}\,min^{-1}$) with that at highest UFH concentrations tested showed a significant difference ($p = 0.0036$). Again, this biphasic shape is consistent with a template-mediated inhibition mechanism. Turning to contact phase factors, similar experiments allowed calculation of AT + UFH reaction rates (Fig. 2). Incremental increases in heparin significantly enhanced the rate of FXIa neutralization by AT up to a maximum k_2 of $1.37 \pm 0.06 \times 10^6\,M^{-1}\,min^{-1}$ (Fig. 2A). Further elevation in [UFH] led to a reduction in reaction rate so that peak k_2 and k_2 at higher heparin concentrations were greatly different ($p < 0.0001$), concordant with template inhibition. Finally, reactions of FXIIa with AT + UFH were performed. By varying UFH, the maximum k_2 measured for FXIIa + AT was $2.89 \pm 0.34 \times 10^5\,M^{-1}\,min^{-1}$ (Fig. 2B). Analyses using hyperbolic and asymptotic curves as models for template and conformational activation mechanisms, respectively, did not give good fits for k_2 versus [UFH] data (R^2 values <0.5). Interestingly, the inhibition rate for FXIIa by AT + UFH was lower than that for the other five coagulation factors being considered.

Comparison of k_2 Values for Inhibition by ATH With Those of AT + UFH—Rates for reaction of coagulation enzymes with ATH were calculated and compared with those for AT + UFH. For each coagulation factor studied, although the AT concentration in ATH or AT + UFH reactions was similar, higher levels of heparin were necessary for maximal inhibition by AT + UFH since (unlike ATH) AT·UFH can dissociate. Supplementation of ATH reactions with UFH to give equivalent heparin concentrations was also not feasible since previous work has shown that UFH significantly impairs ATH inhibition of coagulation factors (*27, 32*). Relative k_2 values for ATH and AT + UFH in reactions with TF-pathway K-dependent enzymes appear in Fig. 3. ATH inhibited thrombin at a rate of $2.58 \pm 0.06 \times 10^9\,M^{-1}\,min^{-1}$, which was significantly faster ($p = 0.004$) than the maximal reaction rate for AT + UFH (Fig. 3A). On the other hand, although inhibition of FXa by ATH ($k_2 = 2.15 \pm 0.13 \times 10^8\,M^{-1}\,min^{-1}$) was statistically faster than reaction of FXa with AT + UFH ($p = 0.035$), the increase was less than 35% (Fig. 3B). ATH inhibition rates for FVIIa ($1.02 \pm 0.03 \times 10^7\,M^{-1}\,min^{-1}$) and FIXa ($2.13 \pm 0.27 \times 10^7\,M^{-1}\,min^{-1}$) were both enhanced ($p < 0.001$ and $p = 0.005$, respectively) compared to the maximum rates measured with AT + UFH (Figs. 3C and 3D). In the case of contact factors, reaction velocity differences between ATH and AT + UFH varied. For FXIa, ATH reacted with a rate ($k_2 = 2.19 \pm 0.08 \times 10^7\,M^{-1}\,min^{-1}$) that was greatly elevated ($p < 0.001$) relative to inhibition by AT + UFH (Fig. 4A). However, neutralization of FXIIa by ATH gave a k_2 value ($3.83 \pm 0.35 \times 10^5\,M^{-1}\,min^{-1}$) that was insignificantly different ($p = 0.1$) from that with AT + UFH (Fig. 4B).

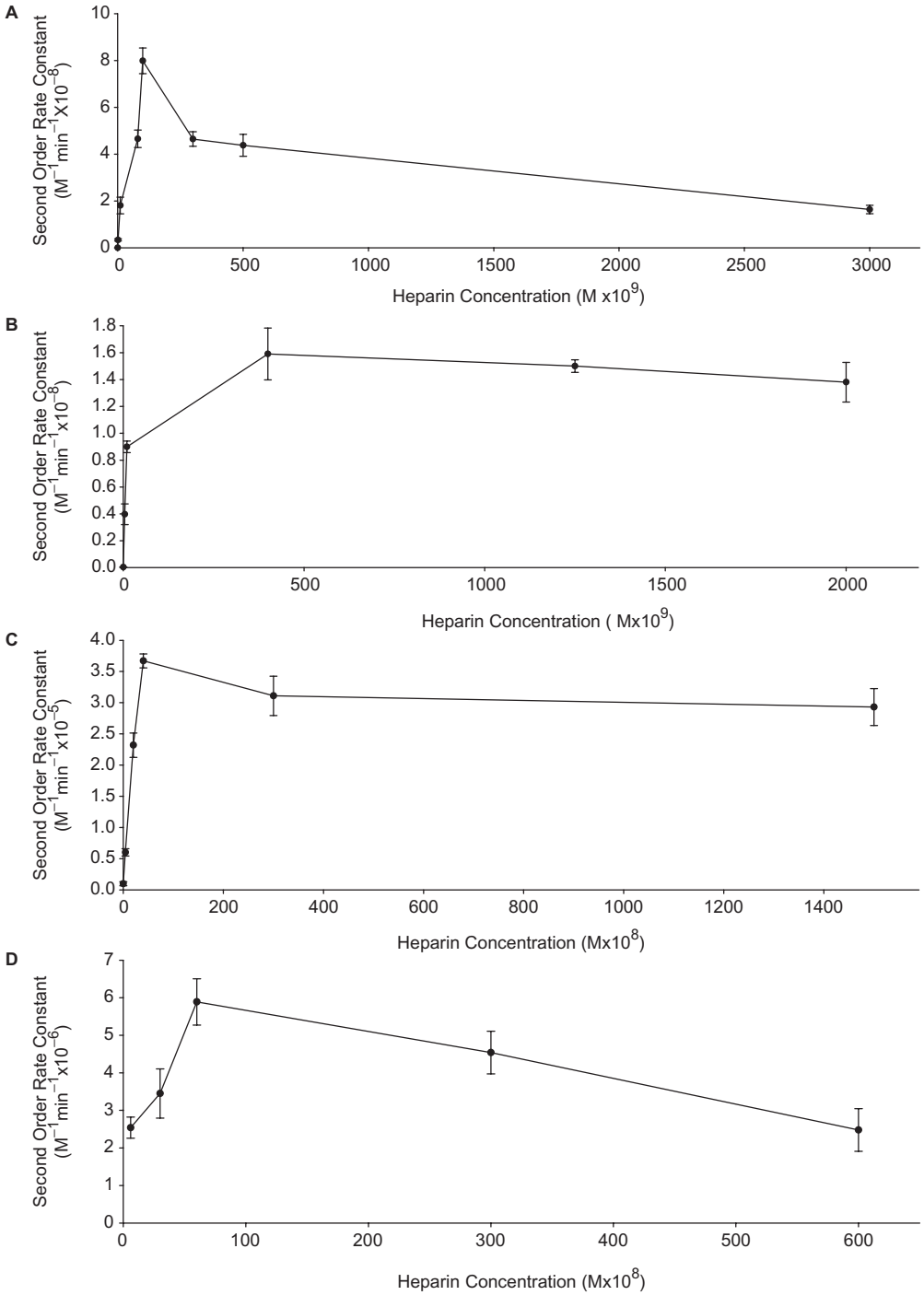

Fig. 1. **Measurement of the second order rate constant (k_2) in reactions of antithrombin (AT) + unfractionated heparin (UFH) with K-dependent coagulation factors.** AT (at 10-times enzyme concentration) + varying concentrations of UFH were reacted with the K-dependent factors: thrombin, factor Xa (FXa), factor VIIa (FVIIa) + tissue factor + phosphatidyl choline/phosphatidyl serine + Ca^{2+}, or factor IXa (FIXa). At different time points, reaction was stopped with polybrene + substrate and the residual enzyme activity measured. Plots were constructed of Ln(activity at time *t*/activity at time 0) versus time and pseudo-first order rate constants (k_1) calculated as –slope. Second order rate constants ($k_2 = k_1/[AT]$) for reactions with thrombin (panel A), FXa (panel B), FVIIa (panel C), and FIXa (panel D) are plotted versus UFH concentration. Values are mean ± SEM.

Heparin-Agarose Binding Studies—In order to obtain objective evidence of whether a template-mediated mechanism (whereby both AT and the enzyme are bridged by the catalyst) may be operating, affinities of the activated coagulation factors for heparin chains were determined. Results from Heparin-Agarose binding to the various coagulation proteins are shown in Table 1. Consonant with the effect of UFH concentration on

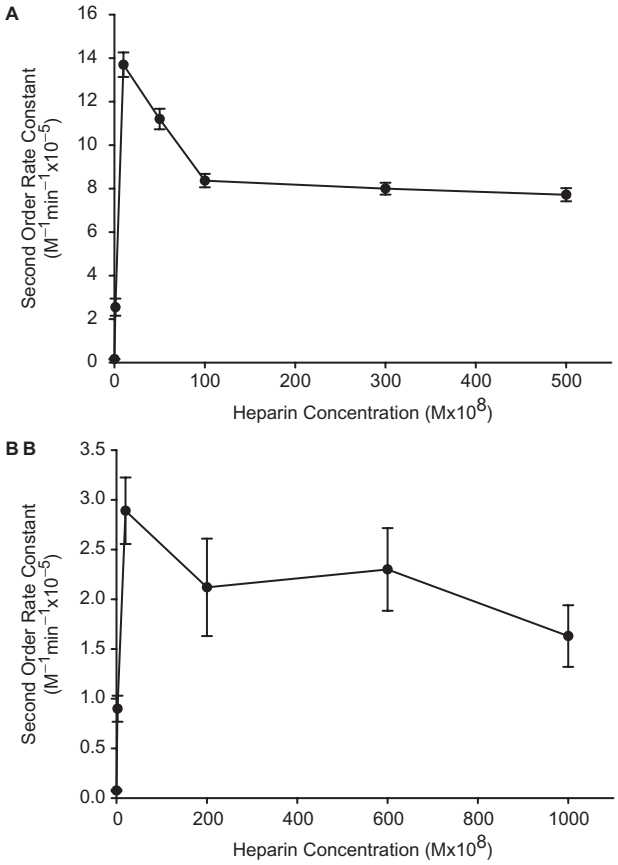

Fig. 2. **Measurement of the second order rate constant (k_2) in reactions of antithrombin (AT) + unfractionated heparin (UFH) with contact phase coagulation factors.** AT (at 10-times enzyme concentration) + varying concentrations of UFH were reacted with contact phase factors: factor XIa (FXIa) or factor XIIa (FXIIa). At different time points, reaction was stopped with polybrene + substrate and the residual enzyme activity measured. Plots were constructed of Ln(activity at time *t*/activity at time 0) versus time and pseudo-first order rate constants (k_1) calculated as –slope. Second order rate constants ($k_2 = k_1$/[AT]) for reactions with FXIa (panel A) and FXIIa (panel B) are plotted versus UFH concentration. Values are mean ± SEM.

the AT inhibition rate, thrombin bound to immobilized heparin and required 1.0 M NaCl for elution. Alternatively, just as FXa displayed an AT inhibition rate versus [UFH] profile consistent with a non-template mechanism, the FXa enzyme remained unbound to the Heparin-Agarose beads under the TSP loading conditions. Interestingly, FVIIa did not exhibit affinity for Heparin-Agarose (Table I). However, relipidated TF successfully bound to the immobilized heparin and was eluted with 0.4 M NaCl in buffer. Similarly, FIXa was adsorbed by Heparin-Agarose and displaced at 0.4 M NaCl concentrations. The two contact factors again showed differing results for heparin interaction (Table I). FXIa bound avidly to the heparin beads and needed at least 0.6 M NaCl for elution. In contrast, FXIIa in TSP did not bind to Heparin-Agarose.

Interaction With hiATH—Experiments were performed to confirm potential inhibition mechanisms that may be

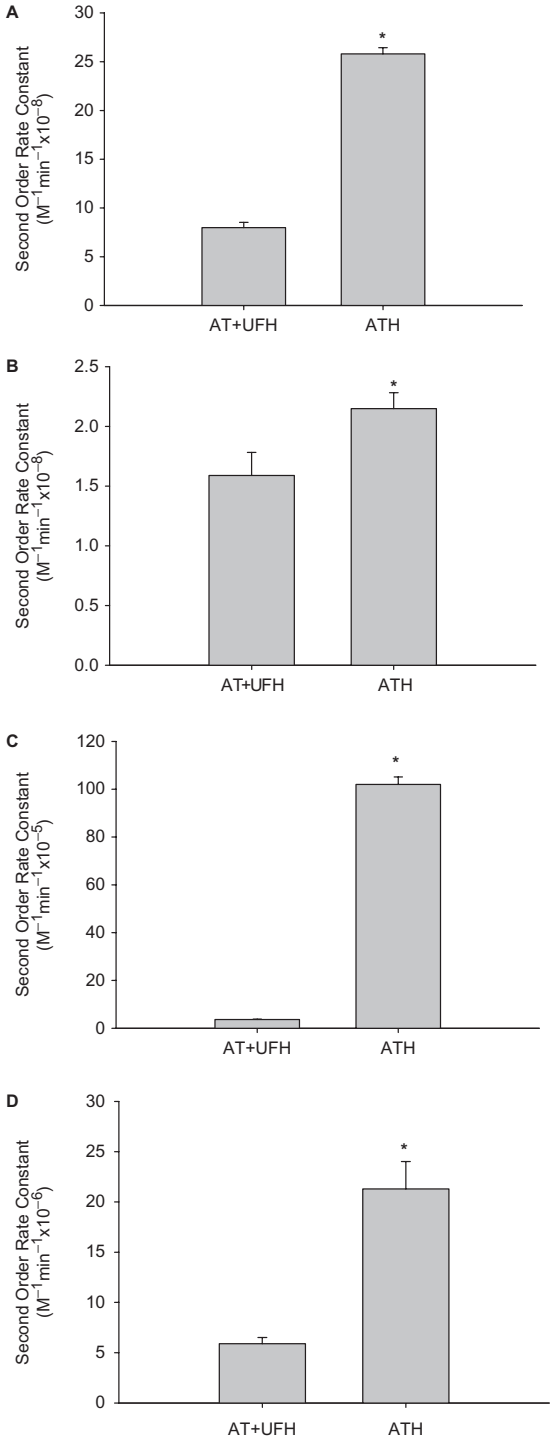

Fig. 3. **Comparison of the maximal second order rate constants (k_2) for reactions of K-dependent coagulation factors with antithrombin (AT) + unfractionated heparin (UFH) and k_2 values for corresponding reactions with covalent antithrombin-heparin (ATH).** Peak k_2 values observed for reactions of K-dependent coagulation factors with AT + UFH (see Fig. 1) are plotted for comparison with rates measured for similar reactions with ATH. Reactions with thrombin, factor Xa (FXa), factor VIIa (FVIIa) + tissue factor + phosphatidyl choline/phosphatidyl serine + Ca^{2+}, and factor IXa (FIXa) are shown in panels A, B, C, and D, respectively. Values are ± SEM. *$p < 0.05$.

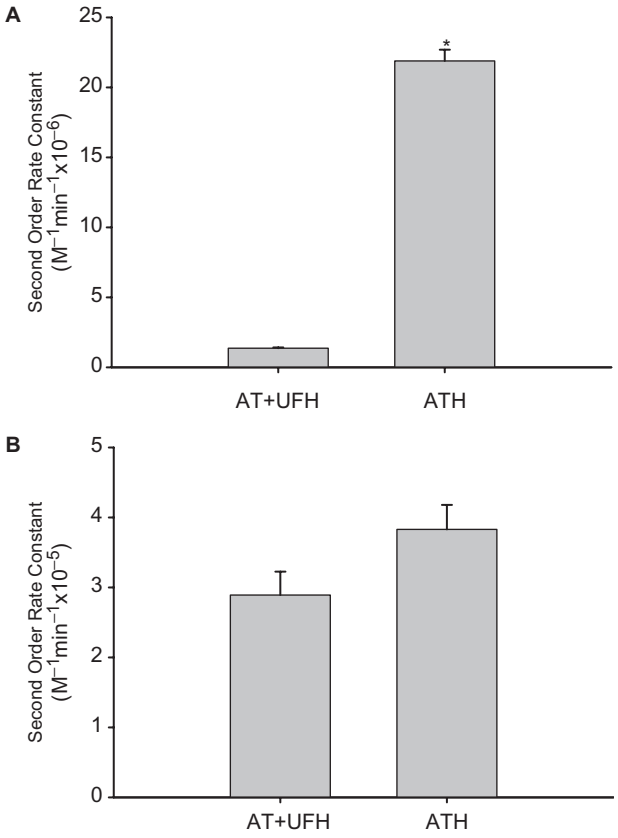

Fig. 4. **Comparison of the maximal second order rate constants (k_2) for reactions of contact phase coagulation factors with antithrombin (AT) + unfractionated heparin (UFH) and k_2 values for corresponding reactions with covalent antithrombin-heparin (ATH).** Peak k_2 values observed for reactions of contact phase coagulation factors with AT + UFH (see Fig. 2) are plotted for comparison with rates measured for similar reactions with ATH. Reactions with factor XIa (FXIa) and factor XIIa (FXIIa) are shown in panels A and B, respectively. Values are ± SEM. $*p < 0.05$.

Table 1. **Binding of coagulation factors to Heparin-Agarose.**

Factor	[NaCl] required to elute bound protein (M)
Thrombin	1.0
Xa	unbound in TSP
VIIa	unbound in TSP
TF	0.4
IXa	0.4
XIa	0.6
XIIa	unbound in TSP

operating involving the ATH heparin moiety. Binding to heparin chains on ATH was evaluated by studying interactions with hiATH molecules in which the modified AT component is unable to react with serine proteinases (*36*). In order to more fully approach the native fluid-phase state, attempts were made to study non-covalent enzyme association with hiATH in free solution. Separation of unbound starting materials from enzyme·hiATH complexes was accomplished by

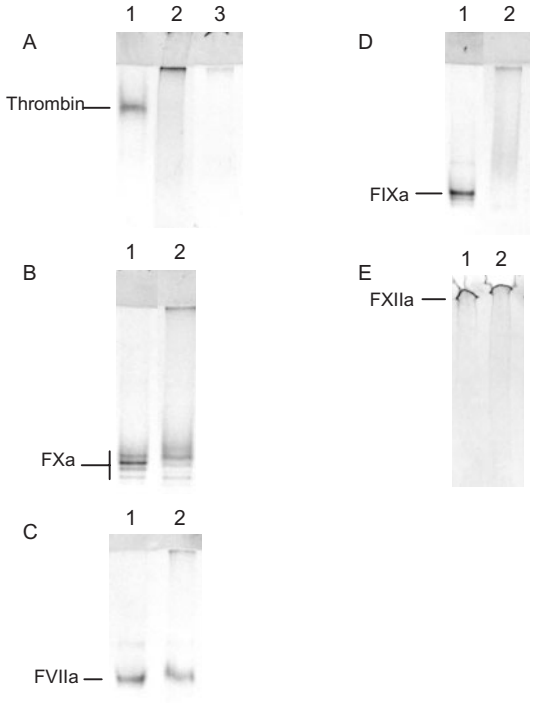

Fig. 5. **Binding of coagulation enzymes to heat-inactivated covalent antithrombin-heparin (hiATH).** HiATH was prepared by heating of ATH at 80°C. Thrombin, factor Xa (FXa), factor VIIa (FVIIa), factor IXa (FIXa), or factor XIIa (FXIIa) were incubated with either buffer (lane 1) or hiATH in buffer (lane 2), followed by non-denaturing PAGE, in panels A, B, C, D, or E, respectively. Gels were stained with Coomassie Blue and dried. Lane 3 (panel A) shows non-denaturing electrophoresis of hiATH alone in buffer.

non-denaturing SDS PAGE. The results are shown in Fig. 5. Similar to the results with Heparin-Agarose chromatography, thrombin readily bound to hiATH, as indicated by disappearance of the characteristic thrombin band when the protease was mixed with hiATH prior to native PAGE (Fig. 5A). By comparison, electrophoresis of FXa + hiATH (Fig. 5B) resulted in only a slight reduction in the intensity of bands for unbound FXa. Mixture of FVIIa with hiATH gave no significant evidence of FVIIa·hiATH complex formation (free FVIIa band was similar in the presence or absence of hiATH (Fig. 5C)). However, FIXa formed a stable complex with hiATH that remained intact during non-denaturing electrophoresis (Fig. 5D). Finally, even with 4% acrylamide porosity, native PAGE of FXIIa revealed only minor migration into the gel (Fig. 5E). Addition of hiATH to FXIIa had no effect on the electrophoretic mobility.

Analysis of hiATH binding to coagulation factors in the free state was hampered, in some cases, by technical issues. Migration into the gel was not possible for FXIa under native conditions due to its basic pI (*39, 40*) and relipidation of TF obscured band patterns. Thus, further assessment of interactions with hiATH was accomplished by performing binding studies with selected coagulation factor proteins immobilized onto Sepharose beads.

Table 2. **Binding of hiATH to immobilized coagulation factors.**

Factor	[NaCl] required to elute bound hiATH (M)
VIIa	unbound in TSP
TF	0.4–0.6
XIa	0.6
XIIa	unbound in TSP

As observed with non-denaturing SDS PAGE, hiATH did not bind to FVIIa-Sepharose (Table 2). TF showed significant hiATH binding, with elution at NaCl concentrations of at least 0.6 M. Thus, consistent with the results for heparin alone, hiATH displayed affinities that were low for FVIIa and significant for TF. With respect to immobilized contact factors, hiATH also showed stable binding to FXIa but no affinity for FXIIa (Table 2).

Inhibition Rate Increase by ATH and Heparin Binding by Coagulation Factors—A comparison was made between AT-heparin covalent linkage effects on rate of enzyme inhibition and ability of the enzyme to bind heparin (compatible with template-mediated activation). Results are presented in Table 3. Reaction rates for ATH were equal or greater than AT + UFH for all coagulation factors compared to AT + UFH. In particular, ATH reacted several-fold more rapidly with thrombin, FVIIa + TF, FIXa and FXIa. Correspondingly, binding to Heparin-Agarose was detected with thrombin, FIXa, and FXIa. Interestingly, although FVIIa did not bind to heparin beads, TF did show significant affinity. Tests with FVIIa·TF mixtures showed slight uptake on Heparin-Agarose of some proteinaceous material that could be displaced at low ionic strength (data not shown). Interaction of hiATH with TF-loaded FVIIa-Sepharose beads resulted in removal of bound TF. In a similar vein, reduced or no enhancement of reaction rate by ATH with FXa and FXIIa, compared to AT + UFH, corresponded to a lack of adsorption of these enzymes to Heparin-Agarose (Table 3). As confirmation that similar interactions were occurring with the conjugate, affinity of coagulation factors for hiATH was the same as that with immobilized heparin. Thus, a clear correspondence exists whereby if covalent linkage of AT and heparin causes a ≥2-fold enhancement in factor inhibition, then that factor likely binds to heparin chains under physiological conditions. As a corollary, lack of inhibition rate enhancement by ATH over AT + UFH corresponds to a lack of coagulation factor affinity for heparin.

Table 3. **Comparison of inhibition rates and binding of coagulant enzymes to heparin.**

Enzyme	k_2 in reaction with ATH divided by k_2 in reaction with AT + UFH	Interaction with Heparin-Agarose[a]	Interaction with hiATH[a]
Thrombin	3.2	+	+
Xa	1.4	–	–
VIIa/TF	28	–/+	–/+
IXa	3.6	+	+
XIa	16	+	+
XIIa	1.3	–	–

[a]Note: +,binds: –, no binding.

DISCUSSION

Anticoagulation by UFH has been a standard of care for prevention and treatment of thrombosis (*42*), especially during surgery (*43*). The mechanism of UFH action has been ascribed to involve only conformational activation of AT towards coagulation enzyme inhibition (*25, 44, 45*) or template mediation with binding to both AT and the protease (*26, 41*). Previous work using a covalent AT complex with heparin (ATH) demonstrated that, relative to non-covalent AT + UFH, inhibition rate was enhanced for thrombin and similar for FXa. In buffer systems, it has been determined that UFH catalyzes AT inhibition of thrombin by a template mechanism and AT inhibition of FXa by conformational activation (*45*). This intriguing acceleration of reaction rate with thrombin but not FXa resulting from conjugation of serpin and heparin suggests that a step in the mechanism may have been altered by covalent linkage. We decided to investigate if the relationship between inhibition rates for ATH and AT + UFH with coagulation factors corresponds to the potential mechanism involved.

Maximal rates determined for AT + UFH neutralization of various coagulation factors were in agreement with values obtained by others (*19, 32, 37, 46–48*). Generally, rates of reaction with AT + UFH or ATH were highest against the enzymes generated at the bottom of the cascade (thrombin and FXa) and lowest against the enzymes which initiate the coagulation pathway (FVIIa, FXIIa, and FXIa). When comparing reaction rates of AT + UFH with those of ATH, a consistent relationship occurred where a more than 40% increase in k_2 for ATH over AT + UFH tended to correspond with enzyme-heparin binding (Table 3). Furthermore, results from binding to hiATH confirmed that enzyme association occurred with the conjugate heparin chain in a similar degree to that of free heparin. Previous investigations have clearly established that template-mediated inhibition mechanisms with AT must involve binding of the heparin catalyst to the protease as well as the inhibitor (*41, 49*). Thus, our results probing interactions with heparin would confirm the likelihood of a template mechanism if binding of enzyme to heparin was observed. Conversely, experiments in which the enzyme did not bind to heparin suggest that conformational activation and not AT·protease bridging may be occurring (*45*). The correspondence of ≥2-fold increase in enzyme inhibition rate for ATH over AT + UFH and evidence of a template mechanism (affinity of the enzyme for heparin) held for thrombin, FIXa, and FXIa. Lack of inhibition rate enhancement due to covalent AT-heparin linkage also correlated with a non-template (no heparin-binding) conformational activation mechanism in the case of FXa and FXIIa. Finally, in the case of FVIIa·TF, heparin template bridging not to FVIIa but to the TF moiety in the complex (Tables 1 and 2) corresponded with the large elevation in k_2 value for ATH relative to AT + UFH (Table 3). Indeed, prediction of inhibition mechanism as either template if AT-heparin conjugation augments inhibition or conformational if the covalent AT-heparin

Table 4. **Comparison of inhibition rate increase of ATH and potential reaction mechanism.**

Enzyme	Inhibition by ATH ≥ 2-fold faster than with AT + UFH	Potential mechanism from heparin binding
Thrombin	Yes	Template
FXa	No	Conformational
FVIIa/TF	Yes	Template
FIXa	Yes	Template
FXIa	Yes	Template
FXIIa	No	Conformational

bonding does not improve reaction rate was validated in this survey of five coagulant enzymes and one enzyme·cofactor complex (Table 4). Previous mechanistic studies for AT + UFH inhibition of coagulation factors are generally harmonious with our findings (*19, 32, 37, 48, 50–52*).

Consideration of the inhibition kinetics and binding interactions for FVIIa·TF allowed for further deductions and hypotheses. It is well known that the physiological action of FVIIa only occurs when bound to TF (*53, 54*). Additionally, neutralization of FVIIa by AT·UFH requires the FVIIa to be TF-bound (*55*). Studies following the steps for FVIIa inhibition have revealed that AT vastly increases the dissociation of FVIIa from TF (*56*) and the FVIIa-AT inhibitor complex formed resists binding to new TF sites (*21*). Comparison of FVIIa·TF reactions with AT to those with AT + UFH show that heparin accelerates inhibition more than 10-fold (*47*), similar to the findings in Fig. 1C. However, compared to pentasaccharide that can only activate AT (no bridging) (*57*), 50-saccharide heparin chains gave only modest increases in second-order association rate constants for reactions with FVIIa·TF (*48*). The lack of heparin binding by FVIIa, reported here (Fig. 5C and Tables 1 and 2) and elsewhere (*52*), is fully consistent with the absence of large-scale template mechanism augmentation of FVIIa inhibition by UFH relative to low molecular weight heparins. Nonetheless, TF bound to heparin immobilized on beads and displayed even higher affinity for hiATH chains. A highly cationic anion binding region on TF, spanning a large area including much of the FVIIa binding site, has been mapped out in detail (*54*). The models reported indicate that only much longer heparin chains have the proper geometry for significant contact with the TF anion exosite in FVIIa.TF complexes. We have previously shown that ATH is comprised of heparin that is vastly longer than UFH (*58*). Given that AT is at the end of the oversized heparin chains in the conjugate, ATH heparin would have a much superior capacity to inhibit FVIIa.TF by either template binding through TF or displacing FVIIa from its cofactor. Plans are being made to further study this fascinating hypothesis through either plasmon resonance or preparation of (hi)ATH.FVIIa.TF crystals for X-ray diffraction.

Our data strongly indicate that in reactions where heparin bridges AT and protease, characteristics that produce increased rates are exemplified by ATH. In template-mediated AT inhibition mechanisms, variation in UFH gives increasing k_2 values up to a peak, beyond which further addition of UFH yields reduced rates. This detrimental effect of UFH overdosing results from the simultaneous presence of both AT·UFH and enzyme·UFH complexes in which heparin can no longer form a bridge (*59*). Since heparin cannot dissociate from the AT in ATH, these non-useful complexes cannot form. Another consequence of free heparin binding is that AT·UFH can dissociate prior to retrieval of the enzyme to form a ternary complex. In fact, it has been established that AT binding to heparin is the rate-determining step in template-mediated or conformational-activated inhibition (*60*). Again, covalent linkage of heparin to the inhibitor eliminates this reaction step completely (*27*). Finally, rates of AT·UFH reaction with proteases involving a template mechanism are dependent on the mean free path of AT·UFH/enzyme diffusion (*27, 61*). In ATH, longer heparin molecules (*58*) effectively occupy a larger region of space, reducing the distance between ATH heparin and free enzyme (*27*). Given the empirical and theoretical basis for mechanistic projections based on ATH versus AT + UFH rate comparisons, studies in other areas of AT inhibition or systems involving different inhibitor·glycosaminoglycan complexes may be pursued. Once data have been accumulated, deductions from selected findings of interest can be confirmed.

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