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Contribution of Exosite Occupancy by Heparin to the Regulation of Coagulation Proteases by Antithrombin

Likui Yang, Chandrashekhara Manithody, Shabir H. Qureshi, and Alireza R. Rezaie*

Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Saint Louis, Missouri 63104

Summary

Heparin promotes the antithrombin (AT) inactivation of factors IXa (fIXa) and Xa (fXa) through a conformational activation of the serpin and also by a template mechanism in the presence of physiological levels of Ca^{2+} . Recently, it was reported that heparin induces conformational changes in the active-sites of fIXa and fXa, raising the possibility that heparin also modulates the reactivity of these proteases with AT by this mechanism. To test this possibility, we prepared an AT mutant in which four critical heparin-binding residues of the serpin (Arg-45, Arg-46, Lys-114, and Arg-129) were replaced with non-basic residues. This mutant lost its affinity for heparin, but retained its normal reactivity with coagulation proteases. Thus, the high-affinity AT-binding pentasaccharide fragment of heparin had no cofactor effect on the reactivity of the AT mutant with coagulation proteases. Full-length heparin-concentration dependence of the AT inhibition of fIXa and fXa revealed that in contrast to a greater than 4–5 orders of magnitude accelerating effect for heparin on the AT inhibition of fIXa and fXa, heparin exhibits a negligible cofactor effect (<2-fold) on the mutant AT inhibition of these proteases. The same results were obtained for the mutant AT inhibition of thrombin and factor VIIa, however, heparin accelerated the mutant AT inhibition of factor XIa ~10-fold. We conclude that, with the exception of factor XIa, heparin-mediated conformational modulation of the active-sites of coagulation proteases makes a minor contribution to the regulation of these proteases by AT.

Keywords

antithrombin; heparin; coagulation; factor Xa; factor IXa; thrombin

Introduction

Antithrombin (AT) is the major serine protease inhibitor (serpin) in plasma that regulates the proteolytic activities of coagulation proteases of both intrinsic and extrinsic pathways (1–3). AT is known to be a slow inhibitor of its target proteases unless it is bound to heparin-like glycosaminoglycans, similar to those found on the surface of vascular endothelium (4,5). This is the basis for the widespread use of heparin as an anticoagulant drug in cardiovascular medicine (6,7). High molecular weight heparins can promote AT inactivation of coagulation proteases by 4–5 orders of magnitude in the presence of physiological concentrations of Ca^{2+} (8). It has been well established that this dramatic cofactor effect of high molecular weight heparins is primarily mediated through (i) a template mechanism by the long-chain heparins bridging the serpin and the protease in one complex and (ii) a conformational activation mechanism by a unique pentasaccharide fragment of heparin altering the structure of AT,

*Address of Corresponding Author: Alireza R. Rezaie, Ph.D., Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1100 South Grand Blvd. St. Louis, MO 63104, Phone: (314) 977-9240, Fax: (314) 977-9205, rezaiear@slu.edu.

thereby improving the reactivity of the serpin with coagulation proteases (1,2,8). It has been demonstrated that the first mechanism accounts for the bulk of the cofactor effect of heparin in the AT inhibition of thrombin, with the second mechanism contributing only approximately two-fold to the acceleration of the protease inhibition by the serpin (8). However, the conformational activation of AT primarily accounts for the accelerating effect of heparin in inhibition of factors IXa (fIXa) and Xa (fXa) (9,10), with the template effect of heparin contributing to promotion of the protease inhibition by AT only in the presence of Ca^{2+} (11, 12). It has been demonstrated that the conformational activation of AT is the primary mechanism by which heparin accelerates the inhibition of factor VIIa (fVIIa) when the protease forms a complex with tissue factor (TF) (8,13,14).

Based on the observation that the interaction of heparin with exosites of fIXa and fXa is associated with a conformational change in the catalytic grooves of these proteases (15,16), it has recently been postulated that heparin may also enhance the reactivity of coagulation proteases with AT by this mechanism (15). However, firm support for this hypothesis is missing and noting that both AT and coagulation proteases contain binding exosites for interaction with heparin, it has not been possible to discriminate the cofactor effect of heparin on the serpin from its effect on the protease. To circumvent this problem, we have constructed and expressed a mutant of AT in which four critical heparin-binding residues of the serpin has been substituted with non-basic residues. This AT mutant does not detectably bind to heparin, but the mutant serpin inhibits all coagulation proteases with a rate that is indistinguishable from that of wild-type AT. Using this mutant in inhibition studies in the absence and presence of a high molecular weight heparin and the pentasaccharide fragment of heparin we demonstrate that a heparin-mediated conformational change in the active-site pocket makes ~10-fold contribution to the AT inhibition of factor XIa. This mechanism, however, does not play a role in the AT inhibition of other coagulation proteases.

Materials and Methods

Expression and purification of AT

Recombinant human AT was expressed in HEK-293 cells using the RSV-PL4 expression/purification vector system as described (17). The same vector system was used to prepare an AT mutant in which the heparin-binding residues Arg-45, Arg-46, Lys-114 and Arg-129 was replaced with either Glu or Ala (AT-4Mut). Both wild-type and mutant serpins were purified from cell culture supernatants by immunoaffinity chromatography using the HPC4 monoclonal antibody linked to Affi-gel 10 (Bio-Rad) as described (17). Concentrations of the AT derivatives were determined from the absorbance at 280 nm using a molar absorption coefficient of $37,700 \text{ M}^{-1} \text{ cm}^{-1}$ and by stoichiometric titration of the serpins with calibrated concentrations of thrombin as described (18). The cDNA fragment coding for the catalytic domain of fXI (fXI-CD) (starting from residues Lys-357, the first residue immediately outside apple-4 domain) was prepared by PCR methods and expressed in HEK-293 cells using the RSV-PL4 expression/purification vector system as described above. The zymogen fXI-CD was purified by the HPC4 immunoaffinity chromatography, activated by factor XIIa and its concentration was calculated by an amidolytic activity assay as described (19).

The plasma proteins thrombin, fIXa, fXa, and fXIa and factor XIIa were purchased from Haematologic Technologies (Essex Junction, VT). The therapeutic unfractionated heparin (average MW ~15 kDa) and the AT-binding pentasaccharide fondaparinux sodium (MW=1.728 kD) (Organon Sanofi-Synthelabo, France) were purchased from Quintiles Clinical Supplies (Mt. Laurel, NJ). The concentration of heparins were based on the AT-binding sites and determined by stoichiometric titration of AT (1 μM) with varying concentrations of heparins (0–5 μM), with monitoring of the interaction by changes in protein fluorescence as described (18). The chromogenic substrates, Spectrozymes FXa (SpFXa) and

TH (SpTH) were purchased from American Diagnostica (Greenwich, CT). S2366 was purchased from Diapharma (West Chester, OH), and CBS 31.39 was purchased from Midwest Bio-Tech. Inc. (Fishers, IN).

Fluorescence measurements

Aminco-Bowman series 2 spectrophotometer (Spectronic Unicam, Rochester, NY) was used for protein fluorescence measurements at 25 °C as described (17). The excitation and emission wavelengths were 280 and 340 nm, respectively. The bandwidths were set at 4 nm for excitation and 8 nm for emission. Titration was performed by the addition of a 1–2 μ L of high concentration of stock solution of heparin into wild-type AT (50 nM) or mutants (200 nM) samples in 0.1 M NaCl, 0.02 M Tris-HCl (pH=7.5) containing 0.1% polyethylene glycol (PEG) 8000 (TBS). Data from at least 3 experiments were analyzed as the ratio of change in the fluorescence intensity of the sample containing heparin to the initial intensity of the control protein lacking the cofactor. The affinity of AT derivatives for heparin was calculated by nonlinear least-squares computer fitting of the data by the quadratic binding equation as described (18).

Inhibition assays

The rate of inactivation of proteases by the AT derivatives in both the absence and presence of pentasaccharide and heparin was measured under pseudo-first order conditions by a discontinuous assay method as described (17). Briefly, in the absence of heparin, 1–5 nM of each protease was incubated with 50–2000 nM AT in 0.1 M NaCl, 0.02 M Tris-HCl (pH 7.5) containing 0.1 mg/mL bovine serum albumin (BSA), 0.1% polyethylene glycol 8000 and 5 mM CaCl_2 (TBS/ Ca^{2+}). All reactions were carried out at room temperature in 50 μ L volumes in 96-well polystyrene plates. After a period of time (5–240 min depending on the rate of the reactions), 50 μ L of chromogenic substrate specific for each protease (SpFXa for fXa, SpTH for thrombin, CBS 31.39 for fXIa, and S2366 for fXIIa) in TBS was added to each well and the remaining enzyme activities were measured by a V_{max} Kinetics Microplate Reader (Molecular Devices, Menlo Park, CA). The reaction conditions with all proteases in the presence of pentasaccharide (H_5) were the same except that AT derivatives (25–2000 nM) were incubated with proteases in the presence of a saturating concentration of H_5 (10 μ M) for 0.5–240 min. The reaction conditions with all proteases in the presence of excess heparin (10–50 μ M) were the same except that concentrations of the AT derivatives ranged from 5–2000 nM and the incubation time was reduced to 0.5–120 min. The observed pseudo-first-order rate constants (k_{obs}) were determined by computer fitting of the time-dependent change of the protease activities to a single exponential function and the second-order association rate constants (k_2) for uncatalyzed and catalyzed reactions were obtained from the slopes of linear plots of k_{obs} vs. the concentrations of AT as described (18).

Heparin concentration dependence of inhibition reactions were determined by incubating each protease (0.5–5 nM) with 5–2000 nM of wild-type or mutant AT and 0–50 μ M unfractionated heparin in 50- μ L reactions in TBS/ Ca^{2+} . Following 0.5–60 min incubations at room temperature, 50 μ L of chromogenic substrate specific for each protease in TBS containing 1 mg/mL Polybrene (to immediately neutralize heparin) was added. The catalyzed k_{obs} values at each heparin concentration were determined from a first-order rate equation, and the k_2 values were calculated by dividing k_{obs} by the serpin concentration. Plots of k_2 as a function of heparin concentration yielded maximal k_2 values and optimal concentrations of heparin for each reaction.

Results

Wild-type and mutant AT derivatives were expressed in HEK-293 cells and purified to homogeneity by the HPC4 immunoaffinity chromatography as described (17). SDS-PAGE

analysis under non-reducing conditions suggested that the recombinant serpins have been purified to homogeneity and that both migrate with a relative molecular mass identical to that of plasma-derived wild-type AT (data not shown). SDS-PAGE analysis further indicated that the mutant forms stable complexes with both fXa and thrombin with no evidence of cleavage by either protease (data not shown). Unlike the high affinity interaction with wild-type AT ($K_D = 1$ nM), neither pentasaccharide nor the full-length heparin exhibited a detectable affinity for AT-4Mut up to 80 μ M polysaccharide as evidenced by monitoring of the intrinsic protein fluorescence (Fig. 1).

Effect of heparin exosite occupancy in fIXa reaction with AT

Heparin has been reported to bind to the protease domain of fIXa to induce conformational changes in the active-site groove, thereby modulating the macromolecular substrate and inhibitor specificity of fIXa (15,20). Thus, it has been hypothesized that the heparin-mediated conformational change in the active-site pocket of fIXa may partially account for the accelerating effect of heparin in inhibition of the protease by AT (15). To determine whether a heparin-induced conformational change in the active-site pocket of fIXa contributes to the high reactivity of fXa with AT, the heparin concentration dependence of inhibition of fIXa by either wild-type AT or AT-4Mut was evaluated. As shown in Fig. 2, the inhibition of fIXa by wild-type AT exhibited a bell-shaped dependence on the heparin concentration in the presence of Ca^{2+} , confirming the previous results that the heparin occupancy of fIXa basic exosite makes a significant contribution to protease inhibition by AT by a template mechanism (12,21,22). In contrast to wild-type AT, however, heparin exhibited a minimal cofactor effect on the AT-4Mut inhibition of fIXa (Fig. 2A). The AT-concentration dependence of fIXa inhibition by AT-4Mut in the absence and presence of 10–50 μ M heparin suggested that heparin accelerates the mutant AT inhibition of fIXa ~ 1.4 -fold (Fig. 2B, Table 1). These results clearly suggest that heparin-mediated modulation of the catalytic pocket makes, if any, a minor contribution to the accelerating effect of heparin ($\sim 10^5$ -fold) in the reaction of fIXa with AT (Table 1). A similar reactivity for fIXa with AT-4Mut was observed in both the absence and presence of pentasaccharide (Table 1).

Effect of heparin exosite occupancy in fXa reaction with AT

Recently, it has been hypothesized that heparin can also allosterically modulate the activity of fXa with macromolecular substrates (16). To determine whether the ligand occupancy of heparin-binding exosite of fXa allosterically modulates the reactivity of fXa with AT, the heparin concentration dependence of the AT and AT-4Mut inhibition of fXa was studied. As shown in Fig. 3, heparin made a significant contribution to the wild-type AT inhibition of fXa by a template mechanism in the presence of Ca^{2+} , however, similar to the reaction with fIXa, the potential modulatory effect of heparin on the active-site pocket of fXa does not notably contribute to the acceleration of the protease inhibition by AT (Table 1). The AT-concentration dependence of fXa inhibition by AT-4Mut in the absence and presence of 10–50 μ M heparin suggested that heparin accelerates the mutant AT inhibition of fXa ~ 1.7 -fold (Fig. 3B, Table 1). Thus, the cofactor effect of heparin on the AT inhibition of fXa is primarily mediated by the combination of a conformational activation of AT and a template mechanism in the presence of Ca^{2+} as has been demonstrated previously (11,23). A similar reactivity for fXa with AT-4Mut was observed in both the absence and presence of pentasaccharide (Table 1).

Effect of heparin exosite occupancy in thrombin reaction with AT

Unlike the reaction with the two proteases described above, the cofactor effect of heparin on the AT inhibition of thrombin is primarily mediated by a template mechanism, with the pentasaccharide-mediated conformational activation of the serpin contributing less than 2-fold to acceleration of the reaction (8). The heparin concentration dependence of inhibition studies

with AT and AT-4Mut indicated that a possible heparin-mediated conformational change in the active-site pocket of thrombin does not contribute to the accelerating effect of heparin ($>10^4$ -fold) in the AT inhibition of thrombin (Fig. 4A). Comparisons of pseudo-first order inhibition rate constants in the absence and presence of 10–50 μ M heparin indicated that a heparin-mediated conformational change in the active site groove may contribute ~ 1.7 -fold to reactivity of thrombin with AT (Table 1). A similar reactivity for thrombin with AT-4Mut was observed in both the absence and presence of pentasaccharide (Table 1).

Effect of heparin exosite occupancy in fXIa reaction with AT

Since both the catalytic and the non-catalytic apple-3 domain of fXIa contain binding sites for heparin, we expressed the catalytic domain of fXIa by itself in mammalian cells and used it for evaluation of the heparin-mediated allosteric effect on the active-site pocket of the protease. The heparin concentration dependence of inhibition studies with wild-type AT and AT-4Mut suggested that heparin accelerates the AT inhibition of fXIa-CD ~ 200 -fold (Fig. 5A, Table 1). Interestingly, heparin also markedly accelerated the AT-4Mut inhibition of fXIa-CD in a saturable manner (Fig. 5A, inset), suggesting that heparin induces a conformational change in the catalytic pocket leading to a substantial improvement in the reactivity of the protease with AT. The same results were obtained when wild-type fXIa was used in the inhibition study (data not presented). The second-order rate constants calculated from the slope of concentration dependence of the AT-4Mut inhibition of fXIa-CD in the absence and presence of 10–50 μ M heparin indicated that the allosteric alteration of the active-site pocket of fXIa by heparin contributes ~ 10 -fold to the acceleration of the reaction with the serpin (Fig. 5, Table 1). The reactivity of fXIa with AT-4Mut was improved ~ 1.2 -fold in the presence of pentasaccharide (Table 1).

Discussion

Heparin can promote the AT inhibition of coagulation proteases by at least three mechanisms. Firstly, heparin can bind to AT to alter the structure of the serpin, thereby facilitating its optimal interaction with the protease by an activation mechanism (9,24). Secondly, heparin chains of sufficient length can bind to both the protease and the serpin, thereby bridging the two molecules together and promoting the interaction by a template mechanism (8,25). Thirdly, the binding of heparin to a protease can induce a conformational change in the active site groove of the protease, thereby improving the complementarity of the residues in and around the active site groove with the serpin. In the case of thrombin, it has been well established that the cofactor effect of heparin is primarily mediated through a bridging mechanism with the conformational activation of the serpin contributing ~ 2 -fold to the inhibition mechanism (8). In the case of fIXa and fXa, the conformational activation of AT by heparin promotes the reactivity of the serpin with both proteases 300–500-fold (8,21). A series of recent studies, have established that the bridging effect of full-length heparins can make a significant contribution (up to 200–300-fold) to the AT inactivation of both fIXa and fXa at the physiological levels of Ca^{2+} (21, 23). In the case of the fVIIa-TF complex, the conformational activation of AT by heparin has been demonstrated to be the primary mechanism by which the polysaccharide accelerates the rate of protease inhibition by the serpin (8). The mechanism of the cofactor function of heparin in the AT inhibition of fXIa appears to resemble that of thrombin, with the high molecular weight heparins accelerating the reaction 500–800-fold and pentasaccharide-mediated conformational activation of the serpin contributing ~ 3 -fold to acceleration of the reaction (8). In this case, however, we recently noted that the template effect of heparin does not account for all of the accelerating effect on the AT inhibition of fXIa (26), possibly suggesting that the binding of heparin to a specific exosite on the protease induces a conformational change in the active-site groove to improve its reactivity with the serpin by a third mechanism.

Recently, two other studies reported that heparin binding to basic exosites of both fIXa and fXa is also associated with conformational changes in the active-site grooves of these proteases, raising the possibility that this mechanism may also contribute to the accelerating effect of heparin in the AT inhibition of these proteases (15). However, it has not been possible to provide firm support for this hypothesis because both the serpin and the proteases contain a binding exosite for interaction with heparin, making it difficult to design a mutagenesis study to discriminate the cofactor effect of heparin on the serpin from its effect on the protease. It follows therefore that the mutagenesis of the residues of the heparin-binding exosite not only eliminates the template effect of heparin but also its potential conformational effect on the active-site pocket of the protease. To circumvent this problem, in this study we prepared the 4Mut derivative of AT which has completely lost its affinity for heparin, but retained its normally reactivity with the coagulation proteases (Table 1). Thus, inhibition studies with this mutant can only report the conformational effect of heparin on the active-site grooves of these proteases. Although analysis of the inhibition data by t-Tests revealed that the fold accelerating effect of heparin on the mutant AT inhibition of both fIXa (1.4-fold) and fXa (1.7-fold) is statistically significant, nevertheless, the results suggest that the contribution from the heparin-mediated conformational change in the active-site pocket to the reactivity of either protease with AT is very small. The hypothesis that heparin-induced conformational change in the active-site of fIXa may contribute to reactivity of the protease with AT was primarily based on the accelerating effect of heparin on the inhibition of fIXa by the kunitz inhibitor, bovine pancreatic trypsin inhibitor which inhibits fIXa by a different mechanism (15). In the case of fXa, although fluorescence studies have observed a heparin-induced conformational change in the active-site groove of the active-site inhibited protease (16), based on our studies here, such a change does not make a notable contribution to the reactivity of the active-site free fXa with AT. Thus, we conclude that although heparin may alter the conformation of the catalytic grooves of fIXa or fXa, nevertheless, a conformational activation of AT and/or a template mechanism accounts for the bulk of the accelerating effect of heparin in the AT inhibition of these proteases.

On the other hand, our data suggests that the heparin-mediated conformational change in the active-site groove of fXIa contributes ~10-fold to improved reactivity of the protease with AT. It is interesting to note that unlike fIXa and fXa, which have basic C-terminal helices that interact with heparin (12,27), the C-terminal helix of fXIa is acidic and not expected to interact with heparin (28). However, the catalytic domain of fXIa has two basic residues on the 220-loop (Arg-222 and Arg-224) that, together with basic residues on the 170-helix, form a patch capable of interaction with heparin (28). We previously demonstrated that the substitution of all three basic residues of the 170-helix of fXIa only partially eliminates the cofactor effect of heparin, with the triple mutant protease exhibiting 18-fold higher reactivity with AT in the presence of heparin (26). In this previous study, the accelerating effect of heparin was saturable and a signature bell-shaped dependence template curve for heparin acceleration of the triple mutant of fXIa was not observed. Thus, we hypothesize that the heparin-mediated conformational change in the active-site groove that improves the reactivity of fXIa with AT ~10-fold is mediated through heparin interacting with basic residues of the 220-loop. The 220-loop of thrombin and vitamin K-dependent coagulation proteases harbor a functionally critical Na^+ -binding site, with the basic residues of this loop contributing to the coordination of Na^+ (29). The corresponding loop in fXIa does not bind Na^+ (29), but appears to interact with heparin to modulate the specificity of the protease with macromolecular substrates and inhibitors (28). Further studies with the 220-loop mutants of fXIa will be required to validate this hypothesis.

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Figure 1.

Binding of heparin to recombinant AT derivatives. The spectral changes were monitored by addition of 1–2 μ L of a concentrated stock solution of heparin to 50–200 nM AT in TBS (pH 7.5) and dissociation constant for wild-type AT ($K_D = 1$ nM) was calculated from the changes of the intrinsic protein fluorescence as described under “Materials and Methods”. The symbols are: ($\circ$) AT wild-type and ($\bullet$) AT-4Mut. Heparin exhibited no detectable affinity for AT-4Mut up to 80 μ M polysaccharide (not shown).

Figure 2.

Heparin concentration dependence of fIXa inhibition by AT derivatives. (A) The heparin-catalyzed k_2 values for the inhibition of fIXa by wild-type AT (○) and AT-4Mut (●) were determined as described under “Materials and Methods”. The fold accelerating effect of heparin was calculated from the ratio of k_2 in the presence of heparin to the same value in the absence of heparin. (B) The pseudo-first order rate constants (k_{obs}) were determined from the time-dependent inhibition of fIXa by different concentrations of AT-4Mut in the absence (○) and presence of heparin (●) in TBS/ Ca^{2+} as described under “Materials and Methods”. Solid lines are best fit of kinetic data to a linear equation. k_2 values from three independent measurements are presented in Table 1.

Figure 3.

Heparin concentration dependence of fXa inhibition by AT derivatives. (A) The heparin-catalyzed k_2 values for the inhibition of fXa by wild-type AT ($\circ$) and AT-4Mut ($\bullet$) were determined and the fold accelerating effect of heparin was calculated from the ratio of k_2 in the presence of heparin to the same value in the absence of heparin. (B) The k_{obs} values were determined from the time-dependent inhibition of fXa by different concentrations of AT-4Mut in the absence ($\circ$) and presence of heparin ($\bullet$) in TBS/ Ca^{2+} . Solid lines are best fit of kinetic data to a linear equation. k_2 values from three independent measurements are presented in Table 1.

Figure 4.

Heparin concentration dependence of thrombin inhibition by AT derivatives. **(A)** The heparin-catalyzed k_2 values for the inhibition of thrombin by wild-type AT ($\circ$) and AT-4Mut ($\bullet$) were determined and the fold accelerating effect of heparin was calculated from the ratio of k_2 in the presence of heparin to the same value in the absence of heparin. **(B)** The k_{obs} values were determined from the time-dependent inhibition of thrombin by different concentrations of AT-4Mut in the absence ($\circ$) and presence of heparin ($\bullet$) in TBS/ Ca^{2+} . Solid lines are best fit of kinetic data to a linear equation. k_2 values from three independent measurements are presented in Table 1.

Figure 5.

Heparin concentration dependence of fXIa-CD inhibition by AT derivatives. (A) The heparin-catalyzed k_2 values for the inhibition of fXIa-CD by wild-type AT (○) and AT-4Mut (●) were determined and the fold accelerating effect of heparin was calculated from the ratio of k_2 in the presence of heparin to the same value in the absence of heparin. The data with AT-4Mut has also been shown in the inset. (B) The k_{obs} values were determined from the time-dependent inhibition of fXI-CD by different concentrations of AT-4Mut in the absence (○) and presence of heparin (●) in TBS/ Ca^{2+} . Solid lines are best fit of kinetic data to a linear equation. k_2 values from three independent measurements are presented in Table 1.

Table 1

Inhibition of coagulation proteases by wild-type and AT-4Mut in the absence and presence of heparin

	-Hep ($M^{-1}s^{-1}$)	+H₅ ($^{-1-1}$)	+Hep ($^{-1-1}$)	+Hep/-Hep (fold)
<u>fIXa</u>				
AT-WT	$(0.68 \pm 0.08) \times 10^2$	$(3.8 \pm 0.3) \times 10^4$	$(1.1 \pm 0.07) \times 10^7$	1.6×10^5
AT-4Mut	$(0.59 \pm 0.11) \times 10^2$	$(0.62 \pm 0.1) \times 10^2$	$(0.82 \pm 0.09) \times 10^2$	1.4
<u>fXa</u>				
AT-WT	$(2.6 \pm 0.2) \times 10^3$	$(6.1 \pm 0.4) \times 10^5$	$(8.4 \pm 0.6) \times 10^7$	3.2×10^4
AT-4Mut	$(3.6 \pm 0.4) \times 10^3$	$3.7 \pm 0.3 \times 10^3$	$(6.1 \pm 0.8) \times 10^3$	1.7
<u>Thrombin</u>				
AT-WT	$(8.5 \pm 0.6) \times 10^3$	$(1.3 \pm 0.1) \times 10^4$	$(8.0 \pm 0.4) \times 10^7$	0.94×10^4
AT-4Mut	$(8.8 \pm 0.5) \times 10^3$	$(8.7 \pm 0.2) \times 10^3$	$(1.7 \pm 0.1) \times 10^4$	1.9
<u>fXIa-CD</u>				
AT-WT	$(2.5 \pm 0.2) \times 10^2$	$(5.1 \pm 0.3) \times 10^2$	$(0.53 \pm 0.06) \times 10^5$	212
AT-4Mut	$(2.0 \pm 0.3) \times 10^2$	$(2.4 \pm 0.2) \times 10^2$	$(2.2 \pm 0.4) \times 10^3$	11

All k_2 values for the AT inhibition of coagulation proteases were determined by a discontinuous assay in TBS/ Ca^{2+} as described under "Materials and Methods". All values (derived from Figs. 2–5) are averages of three independent measurements $\pm$ S.D. The 1.4-fold and 1.7-fold accelerating effects of heparin on the AT-4Mut inhibition of both fIXa and fXa were statistically significant (t-Tests) at p values of <0.05 . H₅, pentasaccharide.