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Role of P2 glycine in determining the specificity of antithrombin reaction with coagulation proteases

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

Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, MO 63104, USA

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ABSTRACT

Structural data suggests that bulky hydrophobic residues at the S2–S4 sub-sites of factor Xa (fXa) restrict the preference of this pocket for small and non-polar residues like Gly at the P2 position of substrates and inhibitors. However, kinetic studies monitoring the cleavage specificity of 10-residue peptides by fXa have identified Phe as the most preferred P2 residue and Gln-Phe-Arg-Ser-Leu-Ser as the most preferred P3–P3′ residues for recognition by fXa. To determine whether this mechanism of specificity is also true for fXa reaction with antithrombin (AT), we prepared two AT mutants having either a Phe at the P2 or Gln-Phe-Arg-Ser-Leu-Ser at the P3–P3′ positions of the reactive center loop. Inhibition kinetic studies indicated that the reactivity of P2-Phe with fXa was significantly (~5-fold) impaired, however, the P3–P3′ mutant exhibited 1.5-fold improved reactivity with the protease, suggesting cooperative effects between P3–P3′ residues influence the P2 specificity of AT. Substitution of Tyr-99 of fXa with a Gly dramatically impaired the reactivity of fXa with wild-type AT, but improved its reactivity with the serpin mutants in the absence, but not in the presence of pentasaccharide. AT with a P2-Phe inhibited thrombin with >150-fold impaired reactivity, however, the defect was restored by either pentasaccharide or by replacing Leu-99 of thrombin with a Gly. The P3–P3′ mutant rapidly inhibited factors VIIa and XIa independent of pentasaccharide. These results indicate that P2-Gly plays a key role in determining the S2 subsite specificity and target protease selectivity of AT in circulation.

Introduction

Antithrombin (AT) is a serine protease inhibitor (serpin) in plasma that regulates the activities of the serine proteases of both intrinsic and extrinsic pathways of the blood coagulation cascade [1–4]. AT inhibits its target coagulation proteases by a branched pathway, suicide substrate inhibition mechanism in which a Michaelis-type enzyme–inhibitor complex, formed in the first reaction step, is converted to a covalent acyl-enzyme intermediate complex in the second step of the reaction [5]. Similar to interaction of serine proteases with true substrates, a typical salt-bridge between Asp-189 at the S1 sub-site of coagulation proteases and an Arg at the P1 position (nomenclature of Schechter and Berger [6]) of the AT reactive center loop (RCL) accounts for the specificity of the initial interaction [5,7]. However, unlike reaction with true substrates, attack of P1-Arg by the catalytic Ser-195 in the second step induces a conformational change in the serpin which traps the protease in the form of inactive acylated complex [8,9]. Since most of the substrates and inhibitors of coagulation proteases contain an Arg at the P1 position, thus other residues surrounding the scissile bond must contribute to determinants of specificity of these proteases [10]. Structural and mutagenesis data have indicated that differences in the P3–P3′ residues of the scissile bonds are partly responsible for determining the specificity of coagulation reactions [11–14]. The molecular basis for such a specificity is known to be due to existence of variant residues in the extended binding pocket of coagulation proteases which can interact with the P3–P3′ residues of the scissile bonds [10,15]. In support of this hypothesis, two residues at positions 99 and 192 of coagulation proteases have been demonstrated to be critical for determining the P2 and P3 recognition specificity of procoagulant and anticoagulant proteases [16,17]. Thus, mutagenesis of both of these residues of coagulation proteases or the P3–P3′ residues of substrates and inhibitors is known to alter the specificity of catalytic reactions [17,18].

Structural and mutagenesis data have indicated that a Gly at the P2 position of the AT RCL is required for an effective interaction of the serpin with fXa in both the absence and presence of heparin [19,20]. The preference for a small residue at the P2 position of AT appears to be due to presence of bulky residues including Tyr-99 and Phe-174 at the P2 binding pocket of fXa which spatially limits the recognition specificity of this pocket for only small and

* Corresponding author. Address: Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1100 South Grand Blvd., St. Louis, MO 63104, USA. Fax: +1 314 977 9205.
E-mail address: rezaiear@slu.edu (A.R. Rezaie).

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non-polar residues, like Gly [17,21]. Consistent with this hypothesis, fXa cleavage sites on the physiological substrates including prothrombin and protease activated receptor-2 also possess Gly at the P2 positions. However, a recent kinetic study monitoring the specificity of the cleavage of 10-residue fluorogenic synthetic peptides with an Arg at the P1 position by fXa identified Phe as the most preferred P2 residue and Gln-Phe-Arg-Ser-Leu-Ser (AT-QFRSLS) as the most preferred P3–P3′ residues for fXa in these substrates [22]. To determine whether the results with the small peptide substrates hold true for interaction of fXa with natural macromolecules, we prepared two RCL mutants of AT in one of which P2-Gly was replaced with Phe and in the other P3–P3′ residues were replaced with optimal residues, identified by the amidolytic activity assay [22]. Characterization of these mutants in inhibition studies with fXa suggest that cooperative effects between P3–P3′ residues influence the P2 recognition specificity of AT and that a Gly at the P2 position is the most preferred residue for interaction with fXa in the presence of pentasaccharide. Further studies showed that AT-QFRSLS rapidly inhibits factors VIIa and XIa independent of pentasaccharide, but reacts slower with thrombin, suggesting a critical role for P3–P3′ residues in modulating the specificity of AT in reaction with different coagulation proteases in circulation.

Materials and methods

Expression and purification of recombinant proteins. Recombinant human AT was expressed in HEK-293 cells using the RSV-PL4 expression/purification vector system as described [13]. The RCL mutants of AT with a Phe substituting for the native Gly at the P2 position (AT-P2-Phe) and Gln-Phe-Arg-Ser-Leu-Ser (AT-QFRSLS) replacing the native P3–P3′ residues (AGRSLN) of AT were constructed by standard PCR mutagenesis methods and expressed using the same vector system. 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) followed by a HiTrap-Heparin (Amersham Pharmacia Bio-tech, Piscataway, NJ) chromatography as described [13]. Concentrations of the AT derivatives were determined from the absorbance at 280 nm using a molar absorption coefficient of 37,700 M^{-1} cm^{-1} and by stoichiometric titration of the serpins with calibrated concentrations of fXa as described [23]. Wild-type and fX mutant, in which Tyr-99 was replaced with a Gly, were expressed in HEK-293 cells, purified to homogeneity and activated by the fX activating enzyme from Russell's viper venom (RVV-X) as described [17]. The expression and purification of thrombin and its Leu-99 to Gly substitution mutant (L99G) have been described [24]. Recombinant activated protein C was prepared as described [18]. Soluble tissue factor (TF) was prepared as described [25].

The plasma proteins factors IXa (fIXa), VIIa (fVIIa) and XIa (fXIa) and RVV-X were purchased from (Haematologic Technologies, Essex Junction, VT). The therapeutic pentasaccharide fondaparinux sodium (MW = 1.728 kDa) was from Organon Sanofi-Synthelabo (France). The concentration of pentasaccharide was based on the AT-binding sites and determined by stoichiometric titration of AT (1 μM) with varying concentrations of pentasaccharide (0–5 μM), with monitoring of the interaction by changes in protein fluorescence as described [23]. The chromogenic substrates, Spectrozymes FXa (SpFXa) PCa (SpPCa) and FVIIa (SpVIIa) 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 [13]. 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 μl of high concentration of stock solution of pentasaccharide (H5) into 50 nM of each AT sample 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 H5 to the initial intensity of the control protein lacking the heparin 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 [23].

Inhibition assays. The rate of inactivation of proteases by the AT derivatives in both the absence and presence of H5 was measured under pseudo-first order conditions by a discontinuous assay method as described [13]. Briefly, in the absence of H5, 1–5 nM of each protease was incubated with 50–2000 nM AT in TBS containing 0.1 mg/mL bovine serum albumin (BSA) and 5 mM $CaCl_2$. All reactions were carried out at room temperature in 50 μL volumes in 96-well polystyrene plates. After a period of time (5–240 min depending on the rate of the reactions), 50 μL of chromogenic substrate specific for each protease (SpFXa for fXa, SpPCa for both thrombin and APC, CBS 31.39 for fIXa, SpVIIa for fVIIa and S2366 for fXIa) 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 a saturating concentration of H5 (1–2 μM) were the same except that concentrations of the AT derivatives ranged from 25 to 400 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 [23].

Results and discussion

Wild-type and mutant AT derivatives were expressed in HEK-293 cells and purified to homogeneity by a combination of HPC4 immunoaffinity and HiTrap-Heparin column chromatography as described [13]. 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 wild-type AT (data not shown). Both mutants formed stable complexes with fXa in both the absence and presence of H5 (data not shown). The binding of H5 to both wild-type and mutant serpins resulted in a similar enhancement in the intrinsic protein fluorescence yielding dissociation constants (K_D) of 11 ± 4 nM for wild-type, 10 ± 2 nM for AT-P2-Phe, and 42 ± 10 nM for AT-QFRSLS (Fig. 1). Thus, the affinity of the AT-P2-Phe mutant for binding to H5 was not affected, however, the affinity of the P3–P3′ site mutant for heparin was impaired ~4-fold, suggesting that the structure of P3–P3′ residues can influence the affinity of AT for its cofactor. This is in agreement with published data showing that the conformation of the RCL is linked to the heparin-binding D-helix [8,26].

Reaction with fXa

AT-P2-Phe inhibited fXa with ~5-fold slower pseudo-first order rate constant (k_{obs}), however, AT-QFRSLS exhibited ~1.5-fold improved reactivity with the protease (Fig. 2A). The latter results support the amidolytic activity data that QFRSLS may be the most

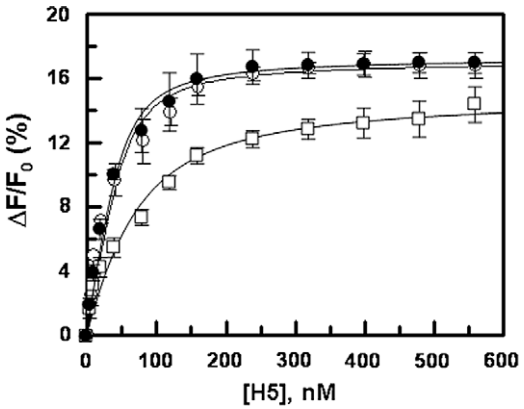

Fig. 1. Binding of pentasaccharide (H5) to recombinant AT derivatives. The spectral changes were monitored by addition of 1–2 μL of a concentrated stock solution of H5 to 50 nM AT in TBS (pH 7.5) and dissociation constants were calculated from the changes of the intrinsic protein fluorescence as described under "Materials and methods". The symbols are: (○) AT-wild-type, (●) AT-P2-Phe, and (□) AT-QFRSLS.

preferred P3–P3′ residues for fXa [22]. Nevertheless, the data with AT-P2-Phe further suggests that there are cooperative effects between P3–P3′ residues and that a P2 preference for Phe by fXa is dependent on the nature of other residues surrounding the scissile bond. This mechanism of specificity may explain how fXa can activate other natural substrates (i.e., factors V and VIII) containing bulky residues like Leu and Ile at the P2 positions. Based on structural and modeling data, it has been hypothesized that the phenolyic side chain of Tyr-99 has conformational plasticity to swing out of its position in order to accommodate P2 residues with bulkier side chains at the S2 sub-site [21]. The likely candidate, influencing the P2 recognition specificity of fXa, is the P3 residue since no significant structural differences appear to exist between other P3–P3′ residues of AT-P2-Phe (AFRSLN) and AT-QFRSLS.

The conformational activation of AT by pentasaccharide is known to accelerate the AT inhibition of fXa ~300-fold [27]. The results presented in Fig. 2B suggest that the reactivity of both AT-P2-Phe and AT-QFRSLS with fXa in the presence of pentasaccharide is ~2- to 3-fold slower than that of wild-type AT (Table 1), suggesting that the native P3–P3′ residues have the most favorable sequence in the activated conformation for interaction with fXa. By contrast to wild-type fXa, a Tyr-99 to Gly (Y99G) mutant of fXa reacted very poorly with wild-type AT, however, the mutant protease reacted with dramatically improved rate constant with both AT mutants containing a Phe at the P2 position (Fig. 2C). Nevertheless, pentasaccharide eliminated the differences in the reactivity of the protease mutant with serpins (Fig. 2D). Thus, in contrast to ~600-fold rate accelerating effect with wild-type AT, the cofactor effect of heparin was reduced to 32- and 6-fold for AT-P2-Phe and AT-QFRSLS, respectively (Table 1). Taken together, these results suggest that Tyr-99 of fXa determines the P2 specificity of the AT RCL and that a Gly at this site is the most preferred residue for recognition by fXa in the heparin-activated conformation.

Reaction with thrombin

The reactivity of AT-P2-Phe with thrombin was dramatically impaired (Fig. 3A) as evidenced by the mutant inhibiting thrombin with ~164-fold slower k_{obs}. Interestingly, however, pentasaccharide which is known to have a minimal effect on the reactivity of AT with thrombin [27], accelerated the AT-P2-Phe inhibition of thrombin ~64-fold (Table 1), suggesting that a Gly at the P2 position of AT is partly responsible for the insensitivity of thrombin to

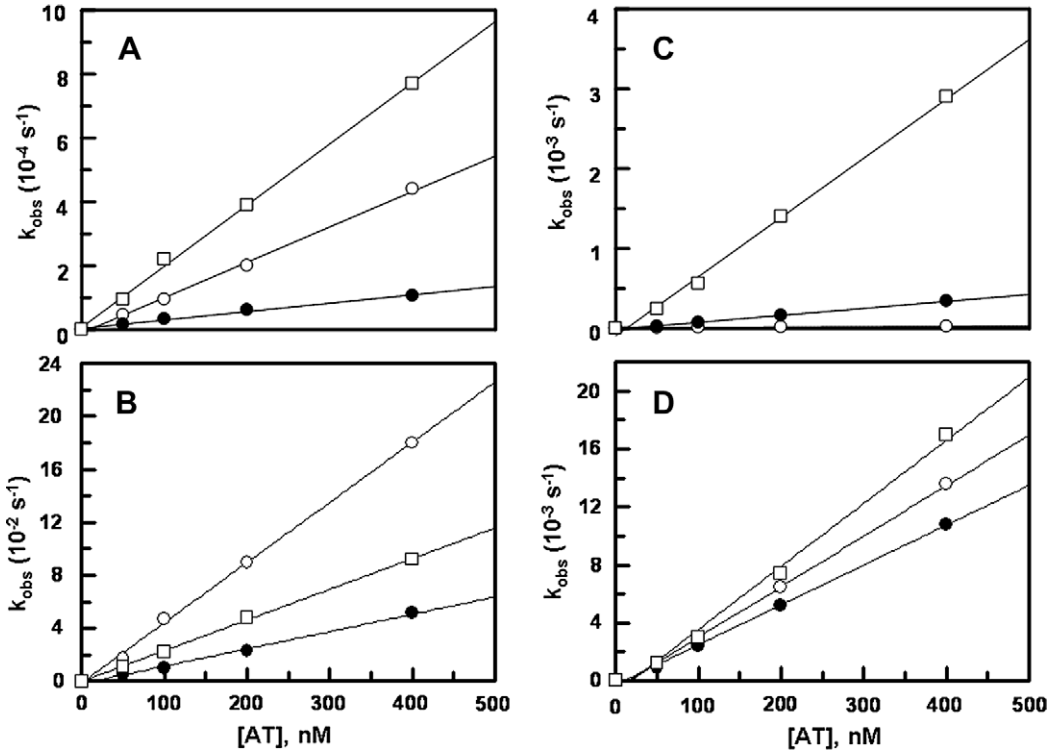

Fig. 2. Inhibition of fXa and fX-Y99G by AT derivatives in the absence and presence of pentasaccharide. (A) The pseudo-first order rate constants (k_{obs}) were determined from the time-dependent inhibition of fXa by different concentrations of AT (○), AT-P2-Phe (●) and AT-QFRSLS (□) in TBS/Ca^{2+} as described under "Materials and methods". (B) The same as A except that k_{obs} values were determined in the presence of pentasaccharide (1 μM). (C) The same as A except that k_{obs} values were determined for fXa-Y99G. (D) The same as C except that k_{obs} values were determined in the presence of pentasaccharide. Solid lines in both panels 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 AT derivatives in the absence and presence of pentasaccharide (H5). k_2 values for the AT inhibition of proteases were determined by a discontinuous assay in TBS/Ca^{2+} as described under "Materials and methods". All values (derived from Figs. 2–4) are averages of three independent measurements ± SD.

	−H5 × 10^2 (M^{-1} s^{-1})	+H5 × 10^5 (M^{-1} s^{-1})	+H5/−H5-fold
fXa			
AT-WT	11.1 ± 1.2	4.5 ± 0.1	405
AT-P2-Phe	2.6 ± 0.1	1.3 ± 0.1	500
AT-QFRSLS	19.2 ± 0.4	2.3 ± 0.2	120
fXa-Y99G			
AT-WT	0.58 ± 0.03	0.35 ± 0.01	603
AT-P2-Phe	8.7 ± 0.4	0.28 ± 0.03	32
AT-QFRSLS	74.3 ± 2.5	0.45 ± 0.02	6
Thrombin			
AT-WT	77.2 ± 6.2	0.13 ± 0.01	1.7
AT-P2-Phe	0.47 ± 0.03	0.03 ± 0.001	64
AT-QFRSLS	28.8 ± 2.8	0.04 ± 0.002	1.4
Thrombin-L99G			
AT-WT	0.19 ± 0.02	0.00036 ± 0.00003	1.9
AT-P2-Phe	81.1 ± 3.6	0.24 ± 0.02	3.0
AT-QFRSLS	788 ± 46	1.2 ± 0.1	1.5
fVIIa-TF			
AT-WT	0.34 ± 0.02	0.029 ± 0.002	85
AT-P2-Phe	1.7 ± 0.3	0.034 ± 0.001	20
AT-QFRSLS	18.3 ± 1.6	0.033 ± 0.004	1.8
fXIa			
AT-WT	3.1 ± 0.2	0.005 ± 0.0006	1.6
AT-P2-Phe	3.2 ± 0.3	0.006 ± 0.0003	1.9
AT-QFRSLS	35.2 ± 1.1	0.045 ± 0.004	1.3

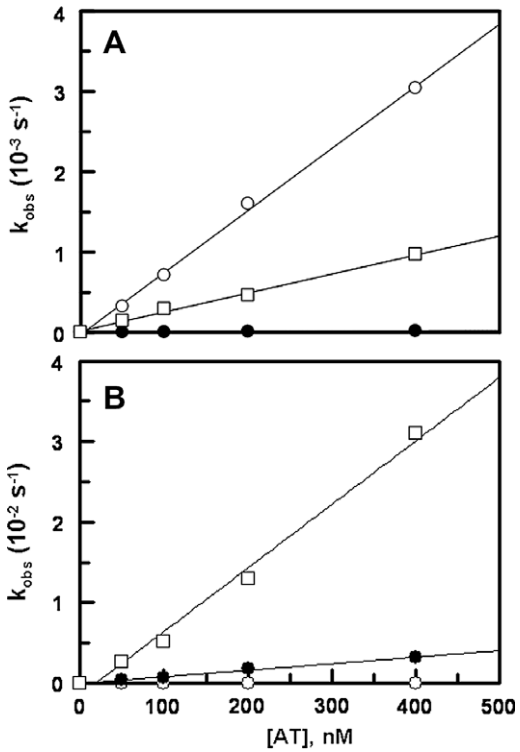

Fig. 3. Inhibition of wild-type and thrombin-L99G by AT derivatives. (A) k_{obs} values for AT (○), AT-P2-Phe (●) and AT-QFRSLS (□) were determined as described under "Materials and methods". (B) The same as A except that k_{obs} values were determined for thrombin-L99G.

recognition of the activated conformation of the serpin. This hypothesis is consistent with a previous report showing that the substitution of P2-Gly of AT with Pro also markedly increases the reactivity of thrombin with the activated conformation of the mutant serpin [19]. In contrast to reaction with AT-P2-Phe, thrombin reacted with AT-QFRSLS with only ~3-fold slower rate constant, and similar to reaction with wild-type AT, the reactivity of thrombin with the activated conformation of the mutant minimally affected (Table 1). Similar to reaction with fXa-Y99G, thrombin-L99G also reacted very poorly with wild-type AT (Fig. 3B). However, the thrombin mutant reacted normally with AT-P2-Phe and its reactivity with AT-QFRSLS was improved ~10-fold (Fig. 3B and Table 1). It was interesting to note that, relative to its reaction with wild-type AT, the reactivity of thrombin-L99G with AT-P2-Phe and AT-QFRSLS was improved 400- and 4000-fold, respectively (Table 1), suggesting a key role for the S2–P2 interaction in modulating the specificity of thrombin reaction with AT.

Structural and mutagenesis data have indicated that the P1-Arg-393 of AT in the native conformation is orienting inward establishing a weak electrostatic interaction with an acidic residue (Glu-255) located on s3C [28,29]. It has been hypothesized that the activation of AT by heparin results in the disruption of this ionic interaction, thereby reorienting P1-Arg to an outward position [26,28,29]. This mechanism of the conformational activation of AT by heparin leads to ~5-fold acceleration of the protease inhibition by the activated serpin [28]. The remaining cofactor activity of heparin in the conformational activation of AT is mediated through exosite-dependent interactions of AT with fXa which is facilitated by heparin exposing cryptic residues on three β-sheets including s1C, s3C and s4C of AT [30–33]. We recently showed that thrombin does not recognize the heparin-activated conformation of AT because it lacks the specific exosite of fXa that recognizes the serpin [34]. Thus, the observation that the reactivity of AT-P2-Phe with thrombin is improved 64-fold in the presence of pentasaccharide supports the hypothesis that, in addition to facilitating exosite interactions, heparin also induces a conformational change in the RCL of AT that involves the P2 residue, however, due to lack of a stabilizing side chain for Gly at this position, it appears that the change in the conformation of the RCL is not detected by thrombin. In this context, the dramatic impairment in the reactivity of AT-P2-Phe with thrombin is most likely due to Phe pointing outward in the native conformation of AT, thus not fitting into the smaller S2 pocket of thrombin [11]. However, the activation of the mutant by pentasaccharide reverses the orientations of the side chains of P1 (Arg-393) and P2 (Phe-392) in the mutant thus rendering thrombin sensitive to the activated conformation of the serpin mutant. The RCL of AT-QFRSLS does not appear to undergo this type of heparin-mediated conformational change (possibly due to P3-Gln), explaining its lower affinity for heparin (Fig. 1).

Reaction with other coagulation proteases

The reactivity of fIXa with AT mutants was not significantly altered, however, k_{obs} for the reaction of fVIIa-TF with AT-QFRSLS was dramatically (~54-fold) improved (Fig. 4A). Interestingly however, in contrast to wild-type AT, which had ~85-fold improved reactivity with fVIIa-TF in the presence of pentasaccharide, the heparin cofactor had minimal accelerating effect on the reaction of the AT mutant with fVIIa-TF (Fig. 4B, Table 1). The reactivity of AT-QFRSLS with fXIa was also improved greater than 10-fold (Fig. 4C). AT-P2-Phe inhibited fVIIa-TF with 5-fold improved reactivity, but exhibited normal reactivity with fXIa (Table 1). These results suggest that the residues of the P3–P3′ site restrict the specificity of the AT reaction with both fVIIa and fXIa and responsible for the slower reactivity of AT with these proteases. In addition to procoagulant proteases, the AT mutant also slowly inhibited APC. Thus, in contrast to no detectable reactivity of AT with APC, AT-QFRSLS inhibited APC with $k_2 = 24$ M^{-1} s^{-1}, suggesting that P2-Gly also contributes to lack of the reactivity of AT with the anti-

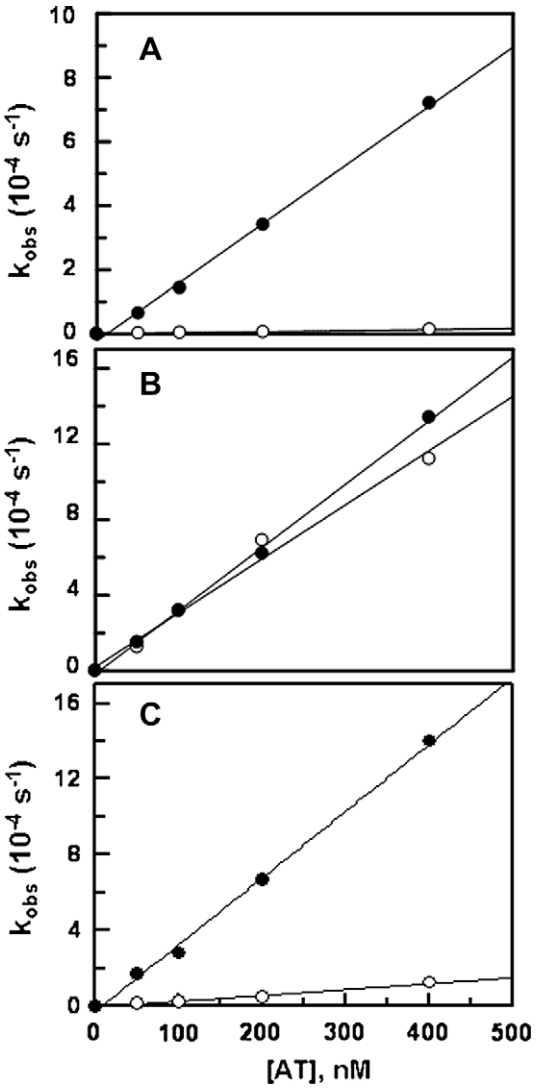

Fig. 4. Inhibition of factors VIIa and XIa by AT derivatives. (A) k_{obs} values for fVIIa-TF by AT (○), AT-QFRSLS (●) were determined as described under "Materials and methods". (B) The same as A except that k_{obs} values were determined in the presence of pentasaccharide. (C) The same as A except that k_{obs} values were determined for factor XIa.

coagulant protease. Taken together, these results suggest that P3–P3′ residues of AT have been adapted to maximally inhibit thrombin in the circulation, but inhibit fIXa and fXa only when it is bound to and activated by specific endothelial glycosaminoglycans. The same structural features retards the reactivity of AT with fVIIa in the initiation phase of the clotting cascade and prevents its reaction with APC in the anticoagulant pathway. P2-Gly plays a key role in enabling AT to exert these regulatory functions.

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The authors have no conflict of interests to declare.

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