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Inhibitory Properties of the P1 Tyr Variant of Antithrombin[†]

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

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

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ABSTRACT: Antithrombin (AT) and protein Z-dependent protease inhibitor (ZPI) are among two physiological serpin inhibitors in plasma that are involved in the regulation of the clotting cascade. Unlike AT, which can inhibit the proteolytic activity of all coagulation proteases, ZPI has narrower protease specificity, inhibiting only factors Xa (fXa) and XIa. Unlike an Arg at the P1 site of the AT reactive center loop (RCL), this residue is a Tyr in ZPI. To investigate the contribution of P1 Tyr in restricting the specificity of ZPI, we engineered an AT mutant in which the P1 Arg of the RCL was replaced with the P1 Tyr of ZPI (AT-R393Y). The reactivity of AT-R393Y with fXa and thrombin was decreased 155- and 970-fold, respectively. However, the serpin mutant inhibited chymotrypsin with an efficiency higher by >4 orders of magnitude. By contrast, chymotrypsin did not exhibit any reactivity with ZPI. The substitution of Asp-189 of fXa with the corresponding residue of chymotrypsin (Ser) did not improve the reactivity of the protease mutant with AT-R393Y; however, the fXa mutant reacted normally with ZPI. These results suggest that the contribution of P1 Tyr to restricting the protease specificity of ZPI is RCL context-dependent and that in addition to P1 Tyr, other structural features within and/or outside the ZPI RCL are involved in determining the protease specificity of the serpin. The results further suggest that thrombin is less tolerant than fXa in accommodating the nonoptimal P1 Tyr of the AT mutant in its active-site pocket.

The proteolytic activity of coagulation proteases is primarily regulated by the serine protease inhibitors (serpins) in plasma (*3*−*5*). Antithrombin (AT)[1] is a major serpin that regulates the activity of coagulation proteases of both intrinsic and extrinsic pathways (*6*). Protein Z (PZ)-dependent protease inhibitor (ZPI) is another serpin which, unlike AT, has a narrower target specificity, thus being capable of inhibiting only factors Xa (fXa) and XIa (fXIa) (*7*, *8*). Both serpins are rather weak inhibitors of their target coagulation proteases unless they form complexes with their specific cofactors. In the case of AT, heparin and heparin-like glycosaminoglycans that line the microvasculature function as cofactors to activate the serpin, thereby dramatically improving its reactivity with coagulation proteases (*3*, *9*). This is the basis for the extensive use of heparin for prophylaxis and treatment of venous thrombosis. Heparin activates AT by binding to a basic exosite on the serpin and inducing a conformational change in the reactive center loop (RCL), thereby facilitating the optimal recognition of AT by coagulation proteases (*9*−*11*). Heparins of sufficient chain length can also bind to basic exosites of coagulation proteases to hold both AT and the protease in one complex, thereby facilitating the interaction by a bridging mechanism (*6*). In the case of ZPI, the vitamin K-dependent PZ, but not heparin, functions as a cofactor to promote the inhibition of fXa bound to negatively charged membrane surfaces (*7*). In contrast to its reaction with fXa, the ZPI inhibition of fXIa is independent of PZ (*8*, *12*). The physiological importance of these serpins to the regulation of blood coagulation can be gleaned from the reports that the deficiency of either serpin is associated with a higher incidence of venous thrombosis (*13*, *14*).

There is growing interest in studying the structure and function of AT and other serpins, with the goal of developing novel serpin-based anticoagulants with potential therapeutic utility. Thus, understanding the mechanism by which the cofactors heparin and PZ bind to their target serpins to accelerate the inhibition of coagulation proteases can facilitate the design of superior anticoagulant serpins capable of effectively regulating the clotting cascade in a manner independent of a cofactor. Indeed, on the basis of the extensive structure−function studies with AT, several AT derivatives capable of rapidly reacting with fXa in a manner independent of a cofactor have been identified (*15*, *16*). However, the exact mechanism by which PZ accelerates the ZPI inhibition of fXa is not well understood. Moreover, the mechanism by which ZPI specifically inhibits fXa and fXIa, but not other coagulation proteases, has not been fully investigated.

Recent mutagenesis and structural data have indicated that, similar to AT and other serpins, ZPI has a typical RCL that is recognized by the catalytic pocket of target proteases (fXa and fXIa) and that the mechanism of the ZPI protease reaction is similar to that of other serpins (*17*−*20*). However, a unique feature that sets ZPI apart from AT and other related serpins is

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*To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1100 S. Grand Blvd., St. Louis, MO 63104. Telephone: (314) 977-9240. Fax: (314) 977-9205. E-mail: rezaiear@slu.edu.

[1]Abbreviations: ZPI, protein Z-dependent protease inhibitor; PZ, protein Z; RCL, reactive center loop; AT, antithrombin; AT-R393Y, AT mutant in which the P1 Arg-393 in the nomenclature of Schechter and Berger (*1*) has been replaced with a Tyr; fXa, activated factor X; fXa-D189S and thrombin-D189S, fXa and thrombin mutants, respectively, in which residue Asp-189 in the chymotrypsin numbering system (*2*) has been substituted with Ser; fIXa, activated factor IX; fXIa, activated factor XI; PEG, polyethylene glycol; BSA, bovine serum albumin.

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that ZPI contains a Tyr, instead of an Arg, at the P1 position of the RCL [nomenclature of Schechter and Berger (*1*)]. P1 Arg in AT and most other serpins, which are specific for coagulation proteases, binds to the primary S1 specificity pocket [Asp-189, chymotrypsin numbering (*2*)] through a salt bridge reminiscent of the interaction of the true substrates with coagulation proteases (*2*, *4*). In this study, we hypothesized that the P1 Tyr of ZPI may primarily be responsible for the narrower protease specificity of the serpin, thus enabling it to interact with the S1 specificity pocket of fXa and fXIa, but not other coagulation proteases. To test this hypothesis, we prepared an AT mutant in which the P1 Arg of the RCL was replaced with the P1 Tyr of ZPI. Moreover, we prepared an fXa mutant in which Asp-189 was replaced with Ser-189 of chymotrypsin. The characterization of the AT mutant in appropriate kinetic assays revealed that P1 Tyr in the context of the ZPI RCL, but not in the context of the AT RCL, determines the target protease specificity of the serpin. We provide further support for this hypothesis by demonstrating that AT with a P1 Tyr rapidly inhibits chymotrypsin, but this residue in the context of the ZPI RCL inhibits fXa but is essentially unreactive with chymotrypsin. These results suggest that in addition to P1 Tyr, other structural features within and/or outside of the RCL are involved in restricting the protease specificity of ZPI.

MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins. Human wild-type AT was expressed in HEK-293 cells using the RSV-PL4 expression/purification vector system as described previously (*21*). The RCL mutant of AT with the P1 Tyr of ZPI substituting the native Arg-393 (AT-R393Y) was constructed by standard PCR mutagenesis methods and expressed using the same vector system. Both wild-type and mutant AT were purified from cell culture supernatants by immunoaffinity chromatography using the HPC4 monoclonal antibody linked to Affi-gel 10 (Bio-Rad) followed by HiTrap-Heparin (Amersham Pharmacia Biotech, Piscataway, NJ) chromatography as described previously (*21*). Concentrations of the AT derivatives were determined from the absorbance at 280 nm using a molar absorption coefficient of 37700 M^{-1} cm^{-1} and by stoichiometric titration of the serpins with calibrated concentrations of either fXa (kinetic method) or heparin (direct binding method) as described previously (*22*). An fX mutant in which Asp-189 was replaced with a Ser (fX-D189S) was expressed in HEK-293 cells, purified to homogeneity, and activated by the fX activating enzyme from Russell's viper venom (RVV-X) as described previously (*23*). The Asp-189 to Ser substitution mutant of thrombin (thrombin-D189S) was prepared as described previously (*24*). The expression and purification of wild-type ZPI and protein Z (PZ) in HEK-293 cells have been described (*18*, *25*). The homogeneity of all recombinant proteins was verified by SDS−PAGE.

The plasma proteins, factors IXa (fIXa), Xa (fXa), and XIa (fXIa), and RVV-X were purchased from Haematologic Technologies (Essex Junction, VT). The therapeutic pentasaccharide fondaparinux sodium (molecular mass of 1.728 kDa) and unfractionated heparin (average molecular mass of ~15 kDa) were purchased from Quintiles Clinical Supplies (Mt. Laurel, NJ). The concentrations of heparins were based on their 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 previously (*22*). Bovine α-chymotrypsin was purchased from Sigma (St. Louis, MO). The chromogenic substrates, S2765, S2238, and S2366, were purchased from Diapharma (West Chester, OH). Spectrozyme CTY (Sp-CTY) was from American Diagnostica (Greenwich, CT), and CBS 31.39 was purchased from Midwest Bio-Tech Inc. (Fishers, IN).

Fluorescence Measurements. The Aminco-Bowman series 2 spectrophotometer (Spectronic Unicam, Rochester, NY) was used for protein fluorescence measurements at 25 °C as described previously (*21*). 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 a high-concentration stock solution of unfractionated heparin or pentasaccharide to each AT sample (50 nM) in 0.1 M NaCl and 0.02 M Tris-HCl (pH 7.5) containing 0.1% polyethylene glycol (PEG) 8000 (TBS). Data from at least three 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 pentasaccharide was calculated by nonlinear least-squares computer fitting of the data by the quadratic binding equation as described previously (*22*).

Inhibition Assays. The rate of inactivation of proteases by AT derivatives in both the absence and presence of the cofactors was measured under pseudo-first-order conditions by a discontinuous assay method as described previously (*21*). Briefly, in the absence of a heparin cofactor, each protease at 1−5 nM was incubated with 50−2000 nM AT in TBS containing 1 mg/mL bovine serum albumin (BSA) and 5 mM $CaCl_2$ (TBS/Ca^{2+}). All reactions were conducted 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 the chromogenic substrate specific for each protease (S2765 for fXa, S2238 for thrombin, CBS 31.39 for fIXa, S2366 for fXIa, and Sp-CTY for chymotrypsin) 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 pentasaccharide (1−2 μM) were the same except that concentrations of the AT derivatives ranged from 25 to 1000 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} versus the concentrations of AT as described previously (*21*).

For inhibition reactions in the presence of the unfractionated high-molecular mass heparin where the k_{obs} values exhibited a saturable dependence on the concentrations of the AT−heparin complex, data were analyzed according to the hyperbolic equation $k_{obs} = k[\text{AT−heparin}]/K_D + [\text{AT−heparin}]$, as described previously (*26*). In this equation, k represents the limiting rate constant for conversion of the intermediate heparin−AT−protease ternary encounter complex to a stable AT−protease complex and K_D is the dissociation constant for binding of the protease to the AT−heparin complex to form a ternary complex.

The inhibition of fXa by ZPI was measured both in the absence and in the presence of PZ on PC/PS vesicles under pseudo-first-order conditions as described previously (*18*). In the

absence of PZ, fXa derivatives (1 nM) were incubated with ZPI (100−250 nM) on PC/PS vesicles (25 μM) in TBS/Ca^{2+} for 30−120 min. In the presence of PZ, the reaction conditions were the same except that fXa (0.5 nM) was incubated with ZPI (10−20 nM) in complex with a saturating concentration of PZ (50 nM) for 15−30 s on PC/PS vesicles (25 μM) in the same TBS buffer. The inactivation reactions were stopped by addition of 50 μL of S2765 (final concentration of 0.25 mM) in TBS containing 50 mM EDTA, and the k_2 value was measured from the remaining enzyme activity as described above.

RESULTS AND DISCUSSION

Unlike AT, which regulates the proteolytic activity of all coagulation proteases, the specificity of ZPI is restricted to reactions with only fXa and fXIa (*8*, *12*). All coagulation proteases have an Asp (residue 189) at their primary specificity pocket, and a salt bridge interaction between this residue and P1 Arg of substrates and inhibitors plays a decisive role in the catalytic specificity of most coagulation reactions (*2*, *4*). Noting that ZPI has a unique residue (Tyr) at its P1 position that is not conserved in AT and other serpins that are specific for coagulation proteases, we hypothesized that among the RCL residues of ZPI, P1 Tyr may primarily be responsible for the narrower target specificity of ZPI. To test this hypothesis, we initially substituted the P1 Tyr of ZPI with an Arg and transfected the mutant construct into mammalian cells. Unfortunately, our repeated attempts to express this mutant failed to yield sufficient quantities of properly folded protein for characterization. Our analysis of the expressed protein via SDS−PAGE revealed that a ZPI mutant containing an Arg at the P1 position cannot be expressed as a full-length active molecule in our mammalian ZPI expression system (*18*). It appears that a ZPI mutant with a P1 Arg leads to the proteolytic cleavage of the serpin by mammalian cells, which has been reported by others (*17*). Noting the importance of ZPI in the regulation of the clotting cascade and the fact that there are very few data about the mechanism of the specificity of this serpin, we took the alternative approach of transferring the P1 specificity of ZPI to that of AT for which we have a reliable expression system (*21*). Thus, we prepared the AT-R393Y mutant, and as described below, by characterizing the inhibitory properties of this mutant with different coagulation proteases and chymotrypsin, we have provided new insight into the role of P1 Tyr in determining the specificity of ZPI.

Expression and Characterization of Recombinant Serpins. Both wild-type and AT-R393Y serpins were expressed in HEK-293 cells and purified to homogeneity by a combination of HPC4 immunoaffinity and HiTrap-Heparin column chromatography as described previously (*21*). SDS−PAGE analysis under nonreducing conditions suggested that the mutant serpin has been purified to homogeneity and that it migrates with a relative molecular mass identical to that of wild-type serpin (Figure 1). Similar to wild-type, AT-R393Y formed stable complexes with both fXa and thrombin, though the extent of complex formation was not efficient in the absence heparin because of the poor reactivity of the mutant with both proteases (see below) in the absence of the cofactor (Figure 1). In contrast to fXa (Figure 1A), no detectable amount of complex was observed for the mutant serpin with thrombin in the absence of heparin (Figure 1B), confirming the kinetic data which showed that the reactivity of AT-R393Y with thrombin has been dramatically impaired. In agreement with the literature, both wild-type and mutant AT

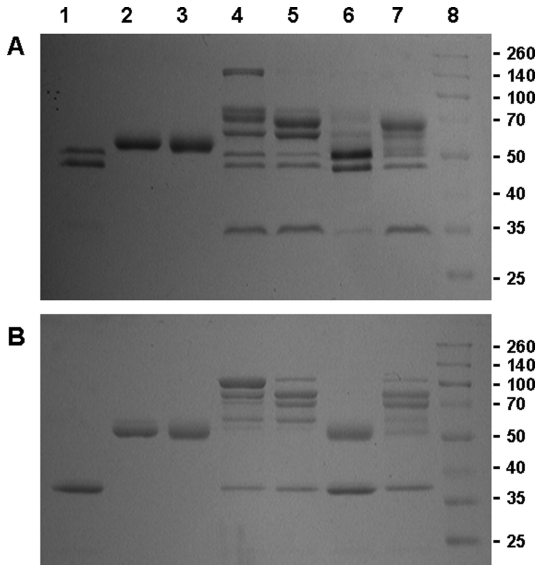

FIGURE 1: SDS−PAGE analysis of the stable protease−AT complexes. AT derivatives (2.5 μM each) were incubated with equimolar concentrations of either fXa (A) or thrombin (B) for 30 min in the absence and presence of a 2-fold molar excess of heparin in 20 μL reaction mixtures at 37 °C. Five microliters of nonreducing sample buffer was added to each sample, boiled for 5 min, and loaded on a 10% SDS gel: (A) lane 1, fXa; lane 2, wild-type AT (AT-WT); lane 3, AT-R393Y; lane 4, AT-WT and fXa; lane 5, AT-WT, fXa, and heparin; lane 6, AT-R393Y and fXa; lane 7, AT-R393Y, fXa, and heparin; lane 8, molecular mass standards (kilodaltons); and (B) same as panel A except that thrombin replaced fXa in the reactions.

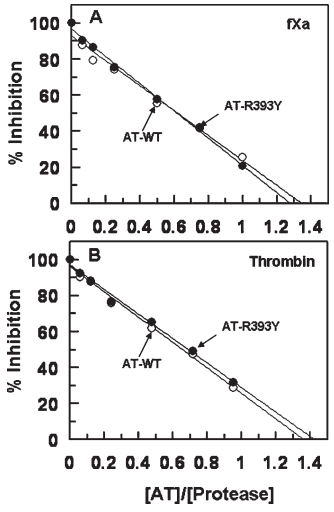

FIGURE 2: Determination of stoichiometries of inhibition of AT-WT and AT-R393Y with proteases in the presence of heparin. A fixed concentration of either fXa [25 nM (A)] or thrombin [25 nM (B)] was incubated with increasing concentrations of AT derivatives [(○) AT-WT and (●) AT-R393Y] in the presence of 0.5 μM heparin at room temperature for 1−8 h. After completion of the reactions, the residual amidolytic activities were measured and plotted vs the [AT]/[protease] ratios. The SI values were determined from the *x*-intercept of the linear regression fit of the kinetic data. SI values of 1.3 ± 0.16 and 1.3 ± 0.07 were obtained for the fXa reactions with AT-WT and AT-R393Y, respectively. The corresponding values for thrombin were 1.4 ± 0.08 for AT-WT and 1.4 ± 0.07 for AT-R393Y.

exhibited a similar stoichiometry of inhibition (SI ∼ 1.3−1.4) with both fXa and thrombin, suggesting that the extent of the reactivity of the proteases with the mutant serpin has not been elevated in the substrate pathway relative to that of wild-type serpin (Figure 2). These results are consistent with the SDS−PAGE

analysis of formation of the complex under nonreducing conditions in the presence of heparin where both fXa and thrombin formed stable complexes with the mutant similar to that observed for their reactions with the wild-type serpin (Figure 1). The binding of heparin and pentasaccharide to both AT and AT-R393Y resulted in similar enhancements of the intrinsic protein fluorescence, yielding dissociation constants (K_D) of 1.7 ± 1.4 nM (wild type) and 1.3 ± 2.3 nM (R399Y) for heparin and 11 ± 4 nM (wild type) and 9 ± 3 nM (R339Y) for pentasaccharide (Figure 3, shown for heparin only). The improvements in K_D values for the interaction of heparins with AT-R399Y are consistent with results of another study which observed ~2−3-fold improvement in the affinity of heparin interaction with the P1 Trp mutant of AT as monitored at an ionic strength of 0.3 or 0.45 (*27*). However, similar studies at higher ionic strengths were not conducted to precisely determine the differences in K_D values for heparin between wild-type and mutant serpins. The basis for the P1 mutations exhibiting enhanced affinity for heparin appears to be due to the mutagenesis-related disruption of a salt bridge that exists between P1 Arg of wild-type AT and the body of the serpin in the native conformation (*28*). Previous results have indicated that heparin activation of AT leads to the disruption of this ionic interaction, suggesting that the conformation of the RCL is linked to the heparin-binding D helix of AT (*29*−*31*).

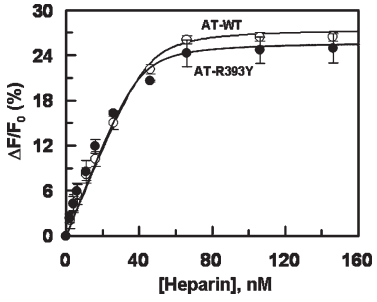

FIGURE 3: Binding of heparin to recombinant AT derivatives. The spectral changes were monitored by addition of 1−2 μL of a concentrated stock solution of unfractionated heparin to 50 nM AT in TBS (pH 7.5) containing 0.1% PEG 8000, and dissociation constants were calculated from the changes in the intrinsic protein fluorescence as described in Materials and Methods: (○) AT-WT and (●) AT-R393Y.

Protease Inhibition. AT-R393Y inhibited fXa and thrombin with ~155- and ~970-fold slower second-order rate constants (k_2), respectively, suggesting that the P1−S1 interaction in both proteases makes a significant contribution to the recognition mechanism of the serpin (Table 1). The results further suggest that the tolerance of the S1 pocket of fXa for a Tyr at the P1 position is much better than that of thrombin, possibly explaining in part the ability of fXa, but not thrombin, to react with ZPI in the absence of a cofactor. As expected, on the basis of its normal affinity for heparin, the fold accelerating effect of heparin cofactors in catalyzing the mutant AT inhibition of coagulation proteases was not changed (Table 1). Similar results have been observed with the heparin-mediated reactions of AT-Trp with both coagulation proteases (*27*). Noting that the P1 Arg of substrates and inhibitors determines the specificity of the S1 pocket (Asp-189) in trypsin-like coagulation proteases, we characterized the properties of AT-R393Y with an fXa mutant in which Asp-189 was replaced with a Ser, a residue found in the S1 pocket of chymotrypsin. Chymotrypsin by virtue of having a Ser at its primary specificity pocket prefers substrates and inhibitors possessing a bulkier hydrophobic residue at the P1 position (i.e., Trp and Tyr). Surprisingly, relative to wild-type AT, the reactivity of the D189S mutant of fXa with AT-R393Y was not changed as the protease mutant reacted with a similar k_2 of 0.3×10^2 M^{-1} s^{-1} with both wild-type and mutant serpins (Table 1). This was in sharp contrast to the reaction of chymotrypsin with AT-R393Y which was improved more than 4 orders of magnitude relative to the reaction of chymotrypsin with wild-type AT (Table 1). The substitution of the P1 Arg of AT with a Trp also converted the mutant serpin to a rapid inhibitor of chymotrypsin (*27*), suggesting that the P1 residue plays a critical

Table 1: Second-Order Rate Constants (k_2) for the Inhibition of Chymotrypsin and Coagulation Proteases by Wild-Type AT and AT-R393Y in the Absence and Presence of Heparin Cofactors[a]

	k_2(−Hep) (M^{-1} s^{-1})	k_2(+H5) (M^{-1} s^{-1})	k_2(+Hep) (M^{-1} s^{-1})
	AT-WT		
fXa	$(30.9 \pm 2.7) \times 10^2$	$(4.6 \pm 0.3) \times 10^5$	$(435 \pm 12) \times 10^5$
fXa-D189S	$(0.3 \pm 0.07) \times 10^2$	$(0.07 \pm 0.003) \times 10^5$	$(10.2 \pm 1.1) \times 10^5$
thrombin	$(87.4 \pm 6.2) \times 10^2$	$(0.11 \pm 0.02) \times 10^5$	$(860 \pm 32) \times 10^5$
thrombin-D189S	ND[b]	ND[b]	$(0.10 \pm 0.02) \times 10^5$
fIXa	$(0.72 \pm 0.03) \times 10^2$	$(0.4 \pm 0.02) \times 10^5$	$(100 \pm 7) \times 10^5$
fXIa	$(2.9 \pm 0.2) \times 10^2$	$(0.5 \pm 0.01) \times 10^3$	$(3.2 \pm 0.3) \times 10^5$
chymotrypsin	$(0.83 \pm 0.07) \times 10^2$	−	−
	AT-R393Y		
fXa	$(0.2 \pm 0.03) \times 10^2$	$(2.8 \pm 0.4) \times 10^3$	$(4.2 \pm 0.2) \times 10^5$
fXa-D189S	$(0.3 \pm 0.04) \times 10^2$	$(7.1 \pm 0.5) \times 10^3$	$(8.1 \pm 0.6) \times 10^5$
thrombin	$(0.09 \pm 0.008) \times 10^2$	$(0.01 \pm 0.001) \times 10^3$	$(0.12 \pm 0.03) \times 10^5$
thrombin-D189S	$(0.1 \pm 0.02) \times 10^2$	−	$(0.46 \pm 0.05) \times 10^5$
fIXa	ND[b]	$(0.04 \pm 0.003) \times 10^3$	$(0.03 \pm 0.002) \times 10^5$
fXIa	ND[b]	ND[b]	$(1.7 \pm 0.3) \times 10^3$
chymotrypsin	$(1.1 \pm 0.4) \times 10^6$	−	−

[a]The second-order rate constants (k_2) in the absence and presence of heparin cofactors were determined from the remaining activities of proteases after incubation with serpins in TBS/Ca^{2+} at room temperature by an amidolytic activity assay described in Materials and Methods. All values are averages of at least three independent measurements ± the standard deviation. H5, pentasaccharide; Hep, unfractionated high-molecular mass heparin. [b]No reactivity was detected after incubation of these proteases with 500 nM serpin for 6 h.

role in selecting the target protease specificity of the serpin. The pentasaccharide fragment of heparin accelerated the reactivity of both wild-type and mutant fXa with both wild-type and mutant AT ~150−250-fold (Table 1), confirming the previous results which showed that the P1 residue of AT contributes minimally to the activation mechanism of the serpin by heparin (*27*). In the presence of heparin, the reactivity of fXa with AT-R393Y or fXa-D189S with either wild-type or mutant AT approached the rate observed for the chymotrypsin reaction with AT-R393Y (Table 1). These results suggest that in contrast to a dominant role for the P1−S1 recognition in determining the specificity of chymotrypsin with AT, the specificity of the interaction of fXa with the serpin is influenced by other residues within or in the vicinity of the active-site pocket and that such interactions are in general nonproductive, explaining the requirement for heparin as a cofactor to overcome the inhibitory interactions of fXa with AT (*32*). Noting that AT-R393Y was converted to a rapid inhibitor of chymotrypsin, we evaluated the reactivity of chymotrypsin with ZPI in which the P1 residue is also a Tyr. Interestingly, we found that chymotrypsin did not exhibit any reactivity with ZPI, and thus, no k_2 value could be estimated for the reaction. By contrast, the D189S mutant of fXa exhibited essentially identical reactivity with ZPI in the absence [$(1.9 \pm 0.2) \times 10^3$ for wild-type fXa and $(2.3 \pm 0.3) \times 10^3$ for fXa-D189S] and presence of PZ [$(7.8 \pm 0.4) \times 10^5$ for wild-type fXa and $(8.3 \pm 0.6) \times 10^5$ for fXa-D189S]. These results clearly suggest that the preference for a Tyr at the P1 position is RCL context-dependent and that other structural features within the ZPI RCL impede the fitting of the serpin into the active-site pocket of chymotrypsin and possibly other nontarget proteases. In support of this hypothesis, thrombin could also react with AT-R393Y (Table 1), albeit with a much slower rate, but the protease was essentially unreactive with ZPI.

A structural feature that may be critical for dictating the serpin specificity of thrombin and other serine proteases is the length of the RCL. Relative to ZPI and most other serpins, the RCL of AT has three insertion residues, Arg-399, Val-400, and Thr-401, at the most C-terminal end of the loop between the P5′ site and s1C sheet (*33*). We previously demonstrated that a longer RCL in AT may contribute to its partially inserted conformation (*16*), which renders it inactive toward fXa in the absence of heparin cofactors (*30*). However, our mutagenesis data also revealed that AT requires a longer RCL to regulate the activity of thrombin. This was evidenced by the observation that deleting the insertion residues trapped the AT in the activated conformation and thus improved the reactivity of the mutant serpin with fXa in the expense of markedly impairing the reactivity of the mutant serpin with thrombin (*16*). Thus, we believe that the basis for the inability of the ZPI RCL not to fit into the active-site pocket of thrombin is not solely due to the P1 Tyr, but rather it is also due to the RCL of ZPI not having an optimal length and mobility to fit into the deep canyonlike active-site pocket of thrombin (*2*).

The reactivity of the D189S mutant of thrombin with wild-type AT was dramatically impaired to the extent that no rate constant for the reaction could be calculated. However, the mutant thrombin exhibited a k_2 of 1×10^4 M^{-1} s^{-1} with AT in the presence of heparin (Table 1). The thrombin mutant was slowly inhibited by AT-R393Y, yielding a k_2 value of 1.1×10 M^{-1} s^{-1} in the absence and a k_2 of 4.6×10^4 M^{-1} s^{-1} in the presence of heparin (Table 1). Thus, converting the S1 primary specificity of thrombin to that of chymotrypsin modestly improved its reactivity with the inhibitor containing a P1 Tyr; however, in agreement with previous data in the literature, the exchange of the S1 specificity residue by itself is not sufficient to convert the specificity of serine proteases to that of the donor protease (*34*, *35*). Nonetheless, the ability of fXa-D189S, but not thrombin-D189S, to react with AT in the absence of heparin is indicative of a higher degree of plasticity for the active-site pocket of fXa, partially explaining the ability of the protease to accommodate the P1 Tyr of ZPI in its S1 pocket. This is supported by the observation that the tick anticoagulant peptide, TAP, a highly specific inhibitor of fXa, also has a Tyr at the P1 position (*36*).

The reactivity of both fIXa and XIa with AT-R393Y was also dramatically impaired so that no k_2 value could be determined for either protease in the absence of a cofactor (Table 1). However, noting that fIXa has an exosite for interaction with the activated conformation of AT (*37*), we found the protease slowly reacted with the mutant AT in the presence of pentasaccharide (Table 1). This was not the case for fXIa since no similar exosite has been identified on fXIa for interaction with the activated conformation of AT. Only in the presence of high-molecular mass heparin could a k_2 for the reaction of fXIa with AT-R393Y be determined which was impaired ~2 orders of magnitude relative to its reaction with wild-type serpin (Table 1). The lack of reactivity of fXIa with AT-R393Y in the absence of a cofactor suggests that P1 Tyr has no role in determining the specificity of the interaction of fXIa with ZPI. Thus, the interaction of other residues within and/or outside of the ZPI RCL with an uncharacterized exosite on fXIa is primarily responsible for the PZ-independent high reactivity of the protease with ZPI. It is worth noting that this exosite must be located on the catalytic light chain of fXIa since we previously demonstrated that both wild-type fXIa and the isolated catalytic domain of the protease react with ZPI with a similar high reactivity in a manner independent of PZ (*12*). Further studies will be required to identify the interactive sites on ZPI and fXIa that facilitate the binary interaction.

Analysis of the Two-Step Reaction of fXa and Thrombin with AT-R393Y. AT inhibits its target proteases, including fXa and thrombin, by a two-step reaction mechanism in which a Michaelis-type encounter complex formed in the initial reaction step is converted to a stable, covalent complex in the second step of the reaction (*26*, *38*). It has been established that high-molecular mass heparins lower the dissociation constant (K_D) for the formation of the initial protease−serpin encounter complex with essentially no effect on the rate constant (k) for formation of the covalent complex in the second step of the reaction (*38*). Noting that the rate constant for the second step of the AT reaction with both fXa and thrombin is relatively high, we found that only by rapid kinetic methods has it been possible to resolve the two-step reaction of AT with either fXa or thrombin (*26*, *38*). To determine the reaction step that has been affected by the substitution of P1 Arg with a Tyr, the heparin-catalyzed reaction of AT-R393Y with both fXa and thrombin was monitored as a function of increasing concentrations of the mutant serpin. Interestingly, because the k values were dramatically impaired, it was possible to resolve the two-step reaction of the mutant serpin with both wild-type and D189S mutant proteases by employing a discontinuous assay method. As shown in Figure 4A, in the presence of a high-molecular mass heparin, the k_{obs} values for the reaction of AT-R393Y with wild-type fXa and the reaction of fXa-D189S with wild-type AT showed a saturable dependence on the concentrations of the heparin−serpin complex, indicating the saturation of an intermediate encounter complex prior to formation of a stable, covalent

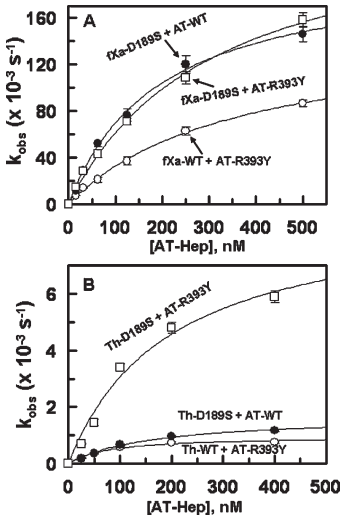

FIGURE 4: Dependence of k_{obs} values for inactivation of coagulation protease derivatives by AT derivatives on the heparin−serpin complex concentration. (A) The k_{obs} values for the heparin-catalyzed inactivation of wild-type fXa by AT-R393Y (○), the heparin-catalyzed inactivation of fXa-D189S by wild-type AT (●), and the heparin-catalyzed inactivation of fXa-D189S by AT-R393Y (□) were determined at increasing concentrations of the heparin−serpin complex (shown on the *x*-axis) in TBS/Ca^{2+} at room temperature by an amidolytic activity assay. (B) Same as panel A except that the inactivation of thrombin and thrombin-D189S was monitored by the same serpins. The solid lines in both panels are least-squares computer fits of data by a hyperbolic equation as described in Materials and Methods. The kinetic parameters are listed in Table 2.

Table 2: Kinetic Constants for the High-Molecular Mass Heparin-Catalyzed Reactions of Wild-Type and Mutant Proteases with AT Derivatives[a]

	K_D ($\times 10^{-9}$ M)	k (s^{-1})	k_2 (M^{-1} s^{-1})
	AT-WT		
fXa-D189S	194 ± 18	0.20 ± 0.01	(1.0 ± 0.1) × 10^6
thrombin-D189S	164 ± 25	0.0017 ± 0.0001	(1.0 ± 0.2) × 10^4
	AT-R393Y		
fXa	355 ± 34	0.15 ± 0.007	(4.2 ± 0.6) × 10^5
fXa-D189S	312 ± 36	0.25 ± 0.01	(8.0 ± 1.2) × 10^5
thrombin	78 ± 16	0.001 ± 0.0001	(1.3 ± 0.4) × 10^4
thrombin-D189S	195 ± 60	0.009 ± 0.001	(4.6 ± 1.9) × 10^4

[a]The kinetic constants were determined from computer fits of the saturable dependence of the observed pseudo-first-order rate constant (k_{obs}) on the AT−heparin complex concentrations (3.5−500 nM) according to a hyperbolic equation described in Materials and Methods. All reactions were conducted in TBS buffer containing 1 mg/mL BSA, 0.1% PEG 8000, and 5 mM Ca^{2+}. In all cases, a molar excess of heparin (1 μM) was used to saturate AT. The second-order rate constants (k_2) were calculated from the k/K_D ratio. Data are derived from Figure 4.

complex. Nonlinear regression analysis of data by the hyperbolic equation given in Materials and Methods yielded K_D values for the ternary complex dissociation constants, and k values for the rate constants for formation of the stable complex (Table 2). The second-order rate constants (k_2), calculated from the ratio of the rate constants, k, to K_D values in all cases (Table 2), are in agreement with values listed in Table 1, determined at subsaturating concentrations of the serpin derivatives. Comparisons of these values with the corresponding values for the ternary wild-type fXa−AT−heparin complex [K_D = 90 nM, and k = 18 s^{-1} (*26*)], as determined previously by rapid kinetic methods, suggest that the defect of mutation in all three reactions is primarily caused by a dramatic impairment in the rate constant in the second step of the reaction (Table 2). These results suggest that the P1−S1 interaction contributes ~100-fold to the energy of the transition-state stabilization in the fXa−AT reaction.

A similar approach was used to analyze the reaction of thrombin and the D189S mutant of thrombin with AT-WT and AT-R393Y. Similar to reactions with fXa, k_{obs} values with both wild-type and mutant thrombin displayed a saturable dependence on the concentrations of the heparin−AT-R393Y complex, (Figure 4B). The nonlinear regression analysis of the kinetic data yielded K_D and k values. The second-order rate constants, calculated from the ratio of the rate constants, k, to K_D values, are listed in Table 2. Similar to reactions with fXa, comparisons of these values with the corresponding values for the ternary wild-type thrombin−AT−heparin complex (K_D = 140 nM, and k = 3.2 s^{-1}), as determined previously by rapid kinetic methods (*39*), suggest that the defect caused by the mutation in all three reactions is also primarily due to a dramatic impairment of the rate constant of the second step reaction; in this case, however, the P1−S1 interaction contributes ~2000-fold to the energy of the transition-state stabilization of the thrombin−AT complex. Thus, the tolerance of thrombin for a Tyr at the P1 position of the serpin is significantly lower than that of fXa, possibly explaining, at least in part, the basis for the inability of ZPI to fit into the catalytic pocket of thrombin. This hypothesis is consistent with the observation in Figure 4, where the reactivities of wild-type fXa and fXa-D189S with mutant and wild-type AT derivatives are similar or nearly identical; however, thrombin reacts with AT-R393Y with a markedly lower rate constant. The substitution of Asp-189 with a Ser in thrombin partially restores the catalytic defect, clearly suggesting that in the case of thrombin the S1−P1 interaction makes a greater contribution to the stabilization of the transition state of the protease−serpin complex. We previously demonstrated that Arg-143 of the autolysis loop of fXa also acts as a secondary binding site for ZPI (*18*). This exosite has been conserved in fXIa, but not in thrombin (*12*). Thus, if the Gla domain-dependent cofactor function of PZ is omitted, the underlying basis for the differential reactivity of fXa and thrombin with ZPI includes (i) differences in the S1−P1 specificity for reaction with a Tyr at the P1 position, (ii) differences in the sensitivity of the two proteases for the length of the RCL, and (iii) the lack of an exosite on thrombin for interaction with a similar complementary site on ZPI.

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