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Role of the Residues of the 39-Loop in Determining the Substrate and Inhibitor Specificity of Factor IXa*

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The activation of antithrombin (AT) by heparin facilitates the exosite-dependent interaction of the serpin with factors IXa (FIXa) and Xa (FXa), thereby improving the rate of reactions by 300- to 500-fold. Relative to FXa, AT inhibits FIXa with ~40-fold slower rate constant. Structural data suggest that differences in the residues of the 39-loop (residues 31–41) may partly be responsible for the differential reactivity of the two proteases with AT. This loop is highly acidic in FXa, containing three Glu residues at positions 36, 37, and 39. By contrast, the loop is shorter by one residue in FIXa (residue 37 is missing), and it contains a Lys and an Asp at positions 36 and 39, respectively. To determine whether differences in the residues of this loop contribute to the slower reactivity of FIXa with AT, we prepared an FIXa/FXa chimera in which the 39-loop of the protease was replaced with the corresponding loop of FXa. The chimeric mutant cleaved a FIXa-specific chromogenic substrate with normal catalytic efficiency, however, the mutant exhibited ~5-fold enhanced reactivity with AT specifically in the absence of the cofactor, heparin. Further studies revealed that the FIXa mutant activates factor X with ~4-fold decreased k_{cat} and ~2-fold decreased K_m, although the mutant interacted normally with factor VIIIa. Based on these results we conclude that residues of the 39-loop regulate the cofactor-independent interaction of FIXa with its physiological inhibitor AT and substrate factor X.

Factor IXa (FIXa)[2] is a vitamin K-dependent plasma serine protease that, upon complex formation with factor VIIIa (FVIIIa), on negatively charged membrane surfaces in the presence of Ca^{2+} (intrinsic Tenase) activates factor X (FX) to factor Xa (FXa) during the blood coagulation process (1–5). FIXa plays an important role in the clotting cascade, because its deficiency is associated with the life-threatening disease, hemophilia B (6). The activity of FIXa toward its physiological substrate FX is very poor in the absence of FVIIIa, however, complex formation with the cofactor improves the catalytic efficiency of FIXa by 4–5 orders of magnitude in the intrinsic Tenase complex (1–5). The proteolytic activity of FIXa in plasma is regulated by the serpin inhibitor, antithrombin (AT) (7–11). Surprisingly, in contrast to a dramatic cofactor-mediated improvement in the activity of FIXa toward FX, FVIIIa has a minimal cofactor effect on the reactivity of the protease with AT or its activity toward small synthetic substrates (12, 13), suggesting that FVIIIa-mediated exosite interactions with FX, involving sites remote from the active-site pocket, play dominant roles in the catalytic reaction (14, 15). In the case of the FIXa reaction with AT, the cofactor function of heparin-like glycosaminoglycans, similar to those lining the surface of the endothelium (16), is required to facilitate the protease recognition of the serpin (17–19).

Heparin accelerates the reactivity of AT with FIXa by two distinct mechanisms: a conformational activation of the serpin and a template mechanism in the presence of physiological levels of Ca^{2+} (17–19). In the first mechanism, the binding of a unique pentasaccharide fragment of heparin to the basic D-helix of AT induces structural changes in the reactive center loop (RCL) and sites remote from the RCL (outside of P6–P3′ sites), thereby facilitating the exosite-dependent interaction of the serpin with the protease (20). The new sites on AT, which are made available by heparin on the serpin (20), interact with two exposed surface loops in the immediate vicinity of the active-site pocket of FIXa. The first surface loop that specifically recognizes the heparin-activated conformation of AT is the autolysis loop (148-loop), with the basic residue Arg[150] of this loop playing a dominant role in the interaction (21, 22). The other loop, which is known to contribute to the recognition of the heparin-activated serpin, is the 39-loop (22). Structural and mutagenesis data have indicated that selected residues of this loop in FIXa and other coagulation proteases contact the P′ side of the RCL in AT and other heparin-binding serpins (22–25). The second mechanism by which heparin accelerates the reactivity of AT with FIXa is through a bridging mechanism where heparins of sufficient chain lengths act as templates on which both the protease and serpin bind in the presence of Ca^{2+}, thereby decreasing the dissociation constant for the formation of a non-covalent Michaelis-type complex (17, 19). Previous results have indicated that heparin accelerates the AT inhibition of FXa by essentially the same two mechanisms (26). Thus, by using a fractionated high molecular weight high affinity hep-

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[2] The abbreviations used are: FIXa, activated factor IX; FIXa-fX[39-loop], factor IXa mutant in which the residues of the 39-loop (residues 31–41) in the chymotrypsin numbering system (29) have been replaced with corresponding residues of FXa; FVIIIa, thrombin-activated factor VIII; FXa, activated factor X; FIXa-S195A and FXa-S195A, factors IXa and Xa in which the catalytic residue, Ser[195], has been replaced with an Ala; AT, antithrombin; RCL, reactive center loop; AT-R399E, AT mutant in which the P6′ residue, Arg[399], in the nomenclature of Schechter and Berger (39) has been replaced with a Glu; H5, pentasaccharide; RVV-X, FX-activating enzyme from Russell's viper venom; PC/PS, phosphatidylcholine and phosphatidylserine; SpFXa, Spectrozyme FXa.

arin (~70 saccharides), we have demonstrated that the conformational activation of AT contributes ~300- to 500-fold and the template mechanism contributes ~100- to 300-fold to acceleration of the AT inhibition of FXa in the presence of physiological levels of Ca^{2+} (27).

Relative to FXa, FIXa reacts with AT with ~40-fold slower rate constant. Structural data suggests that differences in the residues of the 39-loop, a variable loop in the vicinity of the active-site cleft of coagulation proteases (28), may be responsible, at least partly, for the slower reactivity of FIXa with AT (22, 25). To test this hypothesis, in this study, we substituted the residues of the 39-loop of FIXa with the corresponding residues of FXa (residues 31–41 in the chymotrypsin numbering system (29)) and expressed the mutant in mammalian cells. The characterization of the catalytic properties of this mutant revealed that residues of the 39-loop impede the heparin cofactor-independent interaction of FIXa with AT, although the same residues contribute to the effective protease activation of the physiological substrate, FX.

EXPERIMENTAL PROCEDURES

Construction, Mutagenesis, and Expression of Recombinant Proteins—The construction, expression, and purification of wild-type FIX in a novel expression/purification vector system in which the P1′–P3′ residues of the first factor XIa cleavage site on the activation peptide of FIX (Ala^{146}-Glu-Ala) have been replaced with the corresponding basic residues of FX (Arg-Lys-Arg) has been described previously (30). This strategy introduces a post-translational processing site for the furin-like enzymes on the activation peptide of FIX, thus the mutant is synthesized as a two-chain zymogen held together by a disulfide bond (30). In this construct, the first 12 residues of the activation peptide of FIX, immediately following the engineered P3′ Arg, have been replaced with the 12-residue epitope for the Ca^{2+}-dependent monoclonal antibody, HPC4, in order to facilitate the purification of recombinant FIX by this antibody as described before (30). An FIX mutant in which the residues of the 39-loop from residues Val^{31} to Phe^{41} (Val^{31}-Val-Leu-Asn-Gly-Lys-Val-Asp-Ala-Phe^{41}) in the chymotrypsin numbering (29) were replaced with the same 39-loop residues of FXa (Ala^{31}-Leu-Leu-Ilu-Asn-Glu-Glu-Asn-Glu-Gly-Phe^{41}) (FIX-$fX^{39\text{-loop}}$) was prepared and expressed in HEK-293 cells using the same expression/purification vector system. The FIX derivatives were activated by the FX-activating enzyme from Russell's viper venom (RVV-X), and the active enzymes were purified to homogeneity and active-site titrated using a calibrated concentration of AT as described before (30). The Ser^{195} to Ala substitution mutant of FIX was constructed using the same vector, activated by RVV-X and purified on SBTI-Sepharose column as described (31). The construction, expression, and purification of the S195A mutant of FXa in HEK-293 cells have been described (32).

Recombinant human AT was expressed in HEK-293 cells using the RSV-PL4 expression/purification vector system as described (33). The RCL mutant of AT, with a Glu substituting the native Arg^{399} at the P6′ position (AT-R399E), was constructed 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 Heparin-Sepharose chromatography as described (33). Concentrations of both wild-type and mutant AT 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 heparin as described (34).

Human plasma-derived proteins FIXa, FXa, FX, and RVV-X were purchased from (Hematologic Technologies, Essex Junction, VT). The therapeutic unfractionated heparin (average molecular mass of ~15 kDa) and the AT-binding pentasaccharide fondaparinux sodium (molecular mass = 1.728 kDa, 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 the protein fluorescence as described (34). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (35). Human recombinant FVIIIa was a generous gift from Dr. Philip Fay (University of Rochester, Rochester, NY). Heparin-Sepharose was purchased from Amersham Biosciences. The chromogenic substrates, Spectrozyme FXa (SpFXa) was purchased from American Diagnostica (Greenwich, CT), and CBS 31.39 (LGR-pNA) was purchased from Midwest Bio-Tech. Inc. (Fishers, IN). The APTT (activated partial thromboplastin time) reagent (Alexin) was purchased from Sigma, and FIX-deficient plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS).

Cleavage of the Chromogenic Substrate—The steady-state kinetics of hydrolysis of CBS 31.39 (0.1–10 mM) by FIXa derivatives (10 nM) was monitored in 0.02 M Tris-HCl (pH 7.5) buffer containing 0.1 M NaCl, 0.1 mg/ml bovine serum albumin (BSA), 33% ethylene glycol, and 5 mM Ca^{2+} as described (30). The rate of hydrolysis was measured at 405 nm at room temperature in 96-well plates by a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) as described (30). The K_m and k_{cat} values for the substrate hydrolysis were obtained by nonlinear regression fits of data to the Michaelis-Menten equation, and the specificity constant was expressed as the ratio of k_{cat}/K_m.

Factor X Activation by FIXa Derivatives—The initial rate of FX activation by the wild-type and mutant FIXa was measured on PC/PS vesicles in the presence of FVIIIa in TBS containing 0.1 mg/ml BSA, 0.1% polyethylene glycol (PEG) 8000, and 5 mM Ca^{2+} (TBS/Ca^{2+}) at room temperature as described (30). First, the ability of the FIXa mutant to interact with FVIIIa was evaluated by incubating FIXa (0.1 nM) with varying concentrations of FVIIIa (0–60 nM) on PC/PS vesicles (40 μM), and the reaction was initiated by addition of FX (300 nM) in TBS/Ca^{2+}. Following 2-min activation in 30-μl reaction volumes in a 96-well assay plate, the reactions were stopped by addition of EDTA to a final concentration of 20 mM, and the rate of FXa generation was measured by an amidolytic activity using SpFXa as described above. The concentration of FXa generated in the activation reactions was determined from a standard curve prepared from the cleavage rate of SpFXa by known concentra-

tions of plasma-derived FXa under exactly the same conditions. The apparent K_d for the interaction of FIXa with FVIIIa was determined by nonlinear regression fits of data to a hyperbolic binding equation as described (30). This assay was also used to analyze the concentration dependence of FX activation by the FIXa mutant. In this case, FIXa (0.1 nM) was incubated with a saturating concentration of FVIIIa (50 nM) on PC/PS vesicles (40 μM), and the reaction was initiated by addition of varying concentrations of FX (6–800 nM) in TBS/Ca^{2+}. Following 2-min activation, EDTA was added to a final concentration of 20 mM, and the rate of FXa generation was measured as described above. The apparent K_m and k_{cat} values were calculated by nonlinear regression fits of data to the Michaelis-Menten equation.

Measurement of the Clotting Activity—Clotting activities of wild-type FIX and its FX-39-loop swap mutant were assessed by an APTT assay using a STart 4 fibrinometer (Diagnostica/Stago, Asnieres, France) and FIX-deficient plasma as described (36). In this assay, 50 μl of APTT reagent was incubated with a mixture of 50 μl of FIX-deficient plasma and 50 μl of different concentrations of recombinant FIX derivatives for 5 min before the initiation of clotting by the addition of 50 μl of 35 mM $CaCl_2$ at 37 °C.

Fluorescence Measurements—Aminco-Bowman series 2 spectrophotometer (Spectronic Unicam, Rochester, NY) was used for the AT protein fluorescence measurements at 25 °C as described (33). 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 1–2 μl of a high concentration stock solution of fondaparinux (H5) into 50 nM of each AT sample in 0.1 M NaCl, 0.02 M Tris-HCl (pH 7.5) containing 0.1% PEG 8000 (TBS). Data from at least three experiments were analyzed as the ratio of change in the fluorescence intensity of the sample containing pentasaccharide to the initial intensity of the control protein lacking the heparin cofactor. The affinity of AT for H5 was calculated by nonlinear least-squares computer fitting of the data by the quadratic binding equation as described (34).

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 rate conditions by a discontinuous assay method as described (21, 33). Briefly, in the absence of H5, 0.5–10 nM of each protease was incubated with 50–2000 nM AT in TBS/Ca^{2+}. All reactions were carried out at room temperature in 50-ml 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 and CBS 31.39 for FIXa), in TBS was added to each well, and the remaining enzyme activities were measured by using a V_{max} Kinetics Microplate Reader. 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 200 nM, and the incubation time was reduced to 0.5–30 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 (21, 33).

Competitive Binding—The competitive effects of the catalytically inactive S195A mutants of FXa and FIXa on the inhibition of their wild-type counterparts by AT derivatives both in the absence and presence of H5 and unfractionated heparin were studied using the same discontinuous inhibition assay as described (37). In this case, the inhibition of FXa (0.5 nM) or FIXa (10 nM) by AT derivatives (5–50 nM) was monitored in the presence of increasing concentrations of the S195A mutants of proteases (0–5 μM) in the absence and presence of a fixed concentration of H5 (25 nM) or heparin (2.5 nM) in TBS/Ca^{2+}. Following 0.5–5 min incubation at room temperature, 50 ml of SpFXa or CBS 31.39 was added to each well, and the remaining activity of proteases was measured as described above. The experimental conditions were set such that ~90% of the enzyme activity in the absence of the competitor was inhibited. K_D for the non-covalent interaction of AT with both proteases was determined from the non-linear regression analysis of the saturable S195A concentration dependence for the recovery of the enzyme activities as monitored by the hydrolysis of chromogenic substrates at 405 nm as described (37).

RESULTS

Expression and Characterization of Recombinant FIXa Derivatives—The wild-type and mutant FIX zymogens were expressed in HEK-293 cells, purified to homogeneity, and activated by RVV-X to active enzymes as described under "Experimental Procedures" and also in Ref. 30. The concentrations of recombinant FIXa and its 39-loop swap mutant were determined by an amidolytic activity assay and active-site titrations using known concentrations of AT in the presence of heparin as described (21, 30). Analysis of the kinetic parameters for the hydrolysis of the chromogenic substrate, CBS 31.39 (LGR-pNA), by either wild-type or the mutant FIXa yielded similar K_m and k_{cat} values, suggesting that replacing the 39-loop of FIXa with the corresponding loop of FXa has no adverse effect on the folding or the reactivity of the active-site pocket of the FIXa mutant (Table 1).

The ability of the FIXa-$fX^{39\text{-loop}}$ mutant to interact with FVIIIa and to catalyze the activation of the natural substrate FX in the intrinsic Tenase complex was also evaluated. As shown in Fig. 1*A*, a FXa generation assay showed that both wild-type and FIXa-$fX^{39\text{-loop}}$ bind to FVIIIa with similar apparent dissociation constants ($K_{d(\text{app})}$) of ~11–14 nM on 40 μM PC/PS vesicles (Table 1). This is consistent with the reported value for the interaction of FIXa with the cofactor under similar experimental conditions (30). However, the FX concentration dependence of substrate activation revealed that the FIXa mutant exhibits ~2-fold lower apparent K_m and ~4-fold decreased k_{cat} for the activation of FX in the presence of a saturating concentration of FVIIIa (Fig. 1*B* and Table 1), suggesting that the residues of the 39-loop contribute to stabilization of the transition state for the cleavage of the FX scissile bond in the catalytic reaction. Consistent with the results obtained in the purified system, the clotting activity of the FIXa mutant was also impaired to a similar extent in an APTT-based clotting assay using FIX-deficient plasma (Fig. 1*C*).

TABLE 1

Kinetic constants for the cleavage of CBS 31.39 (LGR-pNA) and activation of FX by the FIXa derivatives in the presence of FVIIIa

The kinetic constants for the hydrolysis of the chromogenic substrate by FIXa were calculated from the cleavage rate of increasing concentrations of CBS 31.39 (0.1–10 mM) by each FIXa derivative (10 nM) in TBS/Ca^{2+} containing 33% ethylene glycol. The kinetic constants for the activation of FX by each FIXa derivative (0.1 nM) in complex with a fixed and saturating concentration of FVIIIa (50 nM) on PC/PS vesicles (40 μM) were calculated as described under "Experimental Procedures." The apparent K_d for the interaction of FIXa derivatives with FVIIIa was calculated using the same experimental methods except that the activation of a fixed concentration of FX (300 nM) was monitored in the presence of increasing concentrations of the cofactor (0–60 nM). These values are derived from Fig. 1. Kinetic values are the average of three measurements ± S.E.

	K_m	k_{cat}	k_{cat}/K_m	$K_{d(app)}$ (FVIIIa)
	M	s^{-1}	$M^{-1}s^{-1}$	*nM*
CBS 31.39				
FIXa	$(2.3 \pm 0.1) \times 10^{-3}$	21.4 ± 2.0	$(9.3 \pm 1.1) \times 10^3$	–
FIXa-fX$^{39\text{-loop}}$	$(1.2 \pm 0.1) \times 10^{-3}$	17.5 ± 1.0	$(14.6 \pm 0.9) \times 10^3$	–
FX				
FIXa	$(7.1 \pm 0.3) \times 10^{-8}$	3.5 ± 0.1	4.9×10^7	13.6 ± 0.7
FIXa-fX$^{39\text{-loop}}$	$(3.8 \pm 0.2) \times 10^{-8}$	1.0 ± 0.06	2.5×10^7	10.8 ± 0.3

Reaction with AT—The second-order inhibition rate constants (k_2) for the reaction of wild-type and mutant FIXa with AT in the absence and presence of pentasaccharide, derived from the linear dependence of the pseudo-first order rate constants on the concentrations of the serpin (Fig. 2), are presented in Table 2. In comparison to the reaction with FXa, AT inhibited FIXa with ~40-fold slower rate constant in the absence of pentasaccharide (Table 2). However, the difference between the reactivities of FIXa-fX$^{39\text{-loop}}$ and FXa with the native conformation of AT was reduced to 8- to 9-fold, because the reactivity of the serpin with the mutant FIXa was improved ~5-fold (Table 2). On the other hand, no significant difference between the reactivities of the wild-type and mutant FIXa with AT was observed in the presence of pentasaccharide, suggesting that the residues of the 39-loop impede the interaction of FIXa with the native, but not with the heparin-activated conformation of the serpin. Relative to FXa, AT inhibited both FIXa derivatives with a ~20-fold slower rate in the presence of pentasaccharide (Table 2). Thus, the conformational activation of AT by pentasaccharide overcomes the inhibitory interaction of the serpin with the 39-loop of wild-type FIXa.

Structural and mutagenesis data have indicated that the residues of the 39-loop of coagulation proteases play a critical role in determining the specificity of the P′ sides of scissile bonds on both substrates and inhibitors (22–25). AT contains an Arg (residue 399) at the P6′ position of the RCL. To determine whether Arg^{399} of AT contributes to the recognition of the serpin by FIXa, and if this residue is a target for the conformational activation by heparin, we prepared an Arg to Glu substitution mutant of AT (AT-R399E) and evaluated its reactivity with proteases in both the absence and presence of pentasaccharide. The AT mutant bound pentasaccharide with a normal affinity (Fig. 3), thus both wild-type and mutant serpins exhibited a dissociation constant of ~15 nM for interaction with the heparin cofactor. In agreement with a role for this residue in interaction with coagulation proteases, the reactivity of AT-R399E with FXa was decreased ~5-fold in the absence of pentasaccharide, but the decline in the reactivity of the serpin mutant with the protease was ~2-fold in the presence of the heparin cofactor (Table 2). Wild-type FIXa did not exhibit any change in its reactivity with the serpin mutant in the absence of pentasaccharide, thus the protease reacted with both serpins with essentially identical rate constants (Table 2). On the other hand, the reactivity of FIXa with the mutant serpin was impaired ~3-fold in the presence of pentasaccharide, thus resulting in a lower accelerating effect (151-fold *versus* 484-fold) for the heparin cofactor in the reaction. Similar to the reaction of FXa with the mutant serpin, the reactivity of the FIXa-fX$^{39\text{-loop}}$ mutant with the mutant serpin was markedly (~15-fold) decreased (Table 2). These results suggest that the Arg^{399} of AT is important for interaction with the 39-loop of FXa, but not with FIXa when the serpin is in its native conformation. However, this residue plays a role in interaction with FIXa in the activated conformation, because the AT mutant reacted with a 3-fold slower rate constant with FIXa in the presence of pentasaccharide.

AT inhibits coagulation proteases by a two-step reaction mechanism in which a Michaelis-type encounter complex that is formed in the first step is converted to a covalent complex in the subsequent second step of the reaction (20, 38). It is known that the bridging effect of high molecular weight heparins can lower the dissociation constant (K_D) for the formation of the initial encounter complex with essentially no effect on the rate constant (k) of the subsequent covalent complex formation (27, 38). Recent results have indicated that this mechanism of the cofactor function is also true for the pentasaccharide-mediated acceleration of the AT inhibition of FXa (37). Thus, binding of heparin to AT induces conformational changes in the RCL and sites remote from the RCL, thereby facilitating the recognition of the serpin by the protease (9, 20, 40). Because both FIXa and FXa utilize similar mechanisms to interact with the activated conformation of AT (21, 36, 40), we decided to utilize the S195A derivatives of FIXa and FXa, which are incapable of participating in the second step of the reaction, as probes to determine whether the protease interaction with the P6′ Arg^{399} of AT influences the formation of the initial Michaelis-type complex. As shown in Fig. 4*A*, the S195A mutant of FXa effectively competed with wild-type FXa for interaction with both wild-type AT and AT-R399E in the presence of pentasaccharide. Under these experimental conditions, the S195A mutant did not influence the reactivity of FXa with either serpin in the absence of pentasaccharide (data not shown). Non-linear regression analysis of data yielded K_D values of 0.45 ± 0.06 μM and 2.1 ± 0.09 μM for the interaction of the inactive FXa mutant with the activated conformation of wild-type AT and AT-399E, respectively (Fig. 4). These results, which are consistent with the kinetic data presented in Table 2, clearly suggest that the basis for the ~5-fold slower reactivity of FXa with the mutant

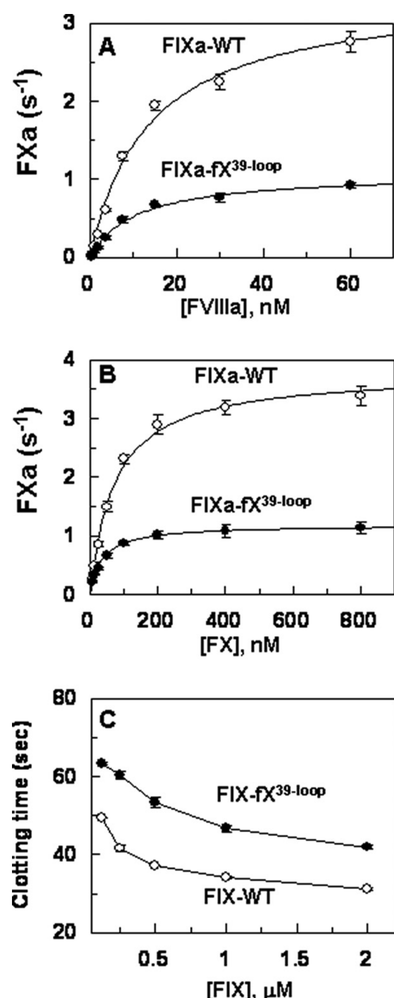

FIGURE 1. **The cofactor and substrate concentration dependence of FX activation by wild-type and mutant FIXa derivatives in the intrinsic Xase complex.** *A*, each FIXa derivative (0.1 nM) was incubated with FX (300 nM) in the presence of increasing concentrations of FVIIIa (0–60 nM) on PC/PS vesicles (40 μM) in TBS/Ca^{2+}. Following 2 min of activation at room temperature the reactions were terminated by the addition of 20 mM EDTA, and the rate of FXa generation was measured by an amidolytic activity assay using SpFXa as described under "Experimental Procedures." *B*, the same as *A* except that the concentration dependence of FX (6–800 nM) activation by the FIXa derivatives (0.1 nM each) was measured in the presence of a saturating concentration of FVIIIa (50 nM) in the same TBS buffer system. The *solid lines* are nonlinear regression analysis of kinetic data from three experiments according to the Michaelis-Menten equation. The kinetic values are presented in Table 1. The *symbols* are: wild-type recombinant FIXa (○) and FIXa-$fX^{39\text{-loop}}$ (●). *C*, the clotting activity of FIX derivatives was analyzed by using an APTT assay with FIX-deficient plasma as described under "Experimental Procedures." The *symbols* are: wild-type recombinant FIX (○) and FIX-$fX^{39\text{-loop}}$ (●).

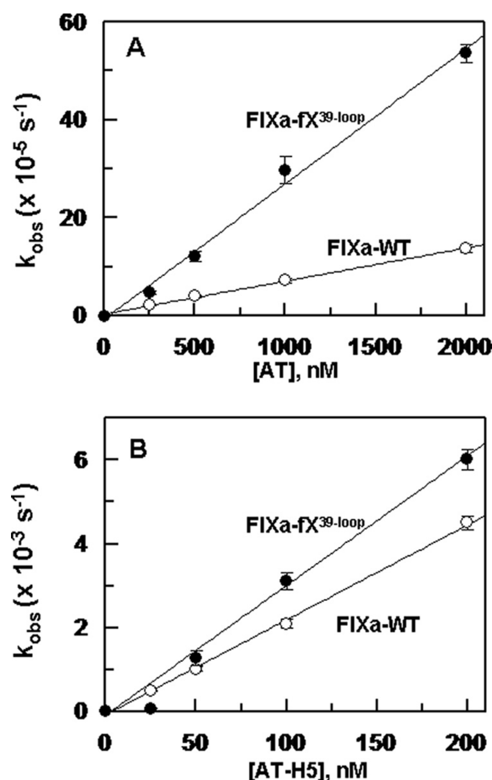

FIGURE 2. **Dependence of k_{obs} values for the reaction of FIXa derivatives on the concentrations of AT.** *A*, the k_{obs} values for the inactivation of wild-type FIXa (○) and FIXa-$fX^{39\text{-loop}}$ (●) by the increasing concentrations of AT was determined in TBS/Ca^{2+} at room temperature by an amidolytic activity assay as described under "Experimental Procedures." *B*, the same as above except that the AT inactivation of the proteases was monitored in the presence of a saturating concentration of pentasaccharide (1–2 μM). The *solid lines* in *A* and *B* are least squares computer fits of the kinetic data from three experiments by a linear equation. The second-order rate constants are presented in Table 2.

serpin is due to a weaker Michaelis-type complex formation in the first step of the reaction. A similar measurement with the S195A mutant of FIXa was not feasible, because an insignificant competitive effect for the inactive protease was observed that remained linear for up to 5 μM, the highest concentration of the mutant that could be used in the reaction (data not shown). Nevertheless, under the same experimental conditions, the inactive FIXa mutant effectively competed with its active counterpart for the Michaelis-type complex formation with both wild-type AT and AT-R399E in the presence of the high molecular weight heparin, yielding K_D values of 0.36 ± 0.04 μM and 0.70 ± 0.08 μM for the non-covalent interaction of the protease with wild-type AT and AT-399E, respectively (Fig. 4*C*). In the presence of heparin, the S195A mutant of FXa effectively inhibited the reversible complex formation of the protease with wild-type AT and AT-R399E with K_D values of 2.2 ± 0.2 nM and 7.9 ± 0.1 nM, respectively (Fig. 4*B*).

DISCUSSION

Relative to other coagulation proteases, FIXa has very poor catalytic activity toward its specific synthetic and physiological macromolecular substrates. However, the catalytic activity of FIXa toward its true substrate, FX, is dramatically enhanced by FVIIIa as the result of the cofactor providing specific exosites for the protease interaction with FX in the intrinsic Tenase complex (1–5, 14). Similarly, the catalytic activity of FIXa toward its pseudo-substrate, AT, is very poor. However, a pentasaccharide fragment of the high affinity heparin binds to the basic D-helix of AT to induce structural changes on the serpin, thereby facilitating the exosite-dependent interaction of the serpin with the protease and promoting the rate of the reaction ~300- to 500-fold (9, 20, 22). Nevertheless, despite similarities in the mechanism of the heparin-catalyzed AT inhibition of both FIXa and FXa (20), the reactivity of the AT-heparin complex with FIXa is slower by an order of magnitude for unknown reasons. The crystal structure of the non-covalent complex of FIXa-S195A with the pentasaccharide-bound AT was recently

TABLE 2

Second-order rate (k_2) constants for the inhibition of FIXa derivatives by the AT derivatives in the absence and presence of pentasaccharide

The second-order rate (k_2) constants in both the absence and presence of pentasaccharide (H5) were determined from the remaining activities of proteases after their incubation with serpins in TBS/Ca^{2+} at room temperature by amidolytic activity assays described under "Experimental Procedures." All values are averages of at least three independent measurements ± S.E., and they are derived from Fig. 2. The last column is the -fold accelerating effect of H5 derived from the ratio of k_2 in the presence of H5 to that in the absence of the cofactor.

	k_2 (−cofactor)	k_2 (+H5)	k_2 (+H)/k_2 (−cofactor)
	$M^{-1} s^{-1}$	$M^{-1} s^{-1}$	*-fold*
AT-WT			
FXa	$(2.5 \pm 0.2) \times 10^3$	$(7.2 \pm 0.9) \times 10^5$	288
FIXa	$(0.64 \pm 0.03) \times 10^2$	$(3.1 \pm 0.5) \times 10^4$	484
FIXa-fX$^{39\text{-loop}}$	$(2.9 \pm 0.3) \times 10^2$	$(3.3 \pm 0.4) \times 10^4$	114
AT-R399E			
FXa	$(0.53 \pm 0.06) \times 10^3$	$(3.5 \pm 0.4) \times 10^5$	660
FIXa	$(0.66 \pm 0.08) \times 10^2$	$(1.0 \pm 0.2) \times 10^4$	151
FIXa-fX$^{39\text{-loop}}$	$(0.19 \pm 0.02) \times 10^2$	$(0.54 \pm 0.05) \times 10^4$	284

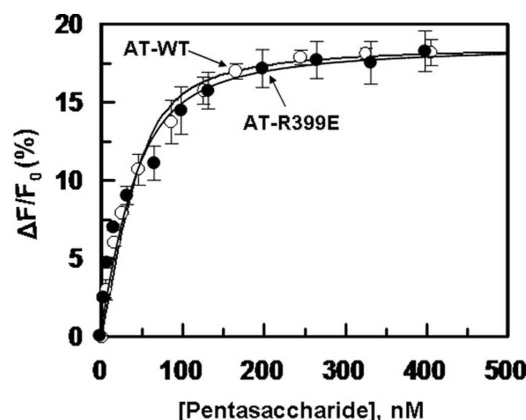

FIGURE 3. **Binding of pentasaccharide to AT derivatives.** The spectral changes were monitored by addition of 1–2 μl of a concentrated stock solution of pentasaccharide (fondaparinux) to 50 nM AT in TBS (pH 7.5) containing 0.1% PEG 8000, and dissociation constants (~15 nM for both serpins) were calculated from the changes of the intrinsic protein fluorescence as described under "Experimental Procedures." The *symbols* are: ○, AT-wild type; and ●, AT-R399E.

solved (22). Analysis of the structural data suggests that the 39-loop of FIXa orients toward the P′ side making several hydrophobic and water-mediated contacts with the side chain of Arg[399] of the serpin (22). Arg[399] is part of a 3-residue insertion loop located at the P6′ site of the AT RCL (22). The residues of the 39-loop of FXa also contact the P′ insertion residues of AT in the crystal structure of FXa-S195A in complex with the pentasaccharide-activated conformer of AT (25). Because there are significant differences in the nature of the residues of the 39-loop between FIXa and FXa (28), it has been hypothesized that the approximately one order of magnitude lower reactivity of FIXa with AT may be due to a non-optimal interaction of this loop with the P′ insertion residues of the serpin. To test this hypothesis, we substituted the 39-loop of FIXa with the corresponding loop of FXa and also replaced Arg[399] of AT with a Glu. A normal amidolytic activity for the FIXa mutant suggested that the mutagenesis has adversely affected neither the folding nor the reactivity of the catalytic triad. However, the FIXa mutant activated FX with ~2-fold lower catalytic efficiency in the intrinsic Tenase complex, suggesting that the residues of the 39-loop contribute to the protease interaction with the natural substrate. This was confirmed by a clotting assay using FIX-deficient plasma. The AT mutant interacted with pentasaccharide with a normal affinity, suggesting that the

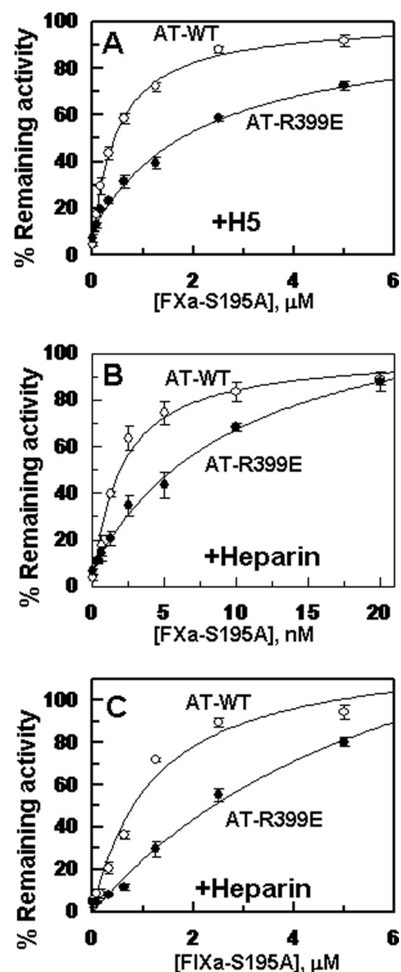

FIGURE 4. **Competitive effects of S195A derivatives of proteases on the interaction of wild-type proteases with AT derivatives.** *A*, the pentasaccharide (H5)-catalyzed inactivation of FXa by wild-type AT (○) and AT-R399E (●) was monitored in the presence of increasing concentrations of FXa-S195A (*x* axis) as described under "Experimental Procedures." *B*, the same as *panel A* except that high molecular weight heparin replaced pentasaccharide in the reaction. *C*, the same as *panel B* except that the competitive effect of FIXa-S195A on the inactivation of FIXa by the AT-heparin complex was monitored. The *solid lines* are nonlinear regression analysis of kinetic data from three experiments according to a hyperbolic binding equation, yielding Michaelis-type dissociation constants of 0.45 ± 0.06 μM and 2.1 ± 0.09 μM for the interaction of FXa (*A*) with either the pentasaccharide-complexed wild-type AT or AT-R399E, respectively. The corresponding values obtained for the protease-serpin complexes in the presence of the high molecular weight heparin in the same order were: 2.2 ± 0.2 nM and 7.9 ± 0.1 nM for FXa (*B*) and 0.36 ± 0.04 μM and 0.70 ± 0.08 μM for FIXa (*C*).

mutagenesis did not lead to a mis-folding of the serpin or its RCL sequence.

The FIXa-fX$^{39\text{-loop}}$ mutant exhibited ~5-fold improvement in its reactivity with the native conformer of AT, suggesting that the residues of the 39-loop of FIXa make inhibitory interactions with AT in the absence of the heparin cofactor. However, both wild-type and mutant FIXa reacted with essentially identical rate constants with the heparin-activated AT, suggesting that the cofactor effect of heparin eliminates the inhibitory interaction of the 39-loop of wild-type FIXa with the serpin. These findings support a recently proposed mechanism for the activation of AT by heparin in which the very low or low rates of reaction between FIXa and FXa, respectively, and AT may result from unfavorable interactions in the native state, and that the dominant contributions to the several hundred-fold activation by heparin result more from removal of such unfavorable interactions than engagement of novel exosites (41). Wild-type FIXa reacted with an essentially identical second-order rate constant with both wild-type AT and AT-R399E in the absence of a cofactor; however, the reactivity of the protease with the mutant serpin was decreased ~3-fold in the presence of pentasaccharide (Table 2). These results support the structural data that Arg^{399} is a target for the conformational activation of the serpin by heparin (22). This observation is also consistent with our previous mutagenesis data showing that the deletion of this residue results in a decline in the reactivity of the protease with the mutant serpin specifically in the presence of pentasaccharide (42). The observation that the reactivities of both wild-type FIXa and FIXa-fX$^{39\text{-loop}}$ with AT remained essentially identical in the presence of pentasaccharide, which is an order of magnitude lower than the rate of the FXa-AT reaction under identical conditions, suggests that the slower reactivity of FIXa with the activated conformation of the serpin is not due to differences in the residues of the 39-loop. It should, however, be noted that the side chain of Lys-36 forms a salt bridge with Glu^{60} in the crystal structure of FIXa (22, 43). Because the side chain of Glu^{60} has lost its electrostatic bonding partner in the FIXa-fX$^{39\text{-loop}}$ mutant, the possibility that the side chain of Glu impedes the interaction of the protease mutant with AT in the presence of pentasaccharide cannot be ruled out. If true, this may explain the inability of the loop swap mutagenesis strategy to convert FIXa to a protease with an FXa-like reactivity with AT in the presence of pentasaccharide. Further mutagenesis studies with Glu^{60} to Ala substitution mutants of wild-type FIXa and FIXa-fX$^{39\text{-loop}}$ will be required to further investigate this question.

The interaction of AT with at least two additional sites on FIXa and FXa is critical for determining the specificity of the reaction in the alternative native and activated conformations of the serpin. The first site is the 99-loop, which has a Tyr at position 99 in both FIXa and FXa. This residue determines the specificity of the P2-binding pocket and is responsible for these proteases exhibiting strong preference for small residues like Gly at the P2 positions of substrates and inhibitors (as in AT) (13, 44, 45). The other site involved in recognizing the serpin is the basic residues of the autolysis loop, in particular Arg^{150}, which play the most critical roles in the protease recognition of the activated conformer of AT (21, 22, 25, 36). Structural and mutagenesis data have indicated that Arg^{150} of the autolysis loop makes productive interactions with a complimentary pocket of AT, located on the strand 3 of β-sheet C on the serpin (21, 25, 40), and that this site becomes accessible for interaction with the proteases only in the presence of pentasaccharide (9, 20, 21, 25). The autolysis loop of both FIXa and FXa utilizes a similar mechanism to interact with the activated conformation of AT (21, 25), however, the 99-loop of FIXa has a basic residue, Lys^{98}, that is not conserved in FXa. It is possible that this residue of the 99-loop in FIXa is involved in restricting the specificity of the protease reaction with AT in the activated conformation. In support of this hypothesis, we have demonstrated that the substitution of this residue with Ala markedly improves the reactivity of the mutant protease with the heparin-activated conformer of AT (17). Moreover, the substitution of the 99-loop of FIXa with the corresponding loop of FXa has been demonstrated to improve the amidolytic activity of the protease, suggesting that the residues of this loop may play a role in restricting the specificity of FIXa in reaction with AT (45). This hypothesis is consistent with other kinetic and modeling data in the literature (46). It is also known that ethylene glycol markedly promotes the catalytic efficiency of FIXa toward synthetic substrates by apparently binding to and altering the conformation of the 99-loop (12, 13). However, we did not observe a significant cofactor activity for ethylene glycol in the FIXa-AT reaction in either the absence or presence of pentasaccharide (data not presented). Thus, further studies will be required to understand the exact role of the 99-loop in determining the specificity of the interaction with the native and activated conformations of AT.

The observation, that the K_D for the reversible Michaelis-type complex formation of FXa-S195A, but not that of FIXa-S195A, with the AT-pentasaccharide complex could be measured using practical and relatively low concentrations of the inactive enzyme, suggests that the affinity of the activated conformer of AT for interaction with FIXa is much weaker than that of FXa, providing the kinetic basis for the slower reactivity of the protease with the activated conformer of the serpin. The comparison of the same results in the presence of heparin, where the K_D values for the interaction of both FXa and FIXa with AT could be measured (Fig. 4), further supports the hypothesis that the slower reactivity of FIXa with AT is due to a weaker K_D for the non-covalent protease-serpin complex formation in the first step of the reaction. Moreover, the observation that the K_D values for the interaction of proteases with AT-R399E were elevated in the presence of heparin cofactors further supports the hypothesis that the P′ side insertion residue of the heparin-activated AT is involved in the interaction of the serpin with the 39-loop of proteases, although it also contributes to determining the specificity of the reaction with FXa in the native conformation (Table 2). These results further support the structural and mutagenesis data that Arg^{399} is a target for the conformational modulation of the serpin by heparin (22, 25) and that the interaction of proteases with this site of AT affects the K_D of complex formation in the first step of the reaction.

In summary, we have demonstrated in this study that the 39-loop of FIXa contributes to the regulation of the specificity

of the protease interaction with the physiological substrate, FX, and the serpin inhibitor, AT. In the latter case, the 39-loop impedes the interaction of the protease with the native conformer of AT. This unique feature of the 39-loop in FIXa is an evolutionary adaptation that is critical for the catalytic function of the protease, thus allowing the protease to assemble into the intrinsic Tenase complex to fulfill its physiological function, which is to amplify thrombin generation during the blood coagulation process. However, the binding of AT to heparin-like glycosaminoglycans lining the endothelium can overcome the inhibitory interaction of the 39-loop with AT, thus enabling the serpin to regulate the activity of the protease at the vascular surface. The presented data further suggest that this loop of FIXa contributes to the effective activation of the substrate, FX, by the protease in the intrinsic Tenase complex.

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