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The role of autolysis loop in determining the specificity of coagulation proteases

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Abstract

We recently demonstrated that the substitution of the autolysis loop (residues 143 to 154 in the chymotrypsin numbering system) of activated protein C (APC) with the corresponding loop of factor Xa (fXa) renders the APC mutant (APC/fX$^{143-154}$) susceptible to inhibition by antithrombin (AT) in the presence of pentasaccharide. Our recent results further indicated, that in addition to an improvement in the reactivity of APC/fX$^{143-154}$ with AT, both the amidolytic and anti-factor Va activities of the mutant APC have also been significantly increased. Since the autolysis loop of APC is five residues longer than the autolysis loop of fXa, it could not be ascertained whether this loop in the mutant APC specifically interacts with the activated conformation of AT or if a shorter autolysis loop is responsible for a global improvement in the catalytic activity of the mutant protease. To answer this question, we prepared another APC mutant in which the autolysis loop of the protease was replaced with the corresponding loop of trypsin (APC/Tryp$^{143-154}$). Unlike an ~500-fold improvement in the reactivity of APC/fX$^{143-154}$ with AT in the presence of pentasaccharide, the reactivity of APC/Tryp$^{143-154}$ with the serpin was improved ~10-fold. These results suggest that both the length and structure of residues of the autolysis loop are critical for the specificity of the coagulation protease interaction with AT. Further factor Va inactivation studies with the APC mutants revealed a similar role for the autolysis loop of APC in the interaction with its natural substrate.

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- Antithrombin
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Introduction

The proteolytic activity of coagulation proteases of both the intrinsic and extrinsic clotting cascades is primarily regulated by the serpin inhibitor antithrombin (AT) in plasma (1-5). However, AT is a poor inhibitor of coagulation proteases unless it is bound to heparin-like glycosaminoglycans found on the surface of the endothelium (1,6). This is the basis for the extensive use of heparin in the prophylaxis and treatment of venous thrombosis (7). High-molecular weight heparins improve the reactivity of AT with coagulation proteases by 3-4 orders of magnitude (8). Such a dramatic co-factor effect for heparin is believed to arise through two different mechanisms. First, the interaction of heparin with a basic region on AT induces a conformational change in the structure of the

serpin, thereby facilitating its recognition by the coagulation enzymes (allosteric activation mechanism) (9,10). Second, heparin can bind to a basic exosite on coagulation proteases, thus being capable of bridging AT and the target protease in one complex, thereby decreasing the dissociation constant for the initial interaction of two proteins (template mechanism) (3,5). The extent of the contribution of each mechanism to the rate-accelerating effect of heparin in protease inactivation by AT has been extensively studied. For certain proteases such as thrombin, the co-factor effect of full-length heparin is mediated primarily through a template mechanism with ~2-fold contribution from the activation mechanism (9). In the case of factors Xa (fXa) and IXa, however, the co-factor effect of full-length heparin is mediated by ~300- to 500-fold enhancement through activation and ~200- to 300-fold enhancement through the template mechanism in the presence of a physiological concentration of Ca^{2+} (8,11-13). The elucidation of these two distinct co-factor mechanisms of heparin has been documented by the observation that a unique pentasaccharide fragment of high-affinity heparin which can bind with equal affinity to AT, but is not capable of bridging the serpin to the protease, enhances the reactivity of fXa with AT ~300-fold by an allosteric mechanism with an ~2-fold co-factor effect on the AT inhibition of thrombin (14,15).

We investigated the mechanism by which coagulation proteases differentially react with the activated conformation of AT and demonstrated that the autolysis loop of both fXa and fIXa (residues 143-154 in chymotrypsin numbering) (16) have several basic residues that specifically interact with the serpin in the presence of pentasaccharide (17-19). Thus, we hypothesized that thrombin does not react with the heparin-activated conformation of AT because the basic residues of fXa are not conserved in the autolysis loop of the protease (17). In support of this hypothesis substitution of the autolysis loop of activated protein C (APC), a protease nonreactive with either the native or activated conformations of AT, with the corresponding sequence of fXa yielded a mutant (APC-$fXa^{143-154}$) which reacted with AT with ~375-fold improved rate constant in the presence of pentasaccharide (17). A similar chimeric mutant of thrombin containing the autolysis loop of fXa from residues 143 to 154 exhibited dramatically improved reactivity with the activated conformation of AT (17). Nevertheless, further characterization of the APC-$fXa^{143-154}$ mutant revealed that the catalytic activity of the mutant protease toward small synthetic substrates and the natural substrate, factor Va (fVa), was also improved (17,20). Since the autolysis loop of APC also has several basic residues, but is five residues longer than the autolysis loop of other vitamin K-dependent coagulation proteases, the general improvement in the catalytic function of the APC mutant in reaction with AT, fVa and small chromogenic substrates raised the possibility that a shorter autolysis loop, but not specific interaction of the residues of this loop with the pentasaccharide-activated conformation of AT, is responsible for the improved reactivity of the mutant with the serpin. This uncertainty was underscored by the observation that thrombin also has an insertion of six residues in its autolysis loop (16).

To address this question, in the present study, we substituted the autolysis loop of protein C with the corresponding shorter and neutral loop of trypsin. Furthermore, we also substituted three basic residues of APC-$fXa^{143-154}$ with Ala. The catalytic properties of these mutants were evaluated with respect to their ability to react with AT in the absence and presence of pentasaccharide and to recognize the target substrate fVa. The results suggest that both the length and the structure of residues of the autolysis loop contribute to determining the specificity of coagulation proteases in their reaction with AT. This hypothesis is also applicable for

the specificity of APC interaction with its natural substrate, fVa.

Material and Methods

Construction, expression and purification of recombinant proteins

The construction, expression and purification of wild-type protein C and the mutant in which the autolysis loop (^{143}Tyr-His-Ser-Ser-Arg-Glu-Lys-Glu-Ala-Lys-Arg-Asn-Arg-Thr-Phe-Val^{154}) of the zymogen was replaced with the corresponding sequence of fX (^{143}Arg-Thr-His-Glu-Lys-Gly-Arg-Gln-Ser-Thr-Arg^{154}) (PC-fX$^{143-154}$) has been described previously (17). A PC-fX$^{143-154}$ variant in which the three basic residues Arg-143, Lys-147 and Arg-150 of the autolysis loop were replaced with Ala residues (PC-fX$^{143-154/3A}$) was constructed by the polymerase chain reaction mutagenesis approach and expressed in the same expression system as described (17). A protein C mutant in which the autolysis loop of the zymogen was replaced with the corresponding sequence of trypsin (^{143}Asn-Thr-Lys-Ser-Ser-Gly-Thr-Ser-Tyr-Pro-Asp-Val^{154}) (PC-Tryp$^{143-154}$) was constructed by the polymerase chain reaction mutagenesis approach and expressed in the same vector system. The amino acid sequence of the autolysis loops (residues 143-154) of trypsin and coagulation proteases discussed in this manuscript is presented in Table 1. Following confirmation of the accuracy of the mutagenesis, the mutant constructs were expressed in human embryonic kidney 293 cells as described (17). Both wild-type and mutant zymogens were purified to homogeneity by immunoaffinity chromatography using the Ca^{2+}-dependent monoclonal antibody HPC4 as described (17, 21). The thrombomodulin (TM) fragment containing epidermal growth factor-like domains 456 (TM456) (21) and protein C inhibitor (PCI) (22) were expressed in mammalian cells as described. All recombinant proteins were tested for homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Human plasma proteins including prothrombin, fVa, fXa, and AT were purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (23). The active AT-binding pentasaccharide fragment of heparin (fondaparinux sodium) was purchased from Quintiles Clinical Supplies (Mt. Laurel, NJ, USA). The normal pooled plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS, USA) and the activated partial prothrombin time (aPTT) reagent, Alexin, was purchased from Sigma (St. Louis, MO, USA). α_1-Antitrypsin was obtained from Athens Research and Technology, Inc. (Athens, GA, USA). The chromogenic substrate, Spectrozyme PCa (SpPCa, H-D-Lys (γ-Cbo)-Pro-Arg-pNA.2AcOH) was purchased from American Diagnostica (Greenwich, CT, USA) and S2238 was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH, USA).

Table 1. Alignment of the sequence of amino acid residues of the autolysis loops of trypsin and coagulation proteases from residues 143 to 154. The chymotrypsin numbering system is used (16).

	143														154
Trypsin	Asn	Thr	Lys	Ser	Ser	Gly	Thr	.	.	.	.	.	Ser	Tyr	Pro	Asp	Val
APC	Tyr	.	His	Ser	Ser	Arg	Glu	Lys	Glu	Ala	Lys	Arg	Asn	Arg	Thr	Phe	Val
Thrombin	Asn	Leu	Lys	Glu	Thr	Trp	Thr	Ala	Asn	Val	Gly	Lys	Gly	Gln	Pro	Ser	Val
FXa	Arg	Thr	His	Glu	Lys	Gly	Arg	.	.	.	.	.	.	Gln	Ser	Thr	Arg
FIXa	Arg	Val	Phe	His	Lys	Gly	Arg	.	.	.	.	.	.	Ser	Ala	Leu	Val

Inactivation by antithrombin

The rate of inactivation of wild-type and mutant proteases by AT in both the absence and presence of pentasaccharide was measured under pseudo-first-order rate conditions by a discontinuous assay method as described (17). In the absence of the co-factor, both wild-type and mutant proteases (2-3 nM) were incubated with plasma AT (0.125-10 μM) in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, containing 1 mg/mL bovine serum albumin (BSA), 0.1% polyethylene glycol 8000 (PEG-8000) and 5 mM Ca^{2+} (TBS/Ca^{2+}). In the presence of pentasaccharide, the reaction conditions were the same except that wild-type and mutant proteases were incubated with AT (0.025-10 μM) in the presence of saturating concentrations of pentasaccharide (1-20 μM) in TBS/Ca^{2+}. All reactions were carried out in 50-mL volumes in 96-well polystyrene plates at room temperature. After 30-300 min, depending on the rate of reactions, 50 μL of the chromogenic substrate (200 μM SpPCa) in TBS was added to each well and the remaining enzyme activity was measured with a V_{max} kinetics microplate reader (Molecular Devices, Menlo Park, CA, USA). The second-order inhibition rate constants for both uncatalyzed and catalyzed reactions were obtained as described (17).

The PCI and α_1-antitrypsin inhibition rates of APC derivatives were measured under pseudo-first-order rate conditions by a discontinuous assay method as described (20). Briefly, 3 nM APC was incubated at room temperature with 50-200 nM PCI or 10-20 μM α_1-antitrypsin in 50-μL reactions in TBS buffer containing 2.5 mM Ca^{2+}, 1 mg/mL BSA and 0.1% PEG-8000.

Hydrolysis of chromogenic substrates

The steady-state kinetics of hydrolysis of SpPCa (15-2000 μM) by both wild-type and mutant APC (5 nM) was measured in TBS/Ca^{2+} at 405 nm at room temperature in a V_{max} kinetic microplate reader as described above. The K_m and k_{cat} values for the substrate hydrolysis were calculated from the Michaelis-Menten equation as described (17).

Zymogen activation

Two milligrams of protein C derivatives was incubated with thrombin (25 μg) in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4 (TBS buffer), containing 5 mM EDTA for 2 h at 37ºC. The APC derivatives were separated from thrombin by an FPLC Mono-Q column developed with a 40-mL linear gradient from 0.1 to 1.0 M NaCl, 20 mM Tris-HCl, pH 7.4, as described (22,24). The concentrations of proteases were determined from the absorbance at 280 nm and extinction coefficient ($E^{1\%}_{1\,cm}$) of 14.5 (assuming a molecular mass of 56 kDa for APC), by an amidolytic activity assay using SpPCa, and by stoichiometric titration of enzymes with known concentrations of PCI as described (22).

The initial rate of wild-type and mutant protein C activation by thrombin was measured in both the absence and presence of TM456 and Ca^{2+} as described (21). In the absence of TM456, the time course of protein C (1 μM) activation by thrombin (5-50 nM) was studied at room temperature in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4 (TBS) containing 1 mg/mL BSA, 0.1% PEG-8000 and 2.5 mM Ca^{2+} in 96-well assay plates. At different time intervals, thrombin activity was quenched by 1 μM AT and the rate of protein C activation was measured from the cleavage rate of SpPCa (200 μM) at 405 nm by a V_{max} kinetic microplate reader as described above. The experimental conditions in the presence of TM456 were the same, with the exception that the activation by thrombin (1.0 nM) was carried out in the presence of a saturating concentration of TM456 (200 nM). The concentration of APC in the reaction mixtures was determined by reference to a standard curve which was

prepared by the total activation of protein C derivatives with the excess thrombin at the time of the experiments.

Measurement of the anticoagulant activity

Anticoagulant activities of the APC derivatives were evaluated both in the purified and plasma-based assay systems. The APC concentration dependence of fVa inactivation was determined by a three-stage assay in the purified system as described (20). Briefly, in the first stage, fVa (5 nM) was incubated with wild-type or mutant APC (1-5 nM) on 25 µM PC/PS vesicles in TBS/Ca^{2+} for 10 min. In the second stage, the remaining fVa activity was determined in a prothrombinase assay from the fVa-catalyzed prothrombin activation by fXa as described (20). The prothrombinase assay was carried out for 30 s with excess prothrombin (1 µM) and a saturating fXa concentration (10 nM) at room temperature. The remaining activity of fVa was determined from the decrease of the rate of thrombin generation as monitored by an amidolytic activity assay in the third stage using 100 µM S2238.

The anticoagulant activities in plasma were evaluated in an aPTT assay using a STart 4 fibrinometer (Diagnostica/Stago, Asnieres, France). Briefly, 0.050 mL TBS lacking or containing 1-20 nM final concentrations of wild-type or mutant APC was incubated with a mixture of 0.05 mL normal pooled plasma plus 0.05 mL aPTT reagent (Alexin) for 5 min before the initiation of clotting by the addition of 0.05 mL of 35 mM $CaCl_2$ at 37ºC as described (20).

Results

Protein C activation

The initial rate of the activation of protein C derivatives by thrombin both in the absence and presence of TM456 is presented in Figure 1. In the absence of TM, the activation of all protein C mutants by thrombin was improved 5- to 8-fold in the presence of Ca^{2+} (Figure 1A). The initial rate of activation of protein C mutants by thrombin in the presence of TM456 was not significantly changed, thus increasing ~50% for both PC-$fX^{143-154}$ and PC-$fX^{143-154/3A}$ and decreasing to a similar extent for the PC-$Tryp^{143-154}$ mutant (Figure 1B). These results suggested that a longer autolysis loop in protein C plays a negative role in its recognition and subsequent activation by thrombin in the absence of TM.

Amidolytic activity

The capacity of autolysis loop mutants of APC to cleave the chromogenic substrate SpPCa was determined to assess the integrity of the catalytic domain active site. SpPCa (Lys-Pro-Arg-pNa) is a tripeptide sequence

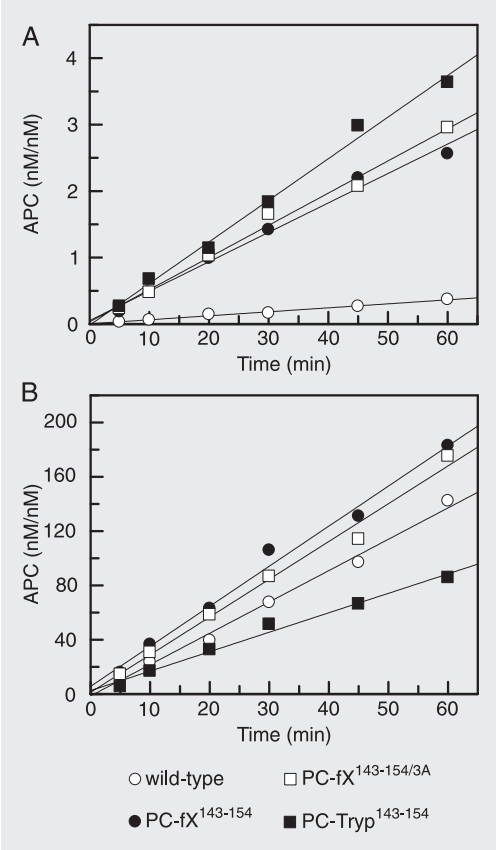

Figure 1. Time course of the initial rate of protein C activation by thrombin in the absence and presence of thrombomodulin 456 (TM456). *A*, Protein C derivatives (1 µM) were incubated with thrombin (10-50 nM) at room temperature in TBS containing 1 mg/mL BSA, 0.1% PEG-8000 and 2.5 mM Ca^{2+}. At indicated time points, small aliquots of the activation reactions were transferred to a 96-well assay plate, the thrombin activity was neutralized by antithrombin, and the rate of activated protein C (APC) generation was determined by an amidolytic activity assay using Spectrozyme PCa as described in Material and Methods. *B*, The same as *A*, except that the time course of activation by thrombin (1 nM) was monitored in the presence of TM456 (200 nM). The data shown are representative experiments of 3 independent and reproducible measurements.

that binds to the S1, S2, and S3 substrate-binding subsites of the catalytic groove, and distortion of these binding sites will alter SpPCa cleavage. Kinetic parameters for the SpPCa cleavage are shown in Table 2. All mutants exhibited improved activity toward SpPCa with an ~2- to 3-fold improvement in K_m for APC-Tryp$^{143\text{-}154}$ and an ~2-fold improvement in k_{cat} for the other two mutant proteases. These results indicate that substitution of the autolysis loop of APC with either fXa or trypsin has no adverse effect on the conformation of the APC S1-S3 substrate-binding sites, but rather the mutagenesis improves the catalytic efficiency of the mutants toward the chromogenic substrate.

Table 2. Kinetic constants for the hydrolysis of Spectrozyme PCa by the APC derivatives.

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
APC wild-type	136.0 ± 25.1	31.1 ± 2.9	0.23 ± 0.06
APC-fXa$^{143\text{-}154}$	115.5 ± 14.4	56.2 ± 4.9	0.49 ± 0.10
APC-fXa$^{143\text{-}154/3A}$	111.2 ± 9.8	51.3 ± 3.0	0.46 ± 0.07
APC-Tryp$^{143\text{-}154}$	43.5 ± 6.8	26.6 ± 3.9	0.61 ± 0.18

The kinetic constants were calculated from the hydrolysis rate of increasing concentrations of SpPCa (0.75 to 2000 μM) by wild-type and mutant activated protein C (APC) derivatives (3 nM) in TBS/Ca^{2+} as described in Material and Methods. The kinetic values are reported as the mean ± SEM for three measurements. SpPCa = H-D-Lys (γ-Cbo)-Pro-Arg-pNA.2AcOH.

Table 3. Second-order inhibition rate constants for the serpin inhibition of activated protein C (APC) derivatives.

	k_2 (AT) (M^{-1} s^{-1})	k_2 (AT-H5) (M^{-1} s^{-1})	k_2 (PCI) (M^{-1} s^{-1})	k_2 (α_1-AT) (M^{-1} s^{-1})
APC wild-type	N/D	2.6 ± 0.6	(1.5 ± 0.3) x 10^3	2.6 ± 0.5
APC-fXa$^{143\text{-}154}$	3.2 ± 1.2	(1.4 ± 0.1) x 10^3	(3.0 ± 0.8) x 10^3	3.0 ± 0.4
APC-fXa$^{143\text{-}154/3A}$	N/D	(4.6 ± 0.4) x 10^1	(1.6 ± 0.2) x 10^3	N/D
APC-Tryp$^{143\text{-}154}$	N/D	(2.5 ± 0.2) x 10^1	(14.1 ± 0.3) x 10^3	26.8 ± 1.4

The APC derivatives (2 to 3 nM) were incubated with antithrombin (AT; 0.025 to 10 μM) in the absence and presence of saturating concentrations of pentasaccharide (H5; 1 to 20 μM), protein C inhibitor (PCI; 50 to 200 nM), and α_1-antitrypsin (α_1-AT; 10 to 20 μM) at room temperature in TBS/Ca^{2+}. The second-order rate constants (k_2) were calculated from the remaining chromogenic substrate activity as described in Material and Methods. Data are reported as the mean ± SD for at least three measurements. N/D = not detected; the incubation of APC with the serpin (~10 μM) for 5 h did not result in a decline in the chromogenic substrate activity of the enzyme.

Inactivation by antithrombin

The anticoagulant protease APC is essentially unreactive with AT in the absence of heparin co-factors (17). However, the incubation of APC with a very high concentration of AT (10 μM) in the presence of pentasaccharide for several hours leads to slow inactivation of the protease by the serpin with an estimated second-order association rate constant (k_2) of 2.6 M^{-1} s^{-1} (Table 3). Similar to the case with the wild type, neither APC-fX$^{143\text{-}154/3A}$ nor APC-Tryp$^{143\text{-}154}$ exhibited detectable reactivity with AT in the absence of pentasaccharide, though APC-fX$^{143\text{-}154}$ was slowly inhibited by the serpin under these conditions (Table 3). On the other hand, the APC-fX$^{143\text{-}154}$ mutant reacted efficiently with AT in the presence of pentasaccharide with a k_2 value of 1.4 x 10^3 M^{-1} s^{-1} (Table 3). Relative to wild-type APC, the reactivity of the other two mutants, APC-fX$^{143\text{-}154/3A}$ and APC-Tryp$^{143\text{-}154}$ with AT in the presence of pentasaccharide was also markedly improved (10- to 20-fold), thus yielding k_2 values of 4.6 and 2.5 x 10^1 M^{-1} s^{-1}, respectively (Table 3). However, in contrast to a greater than 500-fold improvement in the reactivity of APC-fX$^{143\text{-}154}$ with the AT-pentasaccharide complex, a considerably lesser improvement of 10- to 20-fold was observed for the other two mutants of APC with the activated conformation of the serpin (Table 3). These results suggest that both the length and positively charged residues of the autolysis loop are critical for determining the specificity of the protease interaction with AT in the presence of pentasaccharide.

Anticoagulant activity

To understand the role of the autolysis loop in the anticoagulant activity of APC, the capacity of the mutants to inactivate fVa or to elevate the plasma clotting time was evaluated. As demonstrated in Figure 2A and consistent with our previous results (20),

the time course of fVa inactivation suggested that the activity of the APC-fX$^{143\text{-}154}$ mutant was improved ~2-fold. The activity of APC-fX$^{143\text{-}154/3A}$ and APC-Tryp$^{143\text{-}154}$ mutants was also slightly improved in this assay (Figure 2A). Comparisons of the anti-fVa activities of APC-fX$^{143\text{-}154}$ and APC-fX$^{143\text{-}154/3A}$ suggests that, similar to interaction with AT, the basic charges of the autolysis loop contribute to the specificity of protease interaction with the co-factor.

Unlike the improved anti-fVa activity of the APC mutant in the purified system, the plasma clotting activity of the APC-fX$^{143\text{-}154}$ mutant was slightly impaired and the activity of APC-fX$^{143\text{-}154/3A}$ and APC-Tryp$^{143\text{-}154}$ mutants was nearly abolished (Figure 2B). To understand the basis for this paradoxical effect in the activity of the mutant in these two systems, the extent of the reactivity of APC derivatives with other plasma inhibitors was also studied. The time course of the incubation of each APC with normal plasma followed by monitoring the amidolytic activity yielded a half-life of ~30 min for wild-type APC at room temperature, which is consistent with the literature (25). Interestingly, the half-life of APC in plasma was decreased to less than 5 min for the mutants, suggesting that the APC mutants became susceptible to inhibition by the plasma inhibitors. Thus, certain structural features in the autolysis loop of APC appear to be responsible, at least partially, for the slow reactivity of the protease with plasma inhibitors. Further studies with other APC-specific plasma serpin inhibitors, PCI and α_1-antitrypsin, in the purified system supported this conclusion since the reactivity of the APC mutants with these inhibitors was significantly improved. Thus, relative to wild-type APC (k_2 = 1.5 ± 0.3 x 10^3 M^{-1} s^{-1}) the reactivity of the APC-fX$^{143\text{-}154}$ mutant with the serpin was increased ~2-fold (k_2 = 3.0 ± 0.8 x 10^3 M^{-1} s^{-1}). A much greater improvement (~10-fold) in the reactivity of APC-Tryp$^{143\text{-}154}$ (k_2 = 1.4 ± 0.3 x 10^4 M^{-1} s^{-1}) with PCI was observed (Table 3). Interestingly, a similar 10-fold improvement was also observed in the reactivity of the APC-Tryp$^{143\text{-}154}$ mutant with α_1-antitrypsin (Table 3), possibly providing a basis for the poor anticoagulant activity of this mutant in the plasma-based clotting assay. Unlike an improved reactivity with AT, the reactivity of APC-fX$^{143\text{-}154/3A}$ with either PCI or α_1-antitrypsin was not improved. Whether the shorter half-life of this mutant is due to its susceptibility to other plasma serpins was not investigated.

Discussion

AT can inhibit the proteolytic activity of all coagulation proteases of the intrinsic and extrinsic cascade (1-5); however, the serpin is essentially unreactive with the anticoagulant protease APC (17). Previously, we dem-

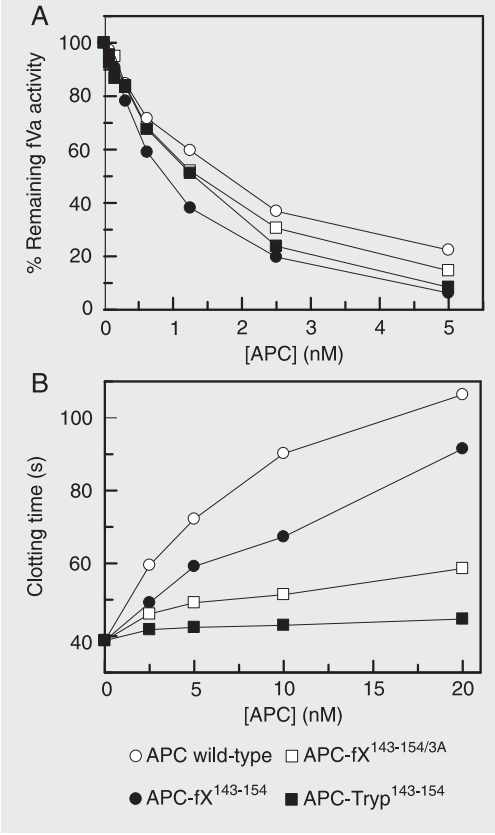

Figure 2. Anticoagulant activity of activated protein C (APC) derivatives in factor Va (fVa) degradation and plasma-based clotting assays. *A*, The time course of fVa (5 nM) inactivation by increasing concentrations of the APC derivatives was carried out on phosphatidylcholine/phosphatidylserine vesicles (25 µM) at room temperature in TBS containing 1 mg/mL BSA, 0.1% PEG-8000 and 5 mM Ca^{2+}. At each time, small aliquots of the inactivation reaction was transferred to a 96-well assay plate and the remaining co-factor activity of fVa was determined by a prothrombinase assay as described in Material and Methods. *B*, The plasma clotting activity of the APC derivatives was determined as a function of different concentration of APC at 37°C as described in Material and Methods. The plots are representative experiments of 2-3 independent and reproducible measurements.

onstrated that the substitution of the autolysis loop of APC with the corresponding loop of fXa renders the mutant protease susceptible to inactivation by AT specifically in the presence of pentasaccharide (17), suggesting that the autolysis loop of fXa carries recognition sites for interaction with the activated conformation of AT. The autolysis loop of fXa contains four basic residues at positions 143, 147, 150, and 154 (Table 1) and the Ala-scanning mutagenesis of these residues has identified Arg-150 as the critical residue that specifically interacts with AT in the presence of pentasaccharide (18). The recent X-ray crystal structure determination of a catalytically inactive mutant of fXa in complex with AT in the presence of pentasaccharide supported the mutagenesis results (26). Nevertheless, in a recent study we noted that, in addition to improvement in the reactivity of the APC-fX$^{143\text{-}154}$ mutant with AT, the activity of this mutant toward both small synthetic and the natural substrate fVa was also improved (20). The autolysis loop of APC is also basic but it is five residues longer than the corresponding loop of fXa (Figure 3). This raised the possibility that it is not only the specific interaction of charged residues of the autolysis loop that determines the specificity, but the shortening of this loop results in a global improvement in the reactivity of the catalytic groove of APC with macromolecular substrates and inhibitors in plasma. To test this possibility, in this study, we substituted the autolysis loop of APC with the corresponding loop of trypsin which has the same number of residues as in fXa, but the loop lacks any charged residue (Table 1). We also substituted Arg-143, Lys-147 and Arg-150 of the APC-fX$^{143\text{-}154}$ mutant with Ala. The observation that, relative to the APC-fX$^{143\text{-}154}$ mutant, the reactivity of both APC-Tryp$^{143\text{-}154}$ and APC-fX$^{143\text{-}154/3A}$ mutants with the AT-pentasaccharide complex was markedly decreased suggested that one or more of the charged residues of the autolysis loop in both fXa and in the APC-fX$^{143\text{-}154}$ specifically interact with the activated conformation of AT. Nevertheless, the observation that, relative to wild-type APC, both APC-Tryp$^{143\text{-}154}$ and APC-fX$^{143\text{-}154/3A}$ mutants exhibited markedly improved inhibition rate constants with AT in the presence of pentasaccharide further suggests that the length of the autolysis loop is also important for the recognition of AT by these proteases. Thus, both the structure of residues of the autolysis loop and its length play pivotal roles in determining the specificity of coagulation protease reactions with the native and activated conformation of AT. The other coagulation protease that exhibits similar reactivity with AT in both the absence and presence of pentasaccharide is thrombin which also contains an autolysis loop that has an insertion of 6 residues (Table 1). Based on data with APC, we hypothesize that a longer autolysis loop in thrombin contributes to the inability of this protease to react with an improved rate constant with the activated conformation of AT. We further hypothesize that a longer autolysis loop is responsible, at least partially, for the resistance of the anticoagulant APC to inactivation by the serpin in the absence and presence of the heparin co-factor.

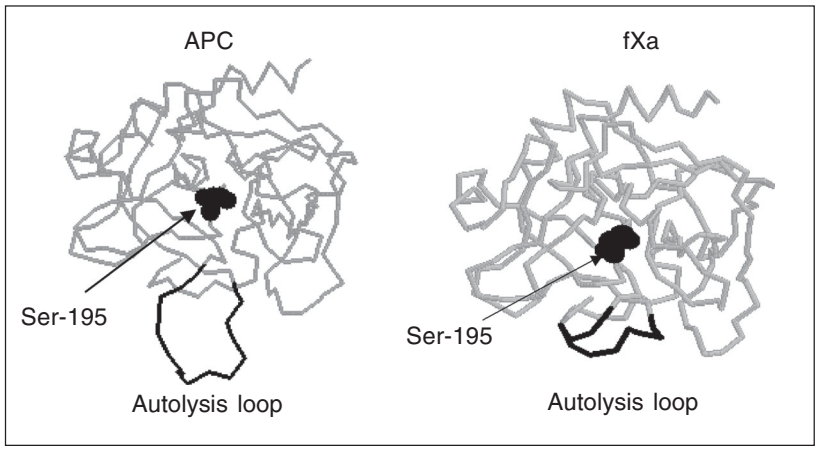

Figure 3. Crystal structures of the catalytic domains of activated protein C (APC) and factor Xa (fXa). Backbone representation of residues 143-154 (black) of the autolysis loop of both APC (left) and fXa (right). The catalytic Ser-195 (black) is shown by an arrow in each protease. The coordinates Protein Data Bank codes 1AUT and 1FJS for APC (27) and fXa (28), respectively, were used to prepare the figures.

The autolysis loop of APC has five basic residues and previous mutagenesis of these residues resulted in impairments in the anticoagulant activity of mutants in both fVa inactivation and plasma-based clotting assays (29), suggesting that the basic residues of this loop are required for the recognition and subsequent proteolytic degradation of the substrate. The observation here that the activity of the APC-fX$^{143\text{-}154}$ mutant containing the autolysis loop of fXa inactivated fVa with an improved activity compared to wild-type APC may seem somewhat contradictory. However, the observation that the activity of the APC-fX$^{143\text{-}154/3A}$ mutant toward fVa was impaired compared to the APC-fX$^{143\text{-}154}$ mutant suggests that the basic residues of the autolysis loop of fXa in the mutant APC functionally substitute for the basic residues of the APC autolysis loop in the mutant protease. Thus, similar to the case with AT, both the length and specific interaction of basic residues of the APC autolysis loop with the substrate fVa contribute to the anticoagulant function of APC.

Despite an improvement in the anti-fVa activity for APC-fX$^{143\text{-}154}$ and normal activities for APC-fX$^{143\text{-}154/3A}$ and APC-Tryp$^{143\text{-}154}$ mutants in the purified system, the anticoagulant activities of these mutants were either impaired or nearly abolished in the plasma-based aPTT assay. This observation raised the possibility that the mutant proteases react with other plasma serpin inhibitors. Indeed the evaluation of the kinetics of inhibition of these mutants after their incubation with normal plasma revealed that the half-life of mutant APC derivatives was considerably shortened. Further studies in the purified system suggested that the reactivity of both APC-fX$^{143\text{-}154/3A}$ and APC-Tryp$^{143\text{-}154}$ mutants with two specific serpin inhibitors of APC, PCI and α_1-antitrypsin, was improved by an order of magnitude. Taken together, these results suggest that a longer autolysis loop in APC plays a regulatory role in the function of APC: it renders the protease resistant to inhibition by plasma serpins at the expense of lowering the catalytic efficiency of APC toward the procoagulant cofactor, fVa. In the latter case, however, we previously demonstrated that the inhibitory effect of a longer autolysis loop in APC can be overcome by the co-factor function of protein S, since both wild-type and APC-fX$^{143\text{-}154}$ inactivated fVa with comparable activities in the presence of protein S (20).

Finally, the observation that all three protein C mutants under study were activated by thrombin at a markedly improved rate in the absence of TM suggests that the unique structural features of the protein C autolysis loop also contribute to the regulation of zymogen activation by thrombin. Thrombin is a poor activator of protein C in the absence of TM under physiological concentrations of calcium (21). The results presented above suggest that the autolysis loop residues of protein C are involved in trapping the zymogen in a conformation that impedes its optimal recognition by thrombin in the absence of TM. Thus, the residues of autolysis loop play critical roles in both zymogenic and enzymatic properties of the protein C anticoagulant pathway.

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