Selective Inhibition of Factor Xa in the Prothrombinase Complex by the Carboxyl-terminal Domain of Antistasin*

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Studies of antistasin, a potent factor Xa inhibitor with anticoagulant properties, were performed wherein the properties of the full-length antistasin polypeptide (ATS-119) were compared with the properties of forms of antistasin truncated at residue 116 (ATS-116) and residue 112 (ATS-112). ATS-119 was 40-fold more potent than ATS-112 in prolonging the activated partial thromboplastin time (APTT), whereas ATS-119 inhibited factor Xa 2.2-fold less avidly and about 5-fold more slowly than did ATS-112. The decreased reactivity of ATS-119 suggests that the carboxyl-terminal domain of ATS-119 stabilizes an ATS conformation with a reduced reactivity toward factor Xa. The observation that calcium ion increases the reactivity of ATS-119 but not that of ATS-112 suggests that calcium ion may disrupt interactions involving the carboxyl terminus of ATS-119. Interestingly, ATS-119 inhibited factor Xa in the prothrombinase complex 2–6-fold more potently and 2–3-fold faster than ATS-112. These differences in affinity and reactivity might well account for the greater effectiveness of ATS-119 in prolonging the APTT and suggest that the carboxyl-terminal domain of ATS-119 disrupts interactions involving phospholipid, factor Va, and prothrombin in the prothrombinase complex. The peptide RPKKKLIPRLS, corresponding to the carboxyl domain of ATS-119 prolonged the APTT and inhibited prothrombinase-catalyzed processing of prothrombin, but it failed to inhibit the catalytic activity of isolated factor Xa. Thus, this novel inhibitor appears to exert its inhibitory effects at a site removed from the active site of factor Xa.

Factor Xa (fXa),1 a serine protease that functions at the intersection of the intrinsic and extrinsic pathway for blood coagulation, activates prothrombin to thrombin. Thrombin in turn activates platelets and converts the soluble plasma protein fibrinogen to the insoluble fibrin matrix of blood clots. The central role of fXa in blood coagulation suggests that fXa inhibitors might have therapeutic utility as anticoagulants. It is important to note, however, that fXa functions in the blood coagulation cascade by inhibiting fXa and other enzymes in the blood coagulation cascade (3, 4). Other fXa inhibitors, such as tick anticoagulant peptide and antistasin (ATS) have been isolated from blood-sucking animals (5, 6). Antistasin, originally isolated from the salivary gland of the Mexican leech Haementeria officinalis (6), is a cysteine-rich polypeptide 119 amino acid residues in length containing a cationic carboxyl-terminal domain that is not part of the two internally homologous domains of antistasin (Fig. 1). Kinetic studies reveal that antistasin is a potent, slow, tight-binding inhibitor of fXa (7). The only other protease inhibited by antistasin appears to be trypsin (IC50, 5 nm) (7). Antistasin, a Kunitz-type protease inhibitor, bears a structural resemblance to the fXa substrate prothrombin, and as expected, the inhibitor undergoes fXa mediated cleavage (at Arg-34) to form a stable covalently bound enzyme-inhibitor complex (7).

Intact antistasin had been successfully expressed in the insect baculovirus host (8). The present study, wherein full-length and two truncated antistasins (from yeast and an African green monkey kidney cell line) are characterized, suggests that the carboxyl-terminal domain of antistasin, which has little influence on inhibitory potency toward isolated fXa, is important for the anticoagulant activity of antistasin. Additionally, a small peptide encompassing the carboxyl-terminal domain of antistasin that selectively inhibits fXa in the prothrombinase complex is identified.

EXPERIMENTAL PROCEDURES

Materials—The chromogenic substrates Spectrozyme Xa (methoxy-carbonyl-n-cyclohexyl-Gly-Gly-Arg-p-nitroanilide) and Chromozyme TH (tosyl-Gly-Pro-Arg-p-nitroanilide) were purchased from American Diagnostica Inc. and Sigma, respectively, and prepared as recommended. The concentrations of both substrates were determined using an ε222 nm = 8.27 × 103 M−1 cm−1 (9). The fluorogenic substrate 7-[N-(t-Boc-Ile-Glu-Gly-Arg)-amido]-4-methylcoumarin (IEGR-AMC) was purchased from Sigma, and its concentration determined using an ε295 nm = 1.72 × 104 M−1 cm−1. Thrombin chromogenic substrate, S2238 (H-O-phenylalanyl-1-pipecolyl-l-arginyl-p-nitroanilide), was purchased from Chromogenix/Pharmacia Hepar (Franklin, OH) and dissolved in water, and its concentration was determined using an ε342 nm = 1.27 × 103 M−1 cm−1. Concentrations of synthetic peptides were determined by quantitative amino acid analysis. Full-length antistasin (ATS-119) and antistasin truncated at residue 116 (ATS-116) were purified using previously described methods from insect and yeast, respectively, that were modified to express recombinant antistasin (8, 10). Antistasin truncated at residue 112 (ATS-112) was expressed in the African green monkey kidney CV-1p cell line by transfection with a pBR322-based plasmid containing human immunodeficiency virus-long terminal repeat promoter, immunoglobulin signal, antistasin cDNA, and SV40

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1 The abbreviations used are: fXa, factor Xa; ATS, antistasin; fVa, factor Va; HBS, HEPES-buffered saline; HBSP, HBS with PEG-8000; IEGR-AMC, N-tert-butyloxyacarbonyl-L-Ile-L-Glu-Gly-L-Arg-7-amido-4-methyl coumarin; PL, phospholipid; AT-III, antithrombin III; PEG, polyethylene glycol.

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Prothrombinase Inhibition by the C Terminus of Antistasin

Inhibition of fXa with Recombinant Antistasin Using a Small Substrate as a Probe—Assay solutions contained recombinant ATS (final concentration, 1 nM), calcium chloride (2 mM) and EIGR-AMC (40 μM, K_m = 750 μM) in HBSP buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, and 0.1% PEG-8000 in a PEG-20,000 precoated 96-well fluorogenic microtitre plate at room temperature. A solution of fXa (final 0.2 nM) containing 0.02 nM hirudin was mixed with the assay solution, and the hydrolysis rate was measured directly from the rate of increase of fluorescence intensity using a fluorescence plate reader (Flurostar, SLT Labinstruments, Salzburg, Austria). Excitation and emission wavelengths were set at 390 and 480 nM, respectively. Figure 1 shows the initial fluorescence, the fluorescence at time t, and the initial and final rate of change of fluorescence, respectively.

Inhibition of fXa Using Prothrombin as Substrate—Truncated forms of fXa (final concentration, 0.5–10 nM) and prothrombin (20 nM–1 μM) were added to an assay solution of fXa (0.025–2 pM), fVa (0.5–5 nM), and phospholipid vesicle (1–5 μM) with calcium chloride (2 mM) in HBSP buffer in a PEG-20,000 precoated 96-well microtitre plate at room temperature. For reactions in the absence of inhibitors, a diluted solution of fXa (final concentration, 0.02–0.05 μM) was used. The rate of thrombin generation appeared to be constant using 0.02–2 pM fXa. At various times (up to 4 h), a 20-μl aliquot of the reaction mixture was removed and quenched with 105 μl of 10 mM EDTA in Tris-buffered saline with PEG-8000. The amount of thrombin generated was determined by an activity assay using 50 μl of S2238 (250 μM) in the quenching buffer. A fit of the data to Equation 4 yielded the pseudo-first order rate constant for reaction of fXa with fXa, where V_t, V_t-1, and P_t represent the initial and final rate of thrombin generation and amount of thrombin generated at time t, respectively.

**Equations:**

\[ F_0 = F_t + V_t \times 1 + (V_t - V_{t-1})(1 - \exp(-k_{obs} \times t)) \times \exp(-k_{obs} \times t) \]

where

- \( F_0 \) is the initial fluorescence,
- \( F_t \) is the final fluorescence,
- \( V_{t-1} \) is the initial rate of fluorescence accumulation,
- \( V_t \) is the final rate of fluorescence accumulation,
- \( k_{obs} \) is the observed second order rate constant for the inhibition of fXa by ATS, and
- \( k_{obs} \) is the observed second order rate constant for the inhibition of fXa by ATS.

**PolyA** terminating signal sequences. Values of antistasin molecular weights were measured with a Finnigan LCQ mass spectrometer.

Human factor Xa (fXa) was prepared from human fX (Hematologic Technologies, Inc., Essex Junction, VT) by the method of Bock et al. (11) or purchased from Hematologic Technologies Inc. fXa concentration was determined using an ε_{280 nm} = 1.16 (mg/ml)^{-1} and a molecular weight of 46,000 (12, 13). The activated fXa was homogeneous (>90%) α-fXa as judged by sodium dodecyl sulfate electrophoresis in the Laemmli buffer system (14); the active site concentration was determined by titration with fluorescein mono-p-guanidino benzene (11) and by titration using the tick anticoagulant peptide double mutant, Y1W/D1OR (K_r = 10 μM) (15). Human fVa was purchased from Hematologic Technologies, Inc. fVa concentration was determined using an ε_{280 nm} = 1.74 (mg/ml)^{-1} and a molecular weight of 168,000 (16). Human prothrombin was isolated from citrated human plasma (17), and the concentration was determined using ε_{280 nm} = 1.38 (mg/ml)^{-1} and a molecular weight of 72,000 (18). Concentrations of recombinant truncated forms of ATS were determined by quantitative amino acid analysis and verified by titration with fXa. Small peptides were prepared by solid-phase synthesis (19) and purified by preparative high pressure liquid chromatography, and fast atom bombardment mass spectrometry. The homogeneity were confirmed by quantitative amino acid analysis, high pressure liquid chromatography, and fast atom bombardment mass spectral analysis.

**The Activated Partial Thromboplastin Time (APTT) Assay—**This assay was performed using a Medical Laboratory Automation coagulation timer (Eletrostat 9000). A 10 μl aliquot of inhibitor or buffer was added to 100 μl of freshly prepared platelet-poor human plasma in a disposable cuvette (n = 2). The resulting sample was mixed with 100 μl of Actin reagent (Baxter Diagnostics Inc., McGaw Park, IL) and 100 μl of 20 mM CaCl_2 at 37 °C according to the standard protocol provided by the reagent manufacturer. Concentrations of inhibitors that doubled clotting times (2× APTT) were reported as the final concentration in the assay mixture.

**Preparation of Phospholipid (PL) Vesicles—**Phospholipids were prepared by a modification of the procedure of Barenholz et al. (20). Synthetic 1,2-dioleoyl-phosphocholine (PC) and 1,2-dioleoyl phosphoserine (PS) (purchased from Avanti Polar Lipids, Inc., Alabaster, AL) were mixed (molar ratio, PC:PS = 75:25) and evaporated to dryness under a nitrogen stream. The resulting residue was suspended in HBSP buffer containing 50 mM HEPES, 150 mM NaCl, pH 7.4, at a phospholipid concentration of 10 mg/ml. The solution was sonicated (Braun-Sonic 2000 or Cole Palmer Ultrasonic Homogenizer) in an ice bath for 6–8 cycles of 1.5 min of sonication spaced by 1-min intervals under a continuous nitrogen stream. The sonicated solution was centrifuged (Beckman XL-90 Ultracentrifuge) in Beckman Ultra-Clear tubes for 3–4 h at 4 °C in an SW 50.1 swinging-bucket rotor, at 190,000 × g (40,000 rpm). The upper 50% of the final solution was collected, and stored under nitrogen at 4 °C. A phosphate assay (21) was used to determine the phospholipid concentration of the vesicle preparation.

**Determination of k_cat and K_m for the Action of fXa on Prothrombin—**Prothrombin was reacted with fXa (0.1 μM-2 μM), fVa (5 μM), and/or phospholipid vesicle (5 μM) with calcium chloride (2 mM) in HBSP buffer in a PEG-20,000 precoated 96-well microtitre plate for 0.5–2 h at room temperature, after which time, processing was quenched by addition of a solution containing 25 mM EDTA and 100 mM tick anticoagulant peptide. The concentration of the active thrombin produced was then determined from the rate of hydrolysis of S2238 (final concentration, 50 μM). Values of k_cat/K_m were derived from the dependence of the rate of thrombin activation on concentrations of the prothrombin and fXa.
Prothrombinase Inhibition by the C Terminus of Antistasin

Characterization of Truncated Forms of Antistasin—Full-length antistasin (ATS-119), an antistasin derivative truncated at residue 116 (ATS-116), and an antistasin derivative truncated at residue 112 (ATS-112) were purified from secreted cell-free products of insect, yeast, and African green monkey kidney cells, respectively, in which recombinant antistasin was expressed (8, 10). The molecular weights of the ATS polypeptides and/or the amino acid sequences of carboxyl-terminal tryptic peptides established the identity of each form of ATS (Table I). Data shown in Table I indicate the substantial effect of carboxyl-terminal truncation on the potency of the truncated forms of ATS in prolonging the APTT clotting time. Surprisingly, the difference in potency of the three truncated forms of ATS was not reflected in their inhibitory activity toward isolated fXa. In fact, the most truncated antistasin (ATS-112), which, relative to the full-length antistasin (ATS-119), exhibited a 40-fold reduction in potency in prolonging the APTT, bound fXa 2.2-fold more tightly and reacted with fXa 5-fold faster than did ATS-119 (Fig. 2 and Table II). Interestingly, calcium ion increased the affinity of all three forms of ATS for fXa by about 5-fold (Fig. 2 and Table II). Additionally, calcium ion increased the value of the rate constant for formation of the fXa-ATS inhibitory complex by about 3-fold for full-length ATS-119, whereas little or no change in the rate constant was observed with ATS-112.

To explore the source of the 40-fold enhanced potency of ATS-119 in prolonging the APTT, we studied the effect of truncated forms of ATS on fXa-catalyzed prothrombin processing in the presence of the other components of the prothrombinase complex. Table III illustrates the well documented increase in catalytic efficiency of fXa upon assembly of the prothrombinase complex. Thus, fVa/Ca and Ca/PL vesicles increased the rate of inhibition by both ATS-119 and ATS-112 (Table IV). Addition of fVa to fXa/Ca/PL leads to a further enhancement in inhibiting prothrombinase than in inhibiting fXa/Ca. The truncated forms ATS-116 and ATS-112 show reduced selectivity toward prothrombinase versus fXa of 6–9-fold and 1.3–1.4-fold, respectively (Tables II and IV). ATS-119 inhibits prothrombinase 2–6-fold more potently and binds to prothrombinase 2–3-fold faster than does truncated ATS-112 (Table IV).

It is interesting to note that PL selectively increases the rate of inhibition of fXa/Ca by ATS-119 so as to alter the relative rate of inhibition of fXa/Ca by ATS-119 and ATS-112 from 0.76 in the absence of PL to 2.5 in the presence of 1 μM PL (Fig. 4). Addition of fVa to fXa/PL leads to a further enhancement in the rate of inhibition by both ATS-119 and ATS-112 (Table IV). Interactions involving the carboxyl-terminal domain of ATS-119 and components of the prothrombinase complex may ac-

### Table I

| ATS variants | Carboxyl terminus | Molecular weight | 2× APTT<sup>a</sup> |
|--------------|------------------|-----------------|---------------------|
| ATS-119      | −R−P−K−R−L−I−P−R−L−S−COOH | 13,328.42<sup>b</sup> | 6                   |
| ATS-116      | −R−P−K−R−L−I−P−COOH    | 13,020.01<sup>c</sup> | 19                  |
| ATS-112      | −R−P−K−R−COOH         | 12,520.41<sup>d</sup> | 240                 |

<sup>a</sup> Values are the final concentrations for doubling APTT clotting times using human platelet poor plasma.  
<sup>b</sup> Calculated value is based on primary structure of ATS 1–112 residues, with all cysteine residues oxidized to disulfide bonds and the amino terminus as pyroglutamate residue.  
<sup>c</sup> Calculated value is based on primary structure of recombinant ATS 1–116 residues, with all cysteine residues oxidized to disulfide bonds, methionine residues oxidized to methionine sulfoxide, and the N terminus as pyroglutamate residue (9).  
<sup>d</sup> Calculated value is based on primary structure of recombinant ATS 1–119 residues, with all cysteine residues oxidized to disulfide bonds and the amino terminus as pyroglutamate residue.

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![Graph A](image1.png)  
![Graph B](image2.png)  

**Fig. 2.** Inhibition of fXa-catalyzed (A) and fXa + calcium ion-catalyzed (B) hydrolysis of the fluorogenic substrate IEGR-AMC (40 μM). The reaction mixture contained 0.2 nM fXa and 1 nM ATS in HBSP buffer at room temperature. Reaction mixture shown in panel B also contained 2 mM CaCl<sub>2</sub>. ○, no ATS; ●, ATS-119; ○, ATS-116; △, ATS-112.
Prothrombinase Inhibition by the C Terminus of Antistasin

Table II
Effects of calcium ion on rate and equilibrium constants for inhibition of factor Xa by truncated forms of antistasin

|        | \( K_m \) | \( k_{cat} \) | \( k_{cat}/K_m \) | Relative activity |
|--------|-----------|---------------|-------------------|-----------------|
| \( \text{ATS-119} \) | 0.08 | 8.2 | 1 | 1 |
| \( \text{ATS-116} \) | 0.14 | 14.0 | 1.7 | 1.7 |
| \( \text{ATS-112} \) | 0.04 | 0.42 | 1.0 | 1.0 |

Table III
Kinetic parameters for prothrombinase processing associated with assembly of the human prothrombinase complex

| \( \text{factor Xa} \) | \( K_m \) | \( k_{cat} \) | \( k_{cat}/K_m \) | Relative activity |
|----------------------|-----------|---------------|-------------------|-----------------|
| \( \text{ATS-119} \) | 0.01 | 1.0 | 1.0 | 1.0 |
| \( \text{ATS-116} \) | 0.02 | 2.0 | 2.0 | 2.0 |
| \( \text{ATS-112} \) | 0.03 | 3.0 | 3.0 | 3.0 |

Fig. 3. Interaction of ATS with \( \text{fXa} \) in the prothrombinase complex using prothrombin as the substrate. The assay contained 0.2 \( \text{nm fXa} \), 2 \( \text{nm CaCl}_2 \), 20 \( \text{nm prothrombin} \) in HBSP buffer at room temperature. Aliquots were removed at the plotted times, and the concentration of activated thrombin was determined using the chromogenic substrate S2238. ●, no ATS; ⊙, ATS-119; ○, ATS-116; △, ATS-112.

Fig. 4. ATS-mediated inhibition of the \( \text{fXa/Ca-catalyzed processing of prothrombin in the presence of PL} \). The reaction mixture contained 2 \( \text{nm fXa} \), 10 \( \text{nm ATS}, 1 \mu \text{M PL}, 2 \text{mm CaCl}_2 \), and 20 \( \text{nm prothrombin} \) in HBSP buffer at room temperature. Aliquots were removed at the plotted times, and the concentration of activated thrombin was determined using the chromogenic substrate S2238. ●, ATS-119; ⊙, ATS-112.

Table IV
Effect of phospholipid and \( \text{fXa} \) on rate and equilibrium constants for inhibition by truncated forms of antistasin prothrombinase-catalyzed processing of prothrombin

|        | \( 1 \mu \text{M PL} \) | \( 5 \mu \text{M PL} \) |
|--------|------------------------|------------------------|
| \( K_m \) | \( k_{cat} \) | \( k_{cat}/K_m \) | \( K_m \) | \( k_{cat} \) | \( k_{cat}/K_m \) |
| \( \text{ATS-119} \) | 8 ± 2 | 4.5 ± 1.5 | 13 ± 4 | 2.5 ± 0.5 |
| \( \text{ATS-116} \) | 17 ± 4 | 2.8 ± 0.5 | 14 ± 5 | 1.7 ± 0.3 |
| \( \text{ATS-112} \) | 33 ± 8 | 2.2 ± 0.7 | 20 ± 10 | 1.4 ± 0.2 |

Parent dissociation constants for inhibitors should be reduced by a factor of \( 1 + |S|/K_m \), where \( |S| \) represents the substrate concentration. Thus, at 0.2 and 1 \( \mu \text{M prothrombin} \), we should observe 2-fold and 6-fold decreases, respectively, in the apparent value of \( K_m \) for inhibition of prothrombinase by antistasin.

In the presence of 5 \( \mu \text{M PL} \) and 5 \( \mu \text{M fXa} \), 8- and 29-fold reductions of inhibitory potency were actually observed for ATS-119 (Table V). Similar effects were observed for ATS-116 (8.5- and 42-fold, respectively) and ATS-112 (19- and 52-fold, respectively). With a lower concentration of PL (1 \( \mu \text{M} \)), prothrombin concentration-dependent decreases of inhibitory potency were somewhat less pronounced (Table V).

Inhibitory Effect of Peptides Derived from the Carboxyl Terminus of Antistasin—To characterize further the anticoagulant effect of the carboxyl terminus of antistasin, the peptides ATS(113–119), ATS(109–119), and ATS(109–112) composed of the indicated residues in antistasin were studied. Additionally, the peptide ATS(119–110), a peptide with the reversed order sequence of ATS(109–119), was studied. ATS(109–112) doubled the APTT and \( \text{fXa} \)-induced clotting time at concentrations of 74 and 120 \( \mu \text{M} \). The shorter peptide ATS(113–119) containing residues truncated in ATS-112, the highly cationic tetrapeptide ATS(109–112), and ATS(119–110), the inverted peptide, did not prolong the clotting time either in the APTT assay at 400 \( \mu \text{M} \) or the \( \text{fXa} \) clotting assay at 500 \( \mu \text{M} \). None of the four peptides inhibited \( \text{fXa} \)-mediated hydrolysis of a small substrate.
at the highest peptide concentration studied (400 nM). ATS(109–119) did, however, inhibit the activation of prothrombin by the prothrombinase complex (Table V). As indicated by the data in Table VI, the inhibitory potency of ATS(109–119) appears to be a complex function of the concentration of fVa and PL wherein maximum inhibition (IC_{50} ~ 27 nM) is observed at approximately 0.1 nM fVa and 0.5 μM PL. Although ATS(109–119) did not inhibit FXa/Ca in the absence of both fVa and PL (Table VI), inhibition of FXa/Ca-catalyzed prothrombin processing by ATS(109–119) does not require assembly of the entire prothrombin complex. As documented in Table VI, ATS(109–119) inhibits the catalytic activity of FXa/Ca in the presence of fVa in the absence of PL and in the presence of PL in the absence of fVa. In contrast to ATS(109–119), none of the other peptides inhibit activation of prothrombin by prothrombinase at the highest concentration tested (400 nM). Finally, 200 μM ATS(109–119) does not inhibit thrombin-catalyzed fibrinopeptide A release from fibrinogen (hence K_{1} > 1 mM), although thrombin slowly (k_{cat}/K_{m} = 1.8 × 10^{3} M^{-1} s^{-1}) cleaves ATS(109–119) at the Arg-117-Leu-118 peptide bond in ATS(109–119).

**DISCUSSION**

The ability of 6 nM ATS-119 to prolong the APTT 2-fold is impressive when one considers that 19 nM hirudin is required to double the APTT with human plasma and that the IC_{50} value of 6 nM ATS-119 to prolong the APTT 2-fold is impressive when one considers that 19 nM hirudin is required to double the APTT with human plasma and that the IC_{50} value of 6 nM ATS-119 to prolong the APTT 2-fold is impressive when one considers that 19 nM hirudin is required to double the APTT with human plasma.

The observation that truncated ATS-112 reacts with isolated FXa faster than does full-length ATS-119 suggests the possibility that the carboxyl-terminal domain of ATS-119 may be stabilizing an antistasin conformation that is unfavorable for binding to FXa. The observation that calcium ion increases the rate of reaction of full-length ATS-119 with isolated FXa but not that of truncated ATS-112 is consistent with the possibility that the carboxyl-terminal domain of ATS-119 may be interacting with certain internal anionic carboxylate groups. Complexation of such carboxylate groups with calcium ion could destabilize their putative interaction with the carboxyl-terminal domain of antistasin and thereby favor an antistasin conformation that reacts more rapidly with isolated FXa. The 5-fold increase in the inhibitory potency toward isolated FXa exhibited by both the full-length and truncated forms of antistasin in the presence of calcium ion may well reflect an effect of calcium ion on the conformation of FXa.

The observation that ATS-119 inhibits prothrombinase more potently and more rapidly than does ATS-112 in a phospholipid and fVa-dependent fashion is consistent with the view that the carboxyl-terminal domain of antistasin interacts with these components of the prothrombinase complex. Thus, in addition to interacting with the active site of FXa, ATS-119 may be disrupting interactions involving FXa, fVa, PL, and prothrombin in the prothrombinase complex. Interestingly, addition of PL and fVa to FXa/Ca increases both affinity for full-length ATS and prothrombin processing activity, albeit fVa and PL enhance prothrombin processing activity to a much greater extent than affinity for ATS. It is tempting to speculate that interactions of ATS with prothrombinase may resemble to some extent those of prothrombin. With regard to this possibility, it is interesting to note that a sequence homology between the carboxyl-terminal domain of ATS-119 and residues 52–62 of prothrombin.

The observation that the antagonist effect of prothrombin on antistasin inhibition was greater than that expected for a competitive antagonist suggests the possibility that at high concentrations, more than one molecule of prothrombin can interact with each prothrombinase complex. Because each prothrombinase complex contains several molecules of PL, the excess prothrombin may bind to and thereby reduce the fraction of PL that is free to interact with ATS in the prothrombinase complex. Further studies of the complex equilibria involving ATS, prothrombin, and the components of the prothrombin complex will be required to properly characterize the antagonism of ATS by prothrombin.

The observation that the peptide ATS(109–119), RP-KRRKLIPLRLS, inhibits prothrombin but not isolated FXa supports the contention that the carboxyl-terminal domain of antistasin does not interact with the active site of FXa, but

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**TABLE V**

The effect of prothrombin on the inhibition of prothrombinase by truncated forms of antistasin

| [II]   | 1 μM PL | 5 μM PL |
|--------|---------|---------|
|        | ATS-119 | ATS-116 | ATS-112 | ATS-119 | ATS-116 | ATS-112 |
| 20 nM  | 6 ± 2   | 12 ± 3  | 39 ± 9  | 9 ± 2   | 13 ± 3  | 33 ± 10 |
| 200 nM | 26 ± 4  | 73 ± 10 | 210 ± 20| 76 ± 10 | 110 ± 20| 640 ± 50|
| 1000 nM| 65 ± 10 | 190 ± 20| 780 ± 80| 260 ± 20| 540 ± 40| 1700 ± 300|

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**TABLE VI**

Inhibition of prothrombinase-catalyzed prothrombin processing by ATS(109–119)

| Factor Va | 0 μM | 0.5 μM | 1 μM | 2 μM | 5 μM |
|-----------|------|--------|------|------|------|
| 0 nM      | >1000| 330    | 480  | >1000| >1000|
| 0.1 nM    | 310  | 27     | 41   | 400  | >1000|
| 0.5 nM    | 300  | 35     | 50   | 1000 | >1000|
| 5 nM      | >1000| 100    | 370  | >1000| >1000|

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**FIG. 5.** Sequence homology of the carboxyl-terminal domain of ATS-119 and residues 52–62 of prothrombin.
rather antagonizes interactions between fXa and its cofactors that give rise to the 10^{7}-fold increase in catalytic efficiency of fXa in prothrombinase. The observation that the peptide ATS(119–109) with the inverted sequence of ATS(109–119) does not inhibit prothrombinase or prolong clotting times makes it difficult to ascribe the inhibition of prothrombinase observed with ATS(109–119) to nonspecific charge-charge interactions. Thus, PRKRLIPRLS exemplifies a new class of synthetic anticoagulants that selectively inhibits the catalytic activity of prothrombinase but not that of fXa.

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