Variegin, a Novel Fast and Tight Binding Thrombin Inhibitor from the Tropical Bont Tick*§

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Tick saliva contains potent antihemostatic molecules that help ticks obtain their enormous blood meal during prolonged feeding. We isolated thrombin inhibitors present in the salivary gland extract from partially fed female Amblyomma variegatum, the tropical bont tick, and characterized the most potent, variegin, one of the smallest (32 residues) thrombin inhibitors found in nature. Full-length variegin and two truncated variants were chemically synthesized. Despite its small size and flexible structure, variegin binds thrombin with strong affinity (Ki ~ 10.4 pM) and high specificity. Results using the truncated variants indicated that the seven residues at the N terminus affected the binding kinetics; when removed, the binding characteristics changed from fast to slow. Further, the thrombin active site binding moiety of variegin is in the region of residues 8–14, and the exosite-I binding moiety is within residues 15–32. Our results show that variegin is structurally and functionally similar to the rationally designed thrombin inhibitor, hirulog. However, compared with hirulog, variegin is a more potent inhibitor, and its inhibitory activity is largely retained after cleavage by thrombin.

Blood coagulation is part of the physiological response to vascular injury, in which circulating zymogens of serine proteases are sequentially activated by limited proteolysis leading to the formation of a fibrin clot. Within this network of reactions, thrombin plays a central role in maintaining the integrity of hemostasis. Thrombin interacts with most of the zymogens and their cofactors, playing multiple procoagulant and anticoagulant roles in blood coagulation (1, 2). As a procoagulant protease, the first traces of thrombin generated in the initiation phase activate factor V (FV) and factor VIII (FVIII) to provide positive feedback leading to the thrombin burst. Thrombin can also activate factor XI, triggering the intrinsic pathway. Thrombin cleaves fibrinogen to fibrin, forming insoluble clots. Fibrin polymers are further strengthened and stabilized through covalent cross-linking driven by thrombin-activated factor XIII. Thrombin also contributes to the generation of a platelet plug, possibly through two mechanisms: (a) it activates platelets by interacting with protease-activated receptors and glycoprotein V, and (b) it prevents destabilization of the platelet plug, by inactivating ADAMTS13, a disintegrin and metalloprotease with a thrombospondin type 1 motif, that cleaves von Willebrand factor. As an anticoagulant protease, thrombin activates protein C in the presence of the cofactor thrombomodulin. Activated protein C (APC) inactivates factor Va and factor VIIIa (FVIIIa), down-regulating the generation of thrombin (1–5).

Thromboembolic disorders are major causes of mortality and morbidity (6). Anticoagulants are pivotal in the prophylaxis and treatment of these disorders. Although heparin and coumarin derivatives (vitamin K antagonists) are the cornerstone of anticoagulation therapy, both classes of drug have well documented limitations, such as a narrow therapeutic window and highly variable dose response. These limitations drive the continual and intense effort to develop new anticoagulants, mainly targeting specific coagulation factors (7). Thrombin represents an ideal target owing to its central role in the coagulation cascade (6, 8). Some examples of direct thrombin inhibitors include hirudin, hirulog (or bivalirudin), and agratroban (7–9). Hirudin is a 65-amino acid protein isolated from the salivary gland of the medicinal leech, Hirudo medicinalis (7, 8, 10). It has a globular N-terminal domain and an acidic C-terminal tail. This C-terminal tail interacts with thrombin exosite-I through electrostatic and hydrophobic interactions. The N-terminal domain binds to an apolar site near the active site of thrombin, obstructing its accessibility (11–13). Hirulog (bivalirudin), a 20-mer polypeptide, is a product of rational design by grafting the hirudin C-terminal tail to an active site binding moiety D-Phe-Pro-Arg-Pro using four Gly residues as spacer (14, 15).
Unlike hirudin and bivalirudin, which are bivalent inhibitors (bind to two distinct sites), argatroban is a univalent inhibitor and binds only to the active site (8).

Over the last few hundred million years, hematophagous arthropods have evolved a rich reservoir of inhibitors for blood coagulation proteases (16, 17). The success of hirudin and bivalirudin demonstrated the feasibility of identifying and developing such anticoagulants. In our search for novel anticoagulants (18–20), we examined the salivary gland extract (SGE) of the tropical bont tick, *Amblyomma variegatum* (Acari: Ixodidae). Here we describe the identification and isolation of a new thrombin inhibitor, variegin. We examined its inhibitory activity, specificity, kinetics, structure-function relationships, and cleavage by thrombin. This 32-residue polypeptide is a potent and specific thrombin inhibitor. The results indicate that its N terminus dictates fast binding kinetics, while central residues bind to the active site and the C terminus binds to exosite-I.

**Experimental Procedures**

**Materials**—Human citrated plasma was provided by the Dept. of Hematology and Transfusionology of the Slovak Institute of Cardiovascular Diseases. Thromboclotin reagent was from Dade AG (Düdingen, Switzerland). Thromboplastin IS reagent and Actin FS Activated PTT reagent were from Dade International Inc. (Miami, FL). 9-Fluorenylmethoxy carbonyl (Fmoc)-l-amino acids, Fmoc-PEG-PS support resin, *N*,*N*-dimethylformamide, 20% v/v piperidine in *N*,*N*-dimethylformamide, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and *N*,*N*-disopropylethylamine (DIPEA) were from Applied Biosystems (Foster City, CA). Trifluoroacetic acid, acetonitrile, 1,2-ethanedi.thiol, thiaoanisole, bovine chymotrypsin, and bovine serum albumin were from Sigma-Aldrich. Human fibrinogen, factor XIIa (FXIIa), tissue plasminogen activator (TPA), urokinase, kallikrein, and bovine trypsin were from Merck Chemicals Ltd. (Nottingham, UK). Human factor IXa (FIXa), factor Xa (FXa), factor Xla (FXla), APC, and plasmin were from Hematologic Technologies, Inc. (Essex Junction, VT). Human factor VIIa (FVIIa) and recombinant α-thrombin were gifts from the Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN) (21, 22). Chromogenic substrates S2222, S2238, S2251, S2288, S2302, S2366, S2444, S2586, and S2765 were from Chromogenix (Milano, Italy). Spectrozyme® FIXa was from American Diagnostica Inc. (Stamford, CT). All other chemicals and reagents used were of analytical grade.

**Salivary Gland Extracts and Estimation of Protein Concentrations**—The extraction procedure of *A. variegatum* SGE and estimation of protein concentrations during fractionation were described previously (23). Briefly, adult female ticks that had been feeding on laboratory rabbits for 9 days (partially fed) were removed and mounted in Petri dishes dorsal side down, and their salivary glands were dissected out into 150 mM NaCl and stored at −70 °C. Prior to use, the frozen salivary glands were incubated at ~90 °C for 5 min, homogenized, and centrifuged at 11,000 × g for 15 min. The supernatant fluids were collected, and the pellets were resuspended in 150 mM NaCl and recentrifuged. Pooled supernatant fluids represented crude SGE. Protein concentration was estimated by the method of Bradford (24).

**Purification of Variegin Isoforms**—Variegin was purified by a three-step reversed-phase HPLC procedure with a Beckman Instruments 126/168 DAD HPLC system (Fullerton, CA). In the first step (Fig. 1A) SGE was loaded onto a Vydac C-4 (5 μm, 250 × 4.6 mm) column (Grace Vydac, Hesperia, CA). Pooled fractions that contained the strongest anticoagulant activity (Fig. 1A, fraction AV-III) were subjected to a second step (Fig. 1B) using a Beckman Ultrasphere C-18 (5 μm, 250 × 4.6 mm) column. Lastly, individual fractions were further purified using a Vydac C-18 (5 μm, 250 × 4.6 mm) column to obtain three fractions of potent antithrombin activity: AV 3/5, AV 5/5, and AV 6/5 (Fig. 1, C and D). The major component in the AV 6/5 fraction was named n-variegin (“n” denotes the native peptide).

**Coagulation Assays**—Thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) assays were used for the initial screens of anticoagulant activities in SGE and fractions. Citrated human plasma (50 μl) was preincubated with a maximum of 5 μl of the SGE or the same volume of 150 mM NaCl (control) at 37 °C for 1 min. After adding the corresponding reagents (TT: 50 μl of Thromboclotin reagent; PT: 100 μl of Thromboplastin IS reagent; APTT: 50 μl of Actin FS Activated PTT added for 3 min and reaction started with 50 μl of 20 mM CaCl2), times required for the formation of fibrin clots were determined visually using a stopwatch.

The activities of crude SGE and the three fractions (AV 3/5, AV 5/5, and AV 6/5) were verified at the Oxford Hemophilia Centre of Churchill Hospital (Oxford, UK). TT, PT, and APTT were performed using an MDA-180 analyzer (Organon Teknika Ltd., Cambridge, UK). Ten microliters of SGE (1 μg/μl) or diluted fractions containing AV 3/5, AV 5/5, and AV 6/5 (concentrations of undiluted fractions were: 0.07 μg/μl, 0.05 μg/μl, and 0.02 μg/μl, respectively) were added to 290 μl of platelet poor plasma, mixed, and incubated for 5 min at 37 °C. The activities were also verified using a Thromboelastograph (TEG) Analyzer (Hemoscope Inc., Skokie, IL). 5 μl of samples was added to 335 μl of citrated whole blood, incubated for 5 min, and the sample was run on the TEG following recalcification.

**Protein Sequence Analysis**—The molecular weights of proteins present in AV 3/5, AV 5/5, and AV 6/5 were determined by Eurosequence (Groningen, the Netherlands) using a BIFLEX (Bruker-Franzen, Bremen, Germany) matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF) mass spectrometer equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. Partial amino acid sequences were determined by N-terminal Edman degradation using an automated sequencer (Model 494, Applied Biosystems). The complete sequence for AV 6/5 was determined by MALDI-MS analysis.

**Peptide Synthesis and Purification**—Three peptides (s-variegin, EP25, and AP18) were synthesized using solid-phase peptide synthesis methods on an Applied Biosystems Pioneer Model 433A Peptide Synthesizer. Fmoc groups of amino acids were removed by 20% v/v piperidine in *N*,*N*-dimethylformamide and coupled using HATU/DIPEA in situ neutralization chemistry. All peptides were synthesized on preloaded polyethylene glycol polystyrene (PEG-PS) resins. Cleavage by a mixture of trifluoroacetic acid/1,2-ethanedi.thiol/thiaoanisole/water released peptide acids (-COOH). Synthetic peptides were purified by reversed
phase-HPLC on an ÄKTA™ purifier (GE Healthcare, Uppsala, Sweden) with a SunFire™ C18 (5 μm, 250 mm × 10 mm, Waters, Milford, MA) column. The purity and mass of all peptides were determined by electrospray ionization mass spectrometry using an API 300 liquid chromatography tandem mass spectrometry system (PerkinElmer Life Sciences Sciex, Selton, CT).

CD Spectroscopy—Far-UV CD spectra (260–190 nm) of n-variegin, s-variegin, EP25, and AP18 dissolved in 10 mM sodium phosphate buffer (pH 7.4) were recorded using a Jasco J-810 spectropolarimeter (Easton, MD). All measurements were carried out at room temperature using 0.1-cm path length cuvettes with a scan speed of 50 nm/min, a resolution of 0.2 nm, and a bandwidth of 2 nm.

Inhibition of Thrombin Amidolytic Activity—All assays for thrombin amidolytic activity on S2238 were performed in 96-well microtiter plates in 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl and 1 mg/ml bovine serum albumin at room temperature. Typically, 100 μl of peptide and 100 μl of thrombin were preincubated for different durations before the addition of 100 μl of S2238. The rates of formation of colored product p-nitroaniline were followed at 405 nm for 10 min with an enzyme-linked immunosorbent assay plate reader. Percentage inhibition was calculated by taking the rate of increase in absorbance in the absence of inhibitor as 0%. Dose-response curves were fitted using Origin software (MicroCal, Northampton, MA) to calculate IC_{50} values and Hill coefficients.

Determination of the Inhibitory Constant K_{i}—The inhibitory constant, K_{i}, was determined using S2238 as substrate. When an enzyme is inhibited by an equimolar concentration of inhibitor, the binding of inhibitor to enzyme causes a significant depletion in the concentration of free inhibitors. This tight-binding inhibition is described by the following equation (25),

\[ V_e = \frac{V_o}{2E_i} \left[ \left( K_i' + l_i - E_i \right)^2 + 4K_i'E_i \right]^{1/2} - \left( K_i' + l_i - E_i \right) \]  

(Eq. 1)

where \( V_e \) is steady-state velocity in the presence of inhibitor, \( V_o \) is velocity observed in the absence of inhibitor, \( E_i \) is total enzyme concentration, \( l_i \) is total inhibitor concentration, and \( K_i' \) is apparent inhibitory constant. For competitive inhibition, \( K_i \) is related to \( K_i' \) by Equation 2,

\[ K_i' = K_i(1 + S/K_m) \]  

(Eq. 2)

where \( K_i' \) increases linearly with \( S, K_i \) is the inhibitory constant, \( S \) is the concentration of substrate, and \( K_m \) is the Michaelis-Menten constant for S2238 (determined to be 3.25 ± 0.56 μM, supplemental Fig. S1, similar to reported values (25, 26)). Both n-variegin and s-variegin were found to be tight binding inhibitors. The data were fitted to these equations using Origin software.

If the rate of interaction of the inhibitor with the enzyme is slow so that the inhibited steady-state velocity is slowly achieved, the progress curve of product formation of this slow binding inhibition is described by Equation 3 (27),

\[ P = V_i t + \left( V_i - V_o \right) \left( 1 - e^{-kt} \right)/k + P_o \]  

(Eq. 3)

where \( P \) is the amount of product formed, \( P_o \) is the initial amount of product, \( V_i \) is final steady-state velocity, \( V_o \) is initial velocity, \( t \) is time, and \( k \) is the apparent first-order rate constant.

There are two possible minimum kinetic mechanisms to describe such slow binding reactions (27, 28) (Schemes 1 and 2). The first is shown in Scheme 1,

\[ E + I \rightleftharpoons EI \]  

Scheme 1

where \( E \) is enzyme, \( I \) is inhibitor, \( EI \) is the stable enzyme-inhibitor complex, \( K_1 \) is the association rate constant, and \( K_2 \) is the dissociation rate constant. In this scheme, slow binding is mainly due to the slow \( K_1 \). The apparent first-order rate constant \( k \) will increase linearly with inhibitor concentration. Alternatively, the second mechanism is shown in Scheme 2,

\[ E + I \rightleftharpoons EI \rightleftharpoons EI* \]  

Scheme 2

where \( EI \) is the initial collision complex, \( K_3 \) is the forward isomerization rate, and \( K_4 \) is the reverse isomerization rate. In this scheme, binding involves rapid formation of an initial collision complex (\( EI \)) that subsequently undergoes slow isomerization to the final enzyme-inhibitor complex (\( EI^* \)). \( k \) increases hyperbolically with inhibitor concentrations. Dissociation constant of \( EI^* \) (denoted \( K_i' \)) can be calculated from Equation 4,

\[ k = K_4 + K_{34}/(I_i + K_i'(1 + S/K_m)) \]  

(Eq. 4)

The overall inhibitory constant \( K_i \) can be calculated from Equation 5.

\[ K_i = K_i'[K_i/(K_3 + K_4)] \]  

(Eq. 5)

EP25 was found to be a slow binding inhibitor following the Scheme 2 mechanism. The data were fitted to these equations using Origin software.

Serine Protease Specificity—The selectivity profile of variegin was examined against 13 serine proteases: fibrinolytic serine proteases (plasmin, TPA, and urokinase), anticoagulant serine protease APC, procoagulant serine proteases (FXIIa, FXIa, FXa, FVIIa, kallikrein, and thrombin), and classic serine proteases (chymotrypsin and trypsin). Effects of s-variegin on these serine proteases were determined by inhibition of their amideolytic activities assayed using specific chromogenic substrates.

Fibrinogen Clotting Time—The abilities of s-variegin, EP25, and AP18 to prolong fibrinogen clotting time were tested using a BBL fibrometer (BD Biosciences, Franklin Lakes, NJ). 200 μl of fibrinogen (final concentration, 3 mg/ml) were incubated with 100 μl of peptides (various concentrations) at 37 °C. Clotting of fibrinogen was initiated by the addition of 100 μl of thrombin (final concentration, 20 nM). All reagents and samples were dissolved in 50 mM Tris buffer (pH 7.4) containing 100 mM NaCl.

Cleavage of S-variegin by Thrombin—S-variegin and EP25 (final concentrations: 150 μM) were incubated with thrombin (final concentration: 5 μM) at both room temperature and
37 °C. After various incubation times, the reactions were quenched with 0.1% trifluoroacetic acid buffer (pH 1.8) and loaded onto a SunFire™ C18 column attached to an ÄKTATM purifier. New peaks other than those present in the chromatogram of 0-min incubation were identified as cleavage products and subjected to electrospray ionization mass spectrometry to verify their masses. The peaks were integrated to calculate the area under the peaks and relative percentage of each peak.

RESULTS

Purification of Variegin Isoforms—Crude SGE of *A. variegatum* exhibited potent anticoagulant activity in all three coagulation assays (PT, APTT, and TT) (Table 1). Potency was in the order TT > APTT > PT, indicating that SGE is a promising source of potent thrombin inhibitor(s). To purify the one or more inhibitors, SGE was fractionated by reversed phase-HPLC (Fig. 1A). After the first step of purification, the most potent anticoagulant fraction (AV-III) was subjected to a second purification step (Fig. 1B). The resulting fractions were screened for antithrombin activity in coagulation and chromogenic sub-

![Figure 1](image_url)

**TABLE 1**

Anticoagulation activities of *A. variegatum* SGE (females fed for 9 days)

Results show the mean of duplicate values. In controls 150 mM NaCl was substituted for SGE.

| PT | APTT | TT |
|----|------|----|
| Control | 15 | 28 | 17 |

| SGE protein (µg) | 0.025 | 50 |
| 0.050 | 105 |
| 0.100 | >180 |
| 0.250 | 15 | 28 |
| 0.500 | 19 | 38 |
| 1.000 | 22 | 45 |
| 2.500 | 40 | >180 |
| 5.000 | >180 |

**FIGURE 1.** Purification of variegin isoforms by reverse phase HPLC. A, in the first step, SGE was fractionated using a gradient of 10–100% acetonitrile over 90 min. Protein concentrations in pooled fractions of AV-I to AV-VIII ranged from 0.08 (AV-I) to 1.39 g/50 l (AV-IV). For TT assays (control clotting time 19 s): NC, no clot after adding 0.01 g of protein/50 l of plasma; ***, prolonged clotting of >1 min after adding <0.01 µg of protein/50 µl of plasma; **, prolonged clotting of >40s (<1 min) after adding <0.01 µg of protein/50 µl of plasma; *, any delay (<40 s) in clotting in comparison with control. For APTT assays (control clotting time = 40 s): NC, no clot after adding <0.01 µg of protein/50 µl of plasma; ****, prolonged clotting of >1 min after adding <0.01 µg protein; ***, prolonged clotting of >1 min after adding <1.0 µg of protein/50 µl of plasma; *, any delay (<1 min) in clotting in comparison with control. For PT assays (control clotting time = 15 s): C, prolonged clotting of >1 min after adding 0.5 µg of protein/50 µl of plasma; O, any delay (<1 min) in clotting in comparison with control. B, fraction AV-III was subjected to a second purification step using a gradient of 10–40% acetonitrile over 60 min. Protein concentrations in fractions ranged from 0.05 to 0.17 g/50 l. The range of fractions with anticoagulant activities (dashed line, assayed by PT, APTT, and TT) was tested for antithrombin activity with S2238. Fractions indicated with asterisks inhibited thrombin amidolytic activity. Two fractions with the strongest activity (retention times of 23.083 and 28.933 min, indicated by arrows) were subjected to a third step of purification (gradient of 10–40% acetonitrile over 60 min) (n = 2). C, the fraction with retention time 23.083 min separated into two main peaks denoted AV 3/5 and AV 5/5. D, the fraction with retention time 28.933 has one main peak and a small “shoulder peak” and was denoted AV 6/5.
strate assays. Two fractions with the strongest activity (retention times, 23.083 and 28.933 min) were further purified in separate runs. The fraction with retention time 23.083 min was separated into two main peaks denoted AV 3/5 and AV 5/5 (Fig. 1C). The fraction with retention time 28.933 had one main peak and with a small "shoulder peak" and was denoted AV 6/5 (Fig.
Novel Class of Thrombin Inhibitors

FIGURE 3. Selectivity profile of variegin. S-variegin was screened against 13 serine proteases: fibrinolytic serine proteases (plasmin, TPA, and urokinase), anticoagulant serine protease APC, procoagulant serine proteases (FXIIa, FXIa, FXa, FVIIIa, kallikrein, and thrombin), and classic serine proteases (chymotrypsin and trypsin). The final concentrations of proteases and substrates are given in parentheses in nanomolar and millimolar, respectively: plasmin (3.61)/S2251 (1.2), TPA (36.9)/S2288 (1), urokinase (40 units/ml)/S244 (0.3), APC (2.14)/S2366 (0.67), FXIIa (20)/S2302 (1), FXIa (0.125)/S2366 (1), FXa (0.43)/S2765 (0.65), FVIIIa (333)/Spectrozyme® FXa (0.4), FVIIIa (460)/S2288 (1), kallikrein (0.93)/S2302 (1.1), α-thrombin (3.33)/S2238 (0.1), chymotrypsin (1.2)/S2586 (0.67) and trypsin (0.87)/S2222 (0.1). Thrombin was tested against three concentrations of s-variegin: stippled bar, 0.01 μM; checked bar, 0.1 μM; and light gray bar, 1 μM. For the other proteases, much higher concentrations of s-variegin were used: solid bar, 1 μM; dark gray bar, 10 μM; and open bar, 100 μM (n = 3, error bar represents ± S.D.).

The anticoagulant activities of these three fractions (AV 3/5, AV 5/5, and AV 6/5) along with crude SGE were verified by PT, APTT, TT, and TEG assays. All four assays revealed that AV 6/5 contained the most potent anticoagulant activity, followed by AV 3/5 and AV 5/5 (Table 2).

Protein Sequence Analysis—Partial sequences of all three fractions were determined by Edman degradation. For AV 6/5 the sequence and molecular weight were completed by MALDI-TOF. MALDI spectrum of AV 6/5 revealed a major m/z signal of 3769.96 Da (monoisotopic mass = 3768.96 Da) and a minor m/z signal of 3777.79 Da (monoisotopic mass = 3776.79 Da). The main component has the sequence SDQGDVAEPKMHKT(hex)APPFDFEAIPEEYLDDES, where the Thr-14 is modified by a hexose moiety. This was named “variegin” and was further characterized. The minor component (3776.79 Da) is almost identical to variegin, with Glu-31 replaced by His. Partial sequences determined by Edman degradation revealed two components in the AV 3/5 fraction (m/z 3953.54 and 3409.57 Da) and three components in AV 5/5 (m/z 3680.23, 3368.94, and 3173.62 Da). All the sequences determined are highly similar to variegin (Fig. 2A). The CD spectrum of variegin is typical of a random-coil protein (supplemental Fig. S2).

BLAST results indicate that variegin does not show similarity to any known proteins in the data base. Interestingly, its C terminus (DFEAIPEEYLDDES) is almost identical to the C terminus of hirudin (residues 55–64: DFEELPEEYL). Thus, we hypothesized that variegin C terminus plays a similar role to hirudin C terminus in binding to thrombin. However, Tyr-63 of hirudin is sulfated (29, 30), while the corresponding Tyr in variegin is not.

Inhibition of Thrombin Amidolytic Activity by N-variegin and Its Ki.—The ability of n-variegin to inhibit thrombin amidolytic activity was assayed with S2238, a small peptidyl substrate that binds only to the active site. N-variegin inhibited the amidolytic activity, and progress curves of inhibition showed that steady-state equilibrium was achieved upon mixing (Fig. 2B). Significant inhibition (~80%) was observed for equimolar concentrations of thrombin and n-variegin (3.33 nM). IC50 and Hill coefficient (mean ± S.D.) of the inhibition were 0.99 ± 0.02 nM and 1.4 ± 0.2, respectively (Fig. 2C). N-variegin is a fast and tight binding competitive inhibitor of thrombin with a Ki (mean ± S.D.) of 10.4 ± 1.4 pM (Fig. 2D).

Synthesis of S-variegin and Variants—For further characterization, three peptides were synthesized, purified, and characterized. Synthetic variegin (SDQGDVAEPKMHKTAPPFDFEAIPEEYLDDES, s-variegin) has the complete sequence of variegin, whereas EP25 (EPKMHKTAPPFDFEAIPEEYLDDES) and AP18 (APPFDFEAIPEEYLDDES) have 7 and 14 residues truncated from the N terminus. Unlike native variegin (n-variegin), Thr is not glycosylated in s-variegin and EP25. CD spectra of s-variegin, EP25, and AP18 are all similar to that of n-variegin, typical of random-coil proteins (supplemental Fig. S2).

Selectivity Profile of Variegin—To determine the specificity, s-variegin was screened against 13 serine proteases, including thrombin. Apart from thrombin, no other serine proteases showed significant inhibition (~5%) even at 1 μM s-variegin.
Inhibition of \(10\%\) was observed at much higher concentrations of s-variegin. The most susceptible proteases are plasmin, trypsin, and FXIa, which were inhibited \(20\) to \(30\%\) by \(100\) \(\mu\)M of s-variegin. In contrast, against thrombin, similar \(30\%\) inhibition was observed at a concentration at least four orders of magnitude lower (\(3.3\) nM) (Fig. 3). Therefore, s-variegin is a specific and potent thrombin inhibitor. On the other hand, negative values were observed for the inhibition of urokinase and FVIIa, suggesting some degree of activation for these enzymes. Although there seems to be a dose-dependent activation for urokinase, similar activation is less evident and inconclusive in the case of FVIIa. Accurate interpretation of the FVIIa data is difficult due to the lack of a dose-dependent relation as well as the overlapping standard deviations.

**Inhibition of Thrombin Amidolytic Activity by S-variegin, EP25, and AP18**—S-variegin is similar to n-variegin in that steady-state equilibrium of inhibition was achieved upon mixing. It was 5-fold less active than n-variegin, and \(30\%\) inhibition was observed at equimolar concentrations of thrombin and s-variegin (3.3 nM). Dose-response curves showed \(IC_{50}\) values of around 5.4 nM and Hill coefficient of around 0.9, independent of preincubation time (0 and 10 min) (Fig. 4A). Hence, s-variegin is also a fast and tight binding inhibitor of thrombin. The absence of Thr glycosylation in s-variegin might account for its weaker activity.

EP25 also inhibited amidolytic activity of thrombin. However, unlike n-variegin and s-variegin, progress curves of inhibition showed two-phase equilibria in the absence of preincubation. Steady-state equilibrium inhibition was achieved relatively slowly, after 20-min preincubation. Dose-response curves of EP25 were dependent on incubation times. Thus the deletion of seven N-terminal residues (SDQGDVA) turned the binding mode from fast to slow. However, potency of EP25 was not affected by the deletion. When the final steady-state equili-
librium was achieved (20-min preincubation) EP25 inhibited thrombin to the same extent as s-variegin. IC₅₀ values (mean ± S.D.) for EP25 and s-variegin are 5.63 ± 0.45 nM and 5.40 ± 0.95 nM, whereas the Hill coefficients (mean ± S.D.) for both inhibitors were 0.98 ± 0.11 and 0.91 ± 0.04, respectively (Fig. 4B).

In contrast, AP18 did not inhibit thrombin amidolytic activity even at 300 μM, suggesting that it did not bind to the active site. Instead, AP18 enhanced thrombin amidolytic activity slightly in a dose-dependent manner (Fig. 4C). This is consistent with the reported behavior of hirudin C terminus (29). In summary, these results suggest that the active site binding moiety on variegin resides within position 8 to 14 (EPKMHKT).

Inhibition of Thrombin Fibrinogenolytic Activity—S-variegin, EP25 and AP18 all prolonged fibrinogen clotting time in a dose-dependent manner (Fig. 4D). Fibrinogen binds to both the active site and exosite-I of thrombin (1, 2). AP18 inhibited fibrinogenolytic but not amidolytic activity of thrombin, and hence we concluded that the C terminus of variegin binds to exosite-I. This observation is consistent with that of hirudin C terminus (29, 30). The difference in activity between s-variegin and EP25 was likely to be due to the slow binding mode of EP25.

Inhibitory Constant $K_i$ of S-variegin and EP25—$K_i$ values of s-variegin and EP25 were determined using S2238 as substrate. S-variegin is a fast and tight binding inhibitor. S-variegin (0.313 nM, 0.625 nM, 1.25 nM, 2.5 nM, 5 nM, 10 nM) was mixed with different concentrations of S2238: 12.5 μM (●), 25 μM (○), 50 μM (▲), 80 μM (◇), 100 μM (●), 150 μM (+), 200 μM (×) and 300 μM (star) to determine $K_i$. Reactions were started with the addition of thrombin (1.8 nM). Data were fitted to Equation 1 (n = 3, error bar represents ± S.D.). $B$, plot of $K_i$ against substrate concentration showed a linear curve, indicating s-variegin competitively inhibited thrombin amidolytic activity on S2238. By fitting the data to Equation 2, the inhibitory constant $K_i$ (mean ± S.D.) was shown to be 146.4 ± 13.6 pm (error bar represents ± S.D.). C, although EP25 also inhibited thrombin at equimolar concentrations if preincubated with thrombin, the initial inhibition without preincubation was weak. $K_i$ of EP25 was determined without preincubation with concentrations at least 8-fold greater than thrombin. Under these assay conditions, binding of EP25 to thrombin does not result in a significant depletion of free EP25 concentration, thus the “tight binding” condition was not considered for data fitting. Progression curves of thrombin (0.9 nM) inhibition by different concentrations of EP25: 7.8 nM (●), 12.5 nM (○), 15.6 nM (▲), 25 nM (◇), 31.3 nM (●), 50 nM (▲), 62.5 nM (◇), 100 nM (●), and 125 nM (●), using S2238 (100 μM) as substrate. The progression curves are non-linear and showed two-phase equilibria typical of slow binding inhibition. Data were fitted to Equation 3 to obtain a $k$ for each concentration of EP25 used (n = 3, error bar represents ± S.D.). D, plot of the apparent first-order rate constant $k$ against EP25 concentrations is a hyperbolic curve described by Equation 4 and hence was fitted to the Equation to obtain a $K_i$ (mean ± S.D.) of 529.7 ± 76.7 pm, representing the dissociation constant of initial collision complex EI. The overall inhibitory constant $K_i$ (mean ± S.D.) was calculated from Equation 5 and was found to be 149.8 ± 30.5 pm (error bar represents ± S.D.).
binding inhibitor of thrombin. Progress curves of inhibition were fitted to Equation 3 to obtain $k$ for each concentration of EP25 (Fig. 5C). $k$, the apparent first-order rate constant for the establishment of the equilibrium between initial collision complex ($E_I$) and final stable complex ($E^*_I$), increased hyperbolically with EP25 concentration (Fig. 5D), as described by Scheme 2. Thus, the binding between EP25 and thrombin involves the isomerization of $E_I$ to $E^*_I$. The dissociation constant of $E_I$ ($K_i$, mean ± S.D., Equation 4) was 529.7 ± 76.7 pm, whereas the overall inhibitory constant $K_i$ (mean ± S.D.) (Equation 5) was 149.8 ± 30.5 pm. Thus $K_i$ of EP25 is essentially the same as $K_i$ of s-variegin (146.4 ± 13.6 pm). These results confirmed that the deletion of seven N-terminal residues did not affect potency but switched the binding mode from fast to slow.

\textit{Cleavage of S-variegin by Thrombin}—Because variegin binds to the thrombin active site, it may be cleaved by thrombin, similar to other serine protease inhibitors (31). Therefore, we examined the cleavage of s-variegin by thrombin and its effects on inhibition. Reversed phase-HPLC analysis showed that s-variegin was indeed cleaved by thrombin at room temperature and 37 °C. At 0 min of incubation only peaks corresponding to uncleaved s-variegin decreased in quantity. Cleavage is almost complete after 180 min incubation. S-variegin (150 nM) was incubated with thrombin (3.33 nM) for various times at room temperature and at various time points assayed for the ability to inhibit thrombin amidolytic activity on 100 μM S2238 (n = 2, error bar represents ±S.D.). S-variegin was present in 30-fold excess of thrombin. Cleavage of s-variegin by thrombin was analyzed with reversed phase-HPLC. Relative percentage of uncleaved s-variegin (light gray bar), cleavage product of mass 1045 Da (representing N-terminal fragment SDQGDVAEPK) (checkered bar) and cleavage product of mass 2582 Da (representing C-terminal fragment MHKTAPPFDFAIEEEYLDDES) (stippled bar) was calculated from the area under the peaks. C, s-variegin was incubated with thrombin (3.33 nM) for up to 24 h at room temperature and at various time points assayed for the ability to inhibit thrombin amidolytic activity on 100 μM S2238 (n = 2, error bar represents ±S.D.). D, similar experiments were carried out replacing s-variegin with EP25. Concentrations of s-variegin or EP25: 10 nM (■), 100 nM (gray bar) and 1000 nM (□) (n = 2, error bar represents ±S.D.). At 100 nm of s-variegin or EP25, the inhibitors were also present in 30-fold excess of thrombin, and hence were used primarily for comparison with cleavage data from HPLC analysis.
increasing incubation time (Fig. 6A). These new peaks had molecular masses of 1045 Da (SDQGDVAEPK) and 2582 Da (MHKTAPPDFEAIPEEYLDDES), respectively, and corresponded to cleavage at the Lys$^{10}$–Met$^{11}$ peptide bond. Cleavage proceeded faster at 37 °C than at room temperature (supplementary Fig. S3).

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**A**

- N-variegain
- S-variegain
- EP25
- AP18
- Hirulog-1
- Hirudin variant 1
- TTI

| N-variegain                  | S-variegain                  | EP25                | AP18                | Hirulog-1                  | Hirudin variant 1 | TTI                  |
|------------------------------|------------------------------|---------------------|---------------------|---------------------------|-------------------|---------------------|
| SDQGDVAEPFKHKTAPPDFEAIPEEYLDDES | SDQGDVAEFHMKTAPPDFEAIPEEYLDDES | EP6MHKTAPPDFEAIPEEYLDDES | APPFDFAIEEYLDDES | [PRP66GGGNGDFEIPPEYLDLDQ | [VVYTDCTESQNLCLCEGSNCQGQNCILGDGEKNCQTCRTFQPSHNDGDFEIPPEYLDQ |

**B**

(i) THROMBIN
(ii) THROMBIN
(iii) THROMBIN
(iv) THROMBIN
(v) THROMBIN
(vi) THROMBIN

K$_{i}$ = 149.8 ± 30.5 pM

**C**

(i) THROMBIN
(ii) THROMBIN
(iii) THROMBIN

K$_{i}$ = 52.3 ± 7.8 pM

**D**

(i) THROMBIN
(ii) THROMBIN

K$_{i}$ = 146.4 ± 13.6 pM
To verify the effect of variegin cleavage, s-variegin and EP25 were incubated with thrombin up to 24 h and at various time points assayed for the ability to inhibit thrombin amidolytic activity. The results showed that both s-variegin and EP25 lost their activity only after prolonged incubation with thrombin (Fig. 6, C and D). Interestingly, at the same temperature (24 °C) and molar ratios (30-fold excess of s-variegin), after 60 min of incubation, ~30% of s-variegin was cleaved, yet no loss of inhibitory activity of s-variegin and EP25 was observed. 24 h of incubation was needed for ~90% loss of inhibitory activity of s-variegin and EP25. In the case of the slow binding inhibition of EP25, percentage inhibition increased with incubation time up to 20 min and then decreased due to cleavage by thrombin (Fig. 6D). Thus, it is likely that the cleavage product(s) retain strong binding to the thrombin active site.

DISCUSSION

Variegin is one of the smallest thrombin inhibitors found in nature. Despite its small size and flexible structure, variegin binds to thrombin with strong affinity. Structure-activity studies revealed the interaction of s-variegin with an extended surface area of thrombin. The thrombin active site binding moiety of variegin is in the region of residues 8–14, and the exosite-I binding moiety is within residues 15–32. The seven N-terminal residue moiety, while not binding directly to thrombin, affected the binding kinetics; when removed, the binding characteristics of variegin changed from fast to slow. Although variegin is cleaved by thrombin, its inhibitory activity was largely retained after cleavage.

Over the years, many thrombin inhibitors have been isolated from hematophagous animals and snake venom. However, no similarities were found in the primary structure of variegin and other thrombin inhibitors. The absence of cysteines, consistent with a flexible structure, also differs from prototypic thrombin inhibitors such as hirudin (compact N terminus, acidic, and extended C terminus) (6, 11–13), rhodniin (double domain Kazal-type inhibitor) (32, 33), ornithodorin (double domain Kunitz-type inhibitor) (34), and theromin (acidic and antistasin-like N terminus, compact C terminus) (35), even though they all bind to the same sites on thrombin (active site and exosite-I) (Fig. 7, A and B). Although variegin residues 19–28 are almost identical to hirudin C terminus, their N termini are completely different (Fig. 7A). Unlike hirudin, variegin is not sulfated at the Tyr residue and has three extra residues at the end. Desulfation of hirudin (25) or its C-terminal peptide (hirugen) (30) retained anti-thrombin activity despite a 10-fold reduction in affinity (25) and activity (30). Our results indicated that AP18 binds to exosite-I and slightly enhanced thrombin amidolytic activity, comparable to the reported behavior of hirudin C terminus (29, 30), suggesting similar roles for these two sequences. This appears to be an example of convergent evolution in two phylogenetically distant lineages.

Variegin is also distinct from other thrombin inhibitors such as hemadin (36, 37), triabin (38, 39), and bothrojaracin (40). Hemadin has a similar structure to hirudin, binding to the thrombin active site with its N terminus, but to exosite-II with its extended C terminus (36, 37). Triabin only inhibits exosite-I and has a similar structure to lipocalins (38, 39). Bothrojaracin, a C-type lectin protein, binds to both exosite-I and exosite-II (40). Only two other thrombin inhibitors of similar size have been reported to date, but they appear to be unrelated to variegin. Despite also having 32 residues, tsetse thrombin inhibitor (TTI), isolated from tsetse fly Glossina morsitans morsitans (41, 42), does not share any sequence similarity with variegin (Fig. 7A). Another low molecular mass thrombin inhibitor (3.2 kDa) was isolated from the camel tick, Hyalomma dromedarii (NTI-1) (43). Unlike variegin, NTI-1 is a weak ($K_i = 11.7 \mu M$) and non-competitive inhibitor of thrombin, binding to only one site on thrombin. Currently, no detailed structural information for NTI-1 is available.

Perhaps variegin is best compared with hirulogs, synthetic bivalent thrombin inhibitors designed by grafting the hirudin C terminus to the active site binding moiety D-Phe-Pro-Arg-Pro through a linker of four Gly residues (Fig. 7A) (14). Although development of hirulogs (marketed as bivalirudin) represents successful rational drug design, variegin demonstrates the ability of nature to produce similar “designs” through evolution. Moreover, the superiority of variegin (s-variegin) and EP25 over hirulogs is evident. Firstly, variegin (s-variegin) and EP25 comprise natural amino acids (hirulogs generally have D-Phe). Second, even without Thr glycosylation, s-variegin and EP25 are stronger inhibitors for thrombin than hirulog-1. For example, EP25 (comparable to hirulog-1 in length) inhibits thrombin with a much stronger affinity ($K_i$ values of EP25 and hirulog-1 are ~149.8 and ~2500 PM (44), respectively). Lastly, although both hirulogs and s-variegin are cleaved by thrombin, s-variegin (and EP25) loses its inhibitory activity toward thrombin at a
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much slower rate than hirulogs. For example, at an inhibitor to thrombin ratio of 3:1, hirulog-1 lost all inhibitory activity toward thrombin amidolytic activity after ~15 min (44), whereas s-variegin and EP25 lost >90% inhibitory activity only after 24-h incubation. Thus variegin may retain the advantages of bivalirudin (i.e. clearance via proteolysis instead of through the renal route as in the case of hirudin (8)) but with improved potency (due to higher affinity for thrombin) and possibly a more sustained effect (by retaining activity after thrombin cleavage), because the half-life of bivalirudin is only 25 min (8).

Because the C termini of variegin and hirulogs, DFE-A(E)IPEEYL, are highly similar (Fig. 7A), we propose that the improved affinity and delayed loss of activity of variegin are mainly due to residues N-terminal to this sequence. Our results indicate that the active site binding moiety on variegin has the sequence EPKMHKT, and thrombin cleaves variegin between Lys and Met. This substrate sequence appears to be different from sequences of most natural substrates of thrombin. For example, Lys being at P1 (nomenclature: substrate residues are numbered from the P1′–P1′′ scissile bond toward the N and C termini, respectively (45)), although possible, is very rarely observed (46). Also, the presence of Glu at P3, Met at P1′, His at P2′, and glycosylated Thr at P4′ are all uncommon (46, 47). Therefore, the identification of this unique active site binding moiety has significant implications for both understanding thrombin substrate preference and the discovery of new leads for developing direct thrombin inhibitors.

Site-directed mutagenesis and intrinsic fluorescence studies suggest the following events during binding of hirudin to thrombin (26, 48): 1) electrostatic steering due to the complementary electrostatic fields of the hirudin C terminus and thrombin exosite-I, 2) ionic tethering through direct interactions between specific residues of hirudin C terminus inducing conformational changes and stabilization of the thrombin-hirudin C-terminal complex, and 3) subsequent binding of hirudin N terminus to the apolar site near the active site. The conformational changes upon binding of hirudin C terminus (step 2) detected in intrinsic fluorescence studies were observed to be the rate-limiting step (48). Hirudin behaved as a slow binding inhibitor in high ionic strength solution (>0.2 M), where ionic interactions were impaired (25). Interestingly, in variegin, the deletion of seven N-terminal residues led to a switch from a fast binding inhibitor to a slow binding inhibitor without any loss of binding affinity. This slow binding observed for EP25 is presumably due to the loss of N-terminal residues instead of impaired ionic tethering observed for hirudin, suggesting a different rate-limiting step. The kinetic studies indicate that the slow binding mode of EP25 probably involves isomerization of the thrombin-EP25 complex. We propose that long range electrostatic interactions between the C terminus of EP25 and thrombin exosite-I allow rapid formation of the initial collision complex (Ef). This leads to subsequent binding of EPKMHKT to the active site in a slow step to form the stabilized enzyme-inhibitor complex (Ep) through short range interactions (step 3 is the rate-limiting step) (Fig. 7C). By contrast, in the full-length variegin, the N terminus, possibly through two negatively charged residues in SDQGDVA, provides an additional electrostatic steering (but probably not tethering) effect to pre-orientate the N terminus close to the active site allowing rapid formation of short-range interactions. The electrostatic steering effect of the N terminus can be facilitated by the presence of the highly basic exosite-II. Exosite-II is located ~10 Å away from the active site (46), a distance that can theoretically be covered by the seven N-terminal residues in an extended conformation (Fig. 7D).

In summary, we present the isolation, characterization, and structure-function relationships of a potent bivalent thrombin inhibitor, variegin. It is a novel class of thrombin inhibitors and provides an excellent platform for the development of new thrombin inhibitors.

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