Hemextin AB Complex, a Unique Anticoagulant Protein Complex from *Hemachatus haemachatus* (African Ringhals Cobra) Venom That Inhibits Clot Initiation and Factor VIIa Activity*

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Yajnavalka Banerjee1 ‡, Jun Mizuguchi1, ‡, Sadaaki Iwanaga2, ‡, and R. Manjunatha Kini1 ‡, ¶

From the 1 Protein Science Laboratory, Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore 117543, the 2 Blood Products Research Department, The Chemo-Sero-Therapeutic Research Institute, Kumamoto 869-1298, Japan, and the 4 Department of Biochemistry, Virginia Commonwealth University Medical Center, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

During injury or trauma, blood coagulation is initiated by the interaction of factor VIIa (FVIIa) in the blood with freshly exposed tissue factor (TF) to form the TF·FVIIa complex. However, unwanted clot formation can lead to death and debilitation due to vascular occlusion, and hence, anticoagulants are important for the treatment of thromboembolic disorders. Here, we report the isolation and characterization of two synergistically acting anticoagulant proteins, hemextins A and B, from the venom of *Hemachatus haemachatus* (African Ringhals cobra). N-terminal sequences and CD spectra of the native proteins indicate that these proteins belong to the three-finger toxin family. Hemextin A (but not hemextin B) exhibits mild anticoagulant activity. However, hemextin B forms a complex (hemextin AB complex) with hemextin A and synergistically enhances its anticoagulant potency. Prothrombin time assay showed that these two proteins form a 1:1 complex. Complex formation was confirmed by size-exclusion chromatography. Using a “dissection approach,” we determined that hemextin A and the hemextin AB complex prolong clotting by inhibiting TF·FVIIa activity. The site of anticoagulant effects was supported by their inhibitory effect on the reconstituted TF·FVIIa complex. Furthermore, we demonstrated their specificity of inhibition by studying their effects on 12 serine proteases; the hemextin AB complex potently inhibited the amidolytic activity of FVIIa in the presence and absence of soluble TF. Kinetic studies showed that the hemextin AB complex is a noncompetitive inhibitor of soluble TF·FVIIa amidolytic activity, with a *Km* of 50 nM. Isothermal titration calorimetric studies showed that the hemextin AB complex binds directly to FVIIa with a binding constant of 1.62 × 10⁴ M⁻¹. The hemextin AB complex is the first reported natural inhibitor of FVIIa that does not require a scaffold to mediate its inhibitory activity. Molecular interactions of the hemextin AB complex with FVIIa/TF·FVIIa will provide a new paradigm in the search for anticoagulants that inhibit the initiation of blood coagulation.

Blood coagulation is an innate response to vascular injury that results from a series of amplified reactions in which zymogens of serine pro-

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1 Recipient of a National University of Singapore research scholarship.

2 To whom correspondence should be addressed. Tel.: 65-6874-5235; Fax: 65-6779-2486; E-mail: dbskinim@nus.edu.sg.

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‡‡ The abbreviations used are: TF, tissue factor; FVIIa, factor VIIa; pNA, p-nitroanilide; 2HCl, dihydrochloride; <Glu, pyroglutamic acid; sTf, soluble tissue factor; HPLC, high pressure liquid chromatography; ITC, isothermal titration calorimetry; TFPI, tissue factor pathway inhibitor; NAPc2, novel anticoagulant peptide c2.
itors of angiotensin-converting enzyme was developed based on bradyki-nin-potentiating peptides from South American snake venoms (15). Inhibitors of platelet aggregation, such as eptifibatide and tirofiban, were designed based on disintegrins, a large family of platelet aggregation inhibitors found in viperid and crotailed snake venoms (16–21). Ancrod (extracted from the venom of the Malayan pit viper) reduces blood fibrinogen levels and has been successfully tested in a variety of ischemic conditions, including stroke (22). To search for new lead molecules, we and others have been focusing on isolating and characterizing pharmacologically active proteins from snake venoms that affect blood coagulation and platelet aggregation. In this study, we report the purification and characterization of a three-finger toxin that mediates anticoagulant activity from the venom of the elapid snake *Hemachatus haemachatus* (African Ringhals cobra). Although it has mild anticoagulant activity, its synergistic interaction with the second three-finger toxin enhances its anticoagulant potency. The anticoagulant protein and its complex specifically inhibit the activation of FX by the TF-FVIIa complex by noncompetitively inhibiting the enzymatic activity of FVIIa.

This is the first report of a naturally occurring FVIIa inhibitor that does not require a scaffold to mediate its inhibitory activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

Lyophilized *H. haemachatus* venom was obtained from African Reptiles and Venoms (Gauteng, South Africa). Thromboplastin with calcium (for prothrombin time assays), Russell’s viper venom (for sphen time assays), thrombin reagent (for thrombin time assays), benzamidine hydrochloride, and 4-vinylpyridine were purchased from Sigma. β-Mercaptoethanol was purchased from Nacalai Tesque (Kyoto, Japan). The chromogenic substrates H-D-Ile-Pro-Arg-p-nitroanilide (pNA) dihydrochloride (2HCl) (S-2288), <Glu-Pro-Arg-pNA-HCl (S-2366), H-D-Phe-phenylethyl-Arg-pNA2HCl (S-2328), H-D-Pro-Phenyl-Arg-pNA-HCl (S-2302), Z-D-Aryl-Gly-Arg-pNA2HCl (S-2765), <Glu-Gly-Arg-pNA-HCl (S-2444), benzoyl-Ile-Glu(Glu-γ-methoxy)-Gly-Arg-pNA-HCl (S-2222), H-D-Val-Leu-Lys-pNA2HCl (S-2251), H-D-Val-Leu-Arg-pNA2HCl (S-2266), and methoxyzuccinyl-Arg-Pro-Tyr-pNA-HCl (S-2586) were from Chromogenix AB (Stockholm, Sweden). Spectrozyme® FIXa (H-D-Leu-phenylalanyl-Gly-Arg-pNA2-AcOH) was obtained from American Diagnostica Inc. (Stamford, CT). All substrates were reconstituted in deionized water prior to use. Recombinant human TF (Innovin) was purchased from Dade Behring (Marburg, Germany). Human plasma was donated by healthy volunteers. All other chemicals and reagents used were of the highest purity available.

**Proteins**

Human plasma-derived FVIIa, FX, and FXa were a gift from the Factor VII Group (Kazuhiro Tomokiy, Yasushi Nakatomi, Teruhiwa Nakashima, and Soutatou Gokudan) of KAKETSUKEN and were purified as described (23, 24). Recombinant human soluble TF (sTF; residues 1–218) was a gift from Dr. Toshiyuki Miyata (National Cardiovascular Center, Suita, Japan), and it was prepared as described (25). Human plasma-derived thrombin, activated protein C, and FIXa were gifts from Hiroshi Kaetsu, Shinji Nakahira, and Takayoshi Hamamoto (KAKETSUKEN), respectively, and prepared as described (23, 26, 27). Three cardiotoxins (CM-14, CM-17, and CM-18 from *Naja naja atrax*) were obtained from Dr. Mitsuhiro Otta (Kobe Pharmaceutical University). Plasma kallikrein and plasmin were purchased from Enzyme Research Laboratories (South Bend, IN). FXa and FXIIa were purchased from Haemtech (Essex Junction, VT). Tissue plasminogen activator and urokinase-type plasminogen activator were purchased from American Diagnostics Inc. α-Chymotrypsin and trypsin were obtained from Worthington.

**Purification of Anticoagulant Protein**

*H. haemachatus* crude venom (100 mg in 1 ml of distilled water) was applied to a Superdex 30 gel-filtration column (1.6 × 60 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.4) and eluted with the same buffer using an ÄKTA purifier system (Amersham Biosciences AB, Uppsala, Sweden). Individual fractions were assayed for anticoagulant activity using prothrombin time (see below). Fractions with potent anticoagulant activity were pooled and subfractionated on a cation-exchange column using the same chromatography system. The anticoagulant fraction was pooled and loaded onto a UNO S6 column (6-ml column volume; Bio-Rad) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). Bound proteins were eluted with a linear gradient of 1 M NaCl in the same buffer. Fractions collected were assayed for anticoagulant activity. The anticoagulant peaks obtained from cation-exchange chromatography were applied to a Jupiter C_{18} column (1 × 25 cm) equilibrated with 0.1% trifluoroacetic acid. The bound proteins were eluted using a linear gradient of 80% acetonitrile in 0.1% trifluoroacetic acid. Individual peaks were collected, lyophilized, examined for anticoagulant activity, and subsequently rechromatographed on a narrow bore PepMap column using a Chromelone microliquid chromatography system (LC Packings, San Francisco, CA).

**Electrospray Ionization Mass Spectrometry**

The homogeneity and mass of the anticoagulant proteins were determined by electrospray ionization mass spectrometry using a PerkinElmer Life Sciences API-300 liquid chromatography/tandem mass spectrometry system. Typically, reverse-phase HPLC fractions were directly used for analysis. Ion spray, orifice, and ring voltages were set at 4600, 50, and 350 V, respectively. Nitrogen was used as a nebulizer and curtain gas. A Shimadzu LC-10AD pump was used for solvent delivery (40% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 50 μl/min. BioMultiview software (PerkinElmer Life Sciences) was used to analyze and deconvolute raw mass spectra.

**Reduction and Pyridylethylation**

Purified proteins were reduced and pyridylethylated using procedures described previously (28). Briefly, proteins (0.5 mg) were dissolved in 500 μl of denaturant buffer (6 M guanidine hydrochloride, 0.25 M Tris-HCl, and 1 mM EDTA (pH 8.5)). After the addition of 10 μl of β-mercaptoethanol, the mixture was incubated under vacuum for 2 h at 37 °C. 4-Vinylpyridine (50 μl) was added to the mixture and kept at room temperature for 2 h. Pyridylethylated proteins were purified on an analytical Jupiter C_{18} column (4.6 × 250 mm) using a gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min.

**N-terminal Sequencing**

N-terminal sequencing of the native and S-pyridylethylated proteins was performed by automated Edman degradation using a PerkinElmer Life Sciences Model 494 pulsed liquid-phase sequencer (Procise) with an on-line Model 785A phenylthiohydantoin-derivative analyzer.

**CD Spectroscopy**

Far-UV CD spectra (260–190 nm) were recorded using a Jasco J-810 spectropolarimeter. All measurements were carried out at room temperature using 0.1-cm path length stopped cuvettes. The instrument optics was flushed with 30 liters of nitrogen gas/min. The spectra were
recorded using a scan speed of 50 nm/min, a resolution of 0.2 nm, and a bandwidth of 2 nm. A total of four scans were recorded and averaged for each spectrum, and the base line was subtracted. The CD spectra of the anticoagulant proteins and their S-pyridylethylated forms were recorded in 50 mM Tris-HCl buffer (pH 7.4).

Reconstitution of the Anticoagulant Complex

Preliminary studies indicated that the active anticoagulant protein interacted with another venom protein, forming a synergistic complex. We reconstituted the complex for various in vitro experiments immediately prior to the experiments by incubating equimolar concentrations of the two proteins (unless mentioned otherwise) at 37 °C for 5 min in 50 mM Tris-buffer (pH 7.4).

Anticoagulant Activity

The anticoagulant activities of *H. haemachatus* venom and its fractions were determined by four coagulation tests using a BBL Fibrometer.

Recalcification Time—The recalcification times were determined according to the method of Langdell *et al.* (29). 50 mM Tris-HCl buffer (pH 7.4; 100 μl), plasma (100 μl), and various concentrations of venom or its fraction (50 μl) were preincubated for 2 min at 37 °C. Clotting was initiated by the addition of 50 μl of 50 mM CaCl₂.

Prothrombin Time—The prothrombin times were measured according to the method of Quick (30). 100 μl of 50 mM Tris-HCl buffer (pH 7.4), 100 μl of plasma, and 50 μl of venom or its fractions were preincubated for 2 min at 37 °C. Clotting was initiated by the addition of 150 μl of thromboplastin with calcium reagent.

Stypven Time—The Stypven times were measured according to the method of Hougie (31). Plasma (100 μl), 50 mM Tris-HCl buffer (pH 7.4; 100 μl), Russell’s viper venom (0.01 μg in 100 μl), and individual proteins or the reconstituted complex (50 μl) were preincubated for 2 min at 37 °C. Clotting was initiated by the addition of 50 μl of 50 mM CaCl₂.

Thrombin Time—The thrombin times were determined according to the method of Lim (32). Individual proteins or the reconstituted complex was incubated with 100 μl of plasma and 100 μl of 50 mM Tris-HCl buffer (pH 7.4) for 2 min at 37 °C in a total volume of 250 μl. Clotting was initiated by the addition of standard thrombin reagent (0.01 NIH unit in 50 μl).

Complex Formation Studies with Size-exclusion Chromatography

The formation of a complex between anticoagulant proteins was examined by gel-filtration chromatography on a Superdex 30 gel-filtration column (1.6 × 60 cm) using an AKTA purifier. The column was equilibrated with 50 mM Tris-HCl buffer in a 250-μl aliquot of the reaction mixture after 15 min of incubation. FXa formed was measured by the hydrolysis of 1 mM S-2222 in Buffer A in a microtiter plate reader at 405 nm. The inhibitory effect on extrinsic tenase activity was determined by adding the individual proteins or the anticoagulant complex 15 min prior to FX addition.

**Serine Protease Specificity**

The selectivity profile of anticoagulant proteins and their complex was examined against 12 serine proteases: procoagulant serine peptidases (FXa, FXa, FXIa, FXIIa, plasma kallikrein, and thrombin), anticoagulant serine protease activated protein C, fibrinolytic serine peptidases (urokinase, tissue plasminogen activator, and plasmin), and classical serine proteases (trypsin and chymotrypsin). Various concentrations of the purified individual proteins or the reconstituted anticoagulant complex were preincubated with each of the enzymes for 5 min at 37 °C, followed by the addition of the appropriate chromogenic substrate.

In a total volume of 200 μl in the individual wells of the microtiter plate, the final concentrations were as follows: FVIIa (300 nM)/S-2288, sTF-FVIIa (30 nM)/S-2288, FXa (0.75 nM)/S-22765, α-thrombin (0.66 nM)/S-2238, plasmin (2 nM)/S-2251, FXa (3 μM)/Spectrozyme® FXa, FXa (0.34 nM)/S-2366, FXIIa (0.4 nM)/S-2302, recombinant tissue plasminogen activator (80 nM)/S-2288, activated protein C (0.34 nM)/S-2366, urokinase/S-2444, plasma kallikrein (0.4 nM)/S-2302, trypsin (2.17 nM)/S-2222, and chymotrypsin (0.4 nM)/S-2586. The kinetic rate of substrate hydrolysis (mOD/min) was measured over 5 min.

**Kinetics of Inhibition**

All studies were performed in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 10 mM CaCl₂, and 1% bovine serum albumin at 37 °C. The kinetics of hydrolysis of the chromogenic substrate S-2288 by sTF-FVIIa was measured prior to examining the inhibitory effects of the individual proteins and the reconstituted anticoagulant complex. Reactions were initiated by the addition of S-2288 (0–5 mM) to the individual wells of a 96-well plate containing FVIIa (30 nM) in complex with sTF (100 nM) in a final volume of 180 μl. Initial reaction velocities were measured as a linear increase in the absorbance at 405 nm over 5 min with a SpectraMax Plus® temperature-controlled microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

The inhibitory potency of the anticoagulant complex was measured over a range of substrate concentrations. Reactions were initiated by the addition of S-2288 to premixed cofactor/enzyme and inhibitor in the wells of a microtiter plate. Reactions with sTF-FVIIa contained 0.025–0.1 μM inhibitor complex and 0–3 mM S-2288. The initial velocities were measured over 5 min under steady-state conditions and were fit by reiterative nonlinear regression to Equation 1, describing a classical noncompetitive inhibitor, to derive the *Kᵢ* value.

\[
1/V = K_m/V_{\text{max}}(1 + [I]/K_i)1/[S] + 1/V_{\text{max}}(1 + [I]/K_i)
\]

(Eq. 1)

**Isothermal Titration Calorimetry (ITC)**

The interaction of the reconstituted anticoagulant complex with FVIIa was monitored with a VP-ITC titration calorimetric system (MicroCal, LLC, Northampton, MA). The instrument was calibrated using the built-in electrical calibration check. FVIIa (10 μM) in 50 mM Tris-HCl buffer and 10 mM CaCl₂ (pH 7.4) in the calorimetric cell was titrated with the reconstituted anticoagulant complex (0.4 mM) dissolved in the same buffer in a 250-μl injection syringe with continual stirring at 300 rpm at 37 °C. All protein solutions were filtered and degassed prior to titration. The first injections presented defects in the
base line, and these data were not included in the fitting process. The calorimetric data were processed and fitted to the single set of identical sites model using MicroCal Origin (Version 7.0) data analysis software supplied with the instrument. The total heat content ($Q$) of the solution (determined relative to zero for the unliganded species) contained in the active cell volume ($V_0$) was calculated according to Equation 2,

$$Q = \frac{nM_t \Delta H V_0}{2} \left( 1 + \frac{X_t}{nM_t} + \frac{1}{nK_a M_t} \right) - \sqrt{\left( 1 + \frac{X_t}{nM_t} + \frac{1}{nK_a M_t} \right)^2 - \frac{4X_t}{nM_t}}$$

(Eq. 2)

where $K$ is the binding affinity constant; $n$ is the number of sites; $\Delta H$ is the enthalpy of ligand binding; and $M_t$ and $X_t$ are the bulk concentrations of macromolecule and ligand, respectively, for the binding $X + M \leftrightarrow XM$. The change in heat ($\Delta H$) measured between the completions of two consecutive injections is corrected for dilution of the protein and ligand in the cell according to standard Marquardt methods. The free energy change ($\Delta G$) during the interaction was calculated using the relationship $\Delta G = \Delta H - T \Delta S = -RT \ln K_w$, where $T$ is the absolute temperature and $R$ is the universal gas constant.

RESULTS

Purification of the Anticoagulant Protein—Crude venom of H. haemachatus exhibited potent anticoagulant activity in both recalcification and prothrombin time assays (Fig. 1, A and B). To purify the anticoagulant protein, the crude venom was size-fractionated by gel-filtration chromatography (Fig. 2A). Fractions corresponding to peaks 2 and 3 contained anticoagulant proteins as determined by prothrombin time assays. Peak 2 corresponded to proteins mostly containing phospholipase A2 that have been characterized previously (33). However, this peak had milder anticoagulant activity compared with peak 3 (Fig. 2A, inset), so we focused on isolating the anticoagulant protein from peak 3, which was fractionated further by cation-exchange chromatography on a UNO S6 column (Fig. 2B). Only peak A (labeled Hemextin A) exhibited mild anticoagulant activity. During our preliminary studies, we found that the anticoagulant activity of peak A was potentiated by peak B (labeled Hemextin B; see below). Because this anticoagulant complex specifically inhibited the extrinsic tenase complex (described...
below), we named it the hemextin AB complex (*Hemachatus* extrinsic tenase inhibitor) and the individual proteins hemextins A and B, respectively. Fractions corresponding to both hemextins A and B were pooled separately and purified by reverse-phase HPLC (Fig. 2, C and D) and capillary liquid chromatography (Fig. 2, E and F). The homogeneity and mass of the individual proteins were determined by electrospray ionization mass spectrometry. The mass spectra of hemextins A and B showed three peaks with mass/charge ratios ranging from 3 to 6 charges (data not shown) and their calculated molecular masses as 6835.00 ± 0.52 and 6792.56 ± 0.32 Da, respectively (Fig. 2, G and H).

**N-terminal Sequence Determination**—We determined the sequence of the first 25 amino acid residues of hemextins A and B by Edman degradation (Fig. 3 A). Conserved cysteine residues in the three-finger toxin family are indicated. B, shown are the CD spectra of native and S-pyridylethylated hemextins A and B.

**CD Spectroscopy**—Both hemextins A and B exhibited negative minima at 215 nm and positive maxima at 194 nm. Thus, similar to other three-finger toxins, both hemextins A and B exhibited a predominantly β-sheet structure (Fig. 3 B). However, the S-pyridylethylated forms of hemextins A and B displayed negative minima at 195 nm, i.e. a predominantly random-coil structure (Fig. 3B). Thus, reduction and pyridylethylolation result in the loss of folding and three-dimensional structure in hemextins A and B.

**Anticoagulant Activity of Hemextins**—The anticoagulant activity of hemextins A and B was determined by the prothrombin time assay (Fig. 4A). Hemextin A prolonged the clotting time and exhibited mild anticoagulant activity, whereas hemextin B did not show any significant effect on the clotting time even at higher concentrations. Interestingly, an equimolar mixture of hemextins A and B exhibited more potent anticoagulant activity, indicating synergism between these proteins (Fig. 4A). Such an increase in the anticoagulant effect could be due either to the inhibition of two separate steps in the coagulation cascade or to the formation of a complex between them. Because hemextin B by itself has no significant effect on prothrombin time, it does not inhibit a separate step; instead, it is likely that hemextins A and B form a complex. S-Pyridylethylated hemextins did not exhibit any anticoagulant activity. An equimolar mixture of S-pyridylethylated hemextins A and B also failed to display any anticoagulant effect (Fig. 4A, inset). Thus, proper folding is important for the interaction between hemextins A and B and their anticoagulant activity.

**Complex Formation between Hemextins A and B**—To investigate the formation of a complex between the two proteins, we employed a titration experiment in the prothrombin time assay. In this experiment, the concentration of hemextin A was kept constant at 4.4 µM, and its anticoagulant activity was monitored with increasing hemextin B concentrations (Fig. 4B). The anticoagulant activity increased with increasing concentrations of hemextin B until the ratio reached 1:1. Further addition did not increase the anticoagulant effect. The results indicate that
hemextins A and B form a 1:1 complex and that complex formation is crucial for potent anticoagulant activity.

Complex formation between hemextins A and B was further confirmed by gel-filtration chromatography. As shown in Fig. 5, the retention time of individual hemextins A and B was ~70 min. However, the reconstituted complex eluted as a major peak with a retention time of ~40 min and as a minor peak with a retention time of ~70 min. The appearance of the major peak with a reduced retention time corresponding to ~27 kDa is consistent with the formation of a tetrameric complex with two molecules each of hemextins A and B. We also reconstituted the hemextin AB complex with S-pyridylethylated forms of the native proteins. However, no change in the retention time of the mixture was observed compared with those of the individual S-pyridylethylated proteins (Fig. 5). These results indicate that proper folding is essential for the formation of the hemextin AB complex.

It is important to note that hemextins A and B do not exist as a complex in the crude venom. Complex formation is probably hindered by the presence of high concentrations of citrate and other salts in the venom. The reconstituted hemextin AB complex could be easily separated into individual components on a cation-exchange column (data not shown).

Site of Anticoagulant Activity—As shown above, hemextin A and its complex with hemextin B prolonged prothrombin time (Fig. 4A). To identify the specific stage in the extrinsic coagulation pathway, we used a simple “dissection approach” (36, 37). We employed three commonly used clotting time assays, viz. prothrombin time, Stypven time, and thrombin time. This approach is based on the principle that initiating the cascade “upstream” from the inhibited step will result in elevated clotting times, whereas initiating the cascade “downstream” from the inhibited step will not affect clotting times. Thus, the anticoagulant action of the individual proteins and the complex can be localized to certain activation step(s) in the cascade (for details, see Refs. 32 and 33). Hemextin A exhibited mild anticoagulant activity by prolonging the clotting time in the prothrombin time assay, but did not prolong
tiritation experiment. The inhibitory activity of hemextin A at 50 μM (the concentration at which the hemextin AB complex inhibited only ~70% of the TF-FVIIa activity) was examined in the presence of increasing concentrations of hemextin B. As shown in Fig. 6B, the inhibitory activity of hemextin A increased with increasing concentrations of hemextin B until the ratio reached 1:1. Further addition did not increase the inhibition. The results indicate that hemextins A and B form a 1:1 complex and that complex formation is crucial for potent anticoagulant activity. These observations further confirm the importance of complex formation between hemextins A and B.

To understand the effect of phospholipids, the inhibitory activity of hemextin A and the hemextin AB complex on FVIIa amidolytic activity was monitored in the presence or absence of STF. In both the cases, we observed potent inhibitory activity in a dose-dependent manner (Fig. 7, A and B).

**Specificity of Inhibition**—To determine the specificity of inhibition, hemextins A and B and their complex were screened against 12 serine proteases. No inhibitory activity was observed against any of the serine proteases with the exception of FVIIa and plasma kallikrein. As with FVIIa, hemextin A and the hemextin AB complex inhibited plasma kallikrein in a dose-dependent manner (Fig. 8). Hemextin B did not inhibit the protease activity of kallikrein. However, the inhibitory potency for FVIIa (in the absence or presence of STF) was at least 50 times higher than for plasma kallikrein.

**Kinetics of Inhibition**—To determine the mechanism of inhibition, we examined the kinetics of hemextin AB complex inhibition of the amidolytic activity of the sTF-FVIIa complex in the presence of S-2288. Kinetic studies revealed that hemextin inhibited sTF-FVIIa activity non-competitively. Lineweaver-Burk plots showed that Kₘ values remained
unaltered, whereas $V_{\text{max}}$ values decreased with increasing concentrations of the inhibitor (Fig. 9A), a characteristic of a noncompetitive inhibitor. The $K_i$ for inhibition was determined to be 50 nM (Fig. 9B). We also calculated the turnover number ($K_{\text{cat}}$, moles of substrate converted to product/mol/enzyme/min) at different concentrations of the inhibitor. As observed in the case of classical noncompetitive inhibitors, the $K_{\text{cat}}$ decreased with increasing concentrations of the hemextin AB complex (data not shown). Because the amidolytic activity of FVIIa alone is very weak (38), we did not study the kinetics of the inhibition of FVIIa amidolytic activity by the hemextin AB complex.

Binding of the Hemextin AB Complex to FVIIa—We studied the interaction between the hemextin AB complex and FVIIa by ITC. We monitored the thermodynamic changes associated with the binding of the hemextin AB complex to FVIIa (Fig. 10). The binding was exothermic, with $\Delta H = -5.445 \text{ kcal mol}^{-1}$, $\Delta G = -4.121 \text{ kcal mol}^{-1}$, and $\Delta S = -4.274 \text{ cal mol}^{-1}$. The calculated $K_a$ for the binding was $1.62 \times 10^5 \text{ M}^{-1}$.

DISCUSSION

Initiation of blood coagulation during injury or trauma is essential for the survival of the organism. However, the formation of unwanted clots has detrimental or debilitating effects and hence the need for anticoagulant therapies. The current anticoagulants used for treating these disorders are nonspecific and have a narrow therapeutic range, necessitating careful laboratory monitoring to achieve optimal efficacy and to minimize bleeding. This is further complicated by other factors such as dietary intake (39). Therefore, novel anticoagulant and antiplatelet agents are being sought. Because FVIIa is the key initiator of blood coagulation and is present in the plasma milieu at very low concentrations, it is an attractive drug target for the design and development of anticoagulants.

So far, only two proteins that specifically inhibit the TF-FVIIa complex have been well characterized, viz. tissue factor pathway inhibitor (TFPI) and nematode anticoagulant peptide c2 (NAPc2). TFPI is an endogenous inhibitor of this complex (40), whereas NAPc2 is an exogenous inhibitor isolated from canine hookworm (Ancylostoma caninum) (41). TFPI is a 42-kDa plasma glycoprotein consisting of three tandem Kunitz-type domains. The first and second units inhibit TF-FVIIa and FXa, respectively. The third Kunitz domain and the C-terminal basic region of the molecule have heparin-binding sites (42). The anticoagulant action of TFPI is a two-stage process. The second Kunitz domain binds first to a molecule of FXa and deactivates it. The first domain then rapidly binds to an adjacent TF-FVIIa complex, preventing further activation of FX (43–45). On the other hand, NAPc2 is an 8-kDa
short polypeptide. Its mechanism of action requires prerequisite binding to FXa or zymogen FX to form a binary complex prior to its interaction and inhibition of membrane-bound TF-FVIIa (41). Therefore, despite the structural differences, both inhibitors form a quaternary complex with TF-FVIIa-FXa. However, in both complexes, the active site of FVIIa is occupied by the respective inhibitors and is not accessible.

Because of the lack of natural inhibitors that specifically interfere with FVIIa activity, a number of artificial inhibitors have been designed and developed. They include proteins that block the association of TF and FVIIa, such as antibodies against TF and FVIIa, TFAA (a TF mutant with reduced cofactor function for FX), FFR-VIIa (inactivated form of FVIIa with 5-fold higher affinity for TF compared with native FVIIa), and peptides derived from TF and FVIIa (47–54). In addition, two series of peptide exosite inhibitors were selected from phage display libraries for their ability to bind to the TF-FVIIa complex (47, 48). They bind to two distinct exosites on the serine protease domain of FVIIa and exhibit ertic and allosteric inhibition (50). Although both peptide classes are potent and selective inhibitors of the TF-FVIIa complex, they fail to inhibit 100% activity even at saturating concentrations. This is overcome either by the fusion of the two peptides (51) or by using a protease switch with substrate phage (49). A number of synthetic compounds have also been designed as active-site inhibitors of FVIIa as well as the TF-FVIIa complex (52, 55–58). A number of naphthylamidines have recently been reported to have FVIIa inhibitory activity (59). They were synthesized by the coupling of amidinobenzaldehyde analogs to a polystyrene resin. However, apart from inhibiting FVIIa activity, these synthetic compounds nonspecifically inhibit the activity of other blood coagulation serine proteases (59).

**Hemextin AB Complex Is a Novel Anticoagulant**—We have reported here the isolation and characterization of two proteins, hemextins A and B, from the venom of *H. haemachatus* that synergistically induce potent anticoagulant activity. Individually, only hemextin A exhibited mild anticoagulant activity, whereas hemextin B had no anticoagulant activity (Fig. 4A). The increase in the anticoagulant potency of hemextin A in the presence of hemextin B (Fig. 4A) indicated probable complex formation between the two proteins. We have shown that 1:1 complex formation is important for potent anticoagulant activity using the prothrombin time assay (Fig. 4B). Complex formation was further confirmed by gel-filtration chromatography (Fig. 5).

Both hemextins A and B belong to the three-finger family of snake venom proteins (Fig. 3A) and not to the family of snake venom serine protease inhibitors. Proteins belonging to this group exhibit a characteristic β-sheet structure (60), also observed in the CD studies (Fig. 3B). It is a well known fact that disulfide bonds associated with cysteine residues are essential structural units in proteins (61). To evaluate the importance of the three-finger fold in both complex formation and anticoagulant activity, we used reduced and subsequently pyridylethylated hemextins A and B. Upon pyridylethylation, they lost their native three-finger fold, as observed in the CD studies (Fig. 3B). The S-pyridylethylated hemextins were functionally inactive (Fig. 4A, inset) and were unable to bind to each other to form the complex, as evident from the gel-filtration studies (Fig. 5). This shows that proper folding of the proteins is important not only for function, but also for complex formation.

Using the dissection approach (36, 37), we identified the site of anticoagulant action of hemextin A and its synergistic complex. Both hemextin A and the hemextin AB complex inhibited the extrinsic tenase complex, but not other steps in the extrinsic pathway. These results were further confirmed by studying the effect of hemextin A and its complex on the reconstituted TF-FVIIa complex. Both hemextin A and the hemextin AB complex inhibited FXa formation by the reconstituted extrinsic tenase complex (Fig. 6A). Furthermore, hemextin A and the hemextin AB complex inhibited the amidolytic activity of FVIIa in both the presence and absence of sTF (Fig. 7, A and B). The hemextin AB complex inhibited with IC_{50} values of ~200 and ~210 nM, respectively. Similar IC_{50} values may be indicative of the fact that hemextin A and the hemextin AB complex do not bind to the cofactor-binding site of FVIIa. The inhibitory activity of hemextin A and the hemextin AB complex may not be due to nonspecific interaction of hemextin A or its complex with the phospholipids in the extrinsic tenase complex, as indicated by their inability to prolong Stypven time, because they failed to inhibit the prothrombinase complex, which is also formed on the phospholipid surfaces. This was further confirmed by determining the inhibitory activity of hemextin A and the hemextin AB complex on the amidolytic activity of the reconstituted extrinsic tenase complex using sTF and FVIIa (Fig. 7B). Furthermore, hemextin A and the hemextin AB complex inhibited the amidolytic activity of FVIIa (Fig. 7A). However, hemextin B did not exhibit any inhibitory activity in the absence of hemextin A. To further characterize the inhibitory properties and to determine the specificity of inhibition, we screened hemextins A and B and the hemextin AB complex against 12 serine proteases. In addition to FVIIa and its complexes, hemextin A and the hemextin AB complex inhibited the amidolytic activity of only kallikrein in a dose-dependent manner (Fig. 8). However, the IC_{50} for the inhibition of kallikrein was ~10 μM, in contrast to that of FVIIa/TF/FVIIa/sTF-FVIIa, which was ~200 nM. Kinetic studies revealed that the hemextin AB complex is a noncompetitive inhibitor of the sTF-FVIIa complex, with a K_{i} of 50 nM. Using ITC studies, we have shown that the hemextin AB complex directly interacts with FVIIa (Fig. 10). The binding interaction between FVIIa and the hemextin AB complex is associated with a negative change in free energy, indicating that this complex formation is favored. The negative change in entropy observed upon binding indicates the formation of a tightly folded complex between the two moieties (62). Thus, these data strongly indicate that the hemextin AB complex is a highly specific inhibitor of FVIIa. To our knowledge, this is the first natural inhibitor of FVIIa.

Some other anticoagulants from snake venoms also inhibit the extrinsic tenase complex. However, they are not as specific. For example, CM-IV, a strongly anticoagulant phospholipase A2 from *Naja nigricollis* venom, prolongs coagulation by inhibiting two successive steps in the coagulation cascade. It inhibits the TF-FVIIa complex by both enzymatic and nonenzymatic mechanisms (63), whereas it inhibits the prothrombinase complex by only a nonenzymatic mechanism (64, 65). Hemextin A and its synergistic complex are the first reported specific inhibitors of FVIIa isolated from snake venom.

The similar dose-dependent inhibition of the TF-FVIIa complex and FVIIa indicates that the hemextin AB complex neither requires TF for its inhibitory activity nor interferes in the binding of TF to FVIIa. Unlike TFPI and NAPc2, it also does not use FXa as a scaffold to bind to FVIIa and thus does not require FX or FXa to inhibit FVIIa. Furthermore, TFPI and NAPc2 bind to the active site of FVIIa. In contrast, as shown by the kinetic studies (Fig. 9), the hemextin AB complex is a noncompetitive inhibitor, unlike competitive inhibitors that bind to the active site. Thus, the hemextin AB complex does not appear to bind to the active site of FVIIa. Therefore, hemextin A and the hemextin AB complex are novel inhibitors of FVIIa and the TF-FVIIa complex.

**Hemextin AB Complex Is a Unique Protein Complex**—Synergism among snake venom toxins is fairly well characterized, particularly among presynaptic neurotoxins. For example, crototoxin isolated from *Crotalus durissus terrificus* venom contains two subunits; the basic sub-
unit is a phospholipase $\Delta_2$ enzyme, whereas the acidic subunit is catalytically inactive (although it is derived from a phospholipase $\Delta_2$–like protein) (66). Individually, only the basic subunit is slightly toxic, whereas the complex exhibits potent toxicity. The acidic subunit appears to act as a chaperone and enhances the specific binding of the basic subunit to the presynaptic site. Similarly, other presynaptic neurotoxins, such as taipoxin from *Oxyuranus scutellatis* (67) and textilotoxin from *Pseudonaja textilis* (68) venoms, contain three and four subunits, respectively. All the subunits are structurally similar to phospholipase $\Delta_2$ enzymes. The noncovalent interactions between the subunits of these toxins are important for their potenti toxicity. Thus, a number of snake venom presynaptic toxins are protein complexes with phospholipase $\Delta_2$ as an integral part. For example, rhodocetin, an antiplatelet protein complex from *Calloselasma rhodostoma* venom, contains two subunits showing structural similarity to C-type lectins (70). Group C prothrombin activators from Australian snakes are procoagulant protein complexes that are structurally and functionally similar to mammalian blood coagulation FX$\alpha$FVa complexes (46, 71, 72). The hemextin AB complex is a unique snake venom protein complex formed by the interaction between two three-finger toxins, in which the anticoagulant activity of hemextin A is poteniated by its synergistic interaction with hemextin B. It should be noted that crude snake venom does not contain the hemextin AB complex. It is not clear when and how this complex is formed.

In summary, we have described a unique anticoagulant protein complex from snake venom that specifically and noncompetitively inhibits FVIIa activity. Our results strongly indicate that the interaction between hemextins A and B is essential for potent anticoagulant activity. This new anticoagulant may help us develop different strategies and therapeutic agents to inhibit the initiation step in blood coagulation.

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REFERENCES

1. Davie, E. W., Fujikawa, K., and Kisiel, W. (1991) *Biochemistry* 30, 10363–10370
2. Davie, E. W. (1995) *Thromb. Haemostasis* 74, 1–6
3. Mann, K. G., Butenis, S., and Brummel, K. (2003) *Arterioscler. Thromb. Vasc. Biol.* 23, 17–25
4. Rapaport, S. I., and Rao, L. V. (1995) *Thromb. Haemostasis* 74, 7–17
5. Morrissey, J. H., Macik, B. G., Neuenschwander, P. F., and Comp, P. C. (1993) *Blood* 81, 734–744
6. Nemerson, Y. (1988) *Blood* 71, 1–8
7. Gustafsson, D., Bylund, R., Antonsson, T., Nilsson, L., Nystrom, J., Eriksson, U., Streeberg, U., and Teger-Nilsson, A. C. (2004) *Nat. Rev. Drug Discov.* 3, 649–659
8. Hirsh, J. (1991) *N. Engl. J. Med.* 324, 1685–1695
9. Hirsh, J. (1991) *N. Engl. J. Med.* 324, 1565–1574
10. Hirsh, J. (1991) *N. Engl. J. Med.* 324, 1685–1875
11. Hirsh, J. (1991) *N. Engl. J. Med.* 324, 1565–1574
12. Hirsh, J. (2001) *Annu. Rev. Med.* 52, 53–58
63. Kini, R. M., and Evans, H. I. (1995) *Toxicon* 33, 1585–1590
64. Stefansson, S., Kini, R. M., and Evans, H. I. (1990) *Biochemistry* 29, 7742–7746
65. Kerns, R. T., Kini, R. M., Stefansson, S., and Evans, H. I. (1999) *Arch. Biochem. Biophys.* 369, 107–113
66. Habermann, E., and Breithaupt, H. (1978) *Toxicon* 16, 19–30
67. Doorty, K. B., Bevan, S., Wadsworth, J. D., and Strong, P. N. (1997) *J. Biol. Chem.* 272, 19925–19930
68. Su, M. J., Coulter, A. R., Sutherland, S. K., and Chang, C. C. (1983) *Toxicon* 21, 143–151
69. Possani, L. D., Martin, B. M., Yatani, A., Mochca-Morales, J., Zamudio, F. Z., Gurrola, G. B., and Brown, A. M. (1992) *Toxicon* 30, 1343–1364
70. Wang, R., Kini, R. M., and Chung, M. C. (1999) *Biochemistry* 38, 7584–7593
71. Rao, V. S., and Kini, R. M. (2002) *Thromb. Haemostasis* 88, 611–619
72. Rao, V. S., Swarup, S., and Kini, R. M. (2003) *Blood* 102, 1347–1354