Ca^{2+}-Induced Binding of Anticoagulation Factor II from the Venom of *Agkistrodon Acutus* with Factor IX

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ABSTRACT:
Anticoagulation factor II (ACF II), a coagulation factor X- binding protein from the venom of Agkistrodon acutus has both anticoagulant and hypotensive activities. Previous studies show that ACF II binds specifically with activated factor X (FXa) in a Ca^{2+}-dependent manner and inhibits intrinsic coagulation pathway. In this study, the inhibition of extrinsic coagulation pathway by ACF II was measured in vivo by prothrombin time assay and the binding of ACF II to factor IX (FIX) was investigated by native polyacrylamide gel electrophoresis and surface plasmon resonance (SPR). The results indicate that ACF II also inhibits extrinsic coagulation pathway, but does not inhibit thrombin activity. ACF II also binds with FIX with high binding affinity in a Ca^{2+}-dependent manner and their maximal binding occurs at about 0.1 mM Ca^{2+}. ACF II has similar binding affinity to FIX and FX as determined by SPR. Ca^{2+} has a slight effect on the secondary structure of FIX as determined by circular dichroism spectroscopy. Ca^{2+} ions are required to maintain in vivo function of FIX Gla domain for its recognition of ACF II. However, Ca^{2+} at high concentrations (>0.1 mM) inhibits the binding of ACF II to FIX. Ca^{2+} functions as a switch for the binding between ACF II and FIX. ACF II extends activated partial thromboplastin time more strongly than prothrombin time, suggesting that the binding of ACF II with FIX may play a dominant role in the anticoagulation of ACF II in vivo. *© 2012 Wiley Periodicals, Inc.* *Biopolymers 97: 818–824, 2012.*
Keywords: Agkistrodon acutus; ACF II; factor IX/X binding protein; factor IX; Ca^{2+}

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INTRODUCTION

Coagulation factors IX and X (FIX and FX) are two pivotal participants in the blood coagulation cascade, in which the two factors are sequentially activated, leading to the formation of insoluble fibrin clots from the soluble fibrinogen.[1] They are all Ca^{2+}-binding proteins and require Ca^{2+} to facilitate their physiological functions. Both proteins possess an N-terminal γ-carboxyglutamic acid (Gla) domain that is essential for the amplification of the coagulation cascade. Snake venoms are rich in a large variety of proteins and enzymes.[2–6] A family of FIX and FX binding proteins (IX/X-bps) has been identified from various kinds of snake venoms.[7–11] All IX/X-bps have anticoagulant activity and are highly homologous with each other. They bind to the Gla domains of FIX and FX in a Ca^{2+}-dependent manner and block the amplification of the coagulation cascade.[12,13]

Anticoagulation factor II (ACF II), a FX binding protein has been purified from the venom of *Agkistrodon acutus*.[9] ACF II forms a 1:1 complex with activated FX (FXa) in a Ca^{2+}-dependent fashion. It markedly prolongs activated partial thromboplastin time (APTT), indicating that it inhibits

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the intrinsic coagulation pathway.[14,15] Recently, we have reported that ACF II is a unique bifunctional protein in the IX/X-bp family that has both anticoagulant and hypotensive effects on the blood of rats.[15] ACF II induces an endothelium-dependent vasodilation via nitric oxide pathway which results in hypotension. ACF II, a naturally occurring anticoagulant and hypotensive reagent, is devoid of hemorrhagic activity, and may be useful as a basis for designing anticoagulant and hypotensive drugs. ACF II has a typical structure of IX/X-bps (PDB code 1Y17) with a homologous sequence to other IX/X-bps.

In this work, the anticoagulant effect of ACF II was measured in vivo by prothrombin time (PT) and thrombin time (TT) assays and the Ca^{2+}-induced binding of ACF II to FIX was investigated by native PAGE and surface plasmon resonance (SPR). Our results demonstrate that ACF II also inhibits the extrinsic coagulation pathway and binds FIX in the presence of 0.1 m*M* Ca^{2+}.

EXPERIMENTAL

Materials

Male Wistar-Imamichi rats (180–250 g, 7–8 weeks old, supplied by Animal Services Center of Anhui Medical University, China) were used in all experiments. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). Lyophilized venom powder was provided by the TUN-XI Snakebite Institute (Anhui, P. R. China). BSA was obtained from Sigma Chemical Company (St Louis, MO). Human FIX was purchased from MyBioSource (San Diego, CA) and its concentration was calculated from its absorption coefficients ($A_{1cm}^{1\%}$=13.3) at 280 nm and its molecular weight (M_r = 56 kD). PT reagent Thromboplastin C Plus and TT reagent Test Thrombin Reagent were purchased from Siemens Healthcare Diagnostics (Deerfield, IL). Chelex-100 was purchased from Bio-Rad Laboratories (Richmond, CA). All other reagents were of analytical reagent grade and produced by the Shanghai Institute of Biochemistry (Shanghai, P.R. China). Milli-Q purified water was used throughout.

Preparation of Protein and Solution

ACF II was purified by a three-step chromatography procedure of anion-exchange chromatography, gel permeation chromatography and cation-exchange chromatography as described previously.[9] The molecular homogeneity of the purified ACF II was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and MALDI-TOF mass spectrometry. The concentration of ACF II was calculated from its absorption coefficient ($A_{1cm}^{1\%}$=31) at 280 nm and its molecular weight (Mr = 29.5 kD). The solution of Ca^{2+} was prepared from $CaCl_2$ in Milli-Q water and standardized by titration with standard EDTA solution. Tris buffer used was freed from any possible contamination of multivalent cations by passage through a column (25 × 3 cm^2) of Chelex-100. Ca^{2+}-free ACF II (apo-ACF II) and Ca^{2+}-free FIX (apo-FIX) were prepared by dialysis of the purified ACF II and FIX against 2 m*M* EDTA in 0.02*M* Tris-HCl (pH 7.4) for 12 h and then extensively against 0.02*M* Tris-HCl (pH 7.4).

In Vivo Anticoagulation Measurements

To determine the effect of ACF II on coagulation after intravenous administration, rats were anaesthetized using a mixture of 25% urethane and 1% α-chloralose (w/v) given intraperitoneally at a dose of 5 ml/kg body weight. ACF II was administered through the caudal vein.[16] Ten min after administration of ACF II, 2 ml of arterial blood was drawn from the abdominal aorta into 3.8% citrate solution and immediately centrifuged. The plasma was collected for analysis of PT and TT. PT and TT were monitored by an Automated Coagulation Analyzer (Sysmex CA-1500, America Dade).[17]

Electrophoresis

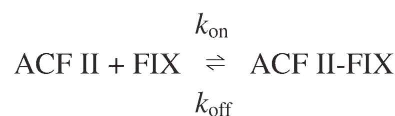
Native PAGE was used for analysis of the Ca^{2+}-induced binding of ACF II with FIX. BSA was used as a non-interacting control protein. It was performed in 3% stacking gel at pH 6.8 and 8% separation gel at pH 8.8. Tris Gly solution (pH 8.8) was used as the electrolyte buffer solution. Both the sample solution and the electrolyte buffer solution as well as polyacrylamide gel contained 0.1 m*M* Ca^{2+} to maintain constant Ca^{2+} concentration throughout the PAGE process.

Surface Plasmon Resonance

SPR was used to analyze the binding between FIX and ACF II. BSA was used as a noninteracting control protein. Measurements were performed at 25°C using a Biacore 3000 instrument. Sensor surfaces were pretreated and then normalized by standard Biacore protocols.[18] Apo-FIX was diluted in immobilization buffer (1 μ*M*, in 10 m*M* sodium acetate, pH 5.0) and immobilized onto one flow cell of a CM5 chip. For coupling of apo-FIX to CM5 sensor surfaces, the surfaces were activated by a 2-min pulse of *N*-ethyl-*N*-(dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (10 μl/min in PBS buffer), followed by injection of the protein, and then deactivated by a 2-min pulse of ethanolamine (pH 8.5, 10 μl/min). ACF II or BSA at various concentrations (0–4 μ*M*) in 0.02*M* Tris–HCl (pH 7.4) containing 0.1 m*M* Ca^{2+} was flowed over the surface (10 μl/min, 20 μl injection with 300-s wash delay). The surface was regenerated between analyte injections with 1*M* NaCl and 1 m*M* EDTA (50 μl at 20 μl/min). The data were transferred into BiaEvaluation 4.1 and the kinetic parameters, on-rate (k_{on}) and off-rate (k_{off}) were obtained for each interaction by a fit of the data to a 1:1 Langmuir model. The association and dissociation constants (K_A and K_D) were determined by the quotients k_{on}/k_{off} and k_{off}/k_{on}, respectively (Scheme 1).

Circular Dichroism Measurements

CD measurements were carried out with a Jasco J-810 spectropolarimeter. The instrument was calibrated with d-10-camphorsulphonic acid.



$$\text{ACF II} + \text{FIX} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{ACF II-FIX}$$

SCHEME 1 The binding of ACF II to immobilized FIX.

All the CD measurements were made at 25°C with a thermostatically controlled cell holder. Far-UV CD spectra were collected between 190 nm and 250 nm with a scan speed of 20 nm/min and a response time of 1 s, in quartz cells of 1 mm path length. The obtained values were normalized by subtracting the baseline recorded for the buffer having same concentration of salts under similar conditions. The data were expressed in mean residue ellipticity [θ] in deg cm^2 $dmol^{-1}$, which is defined as $[\theta] = 100\theta_{obs}(lc)^{-1}$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeter. The secondary structure contents were estimated from the CD spectra by the LINCOMB program.[19]

RESULTS

Effect of ACF II on PT and TT In Vivo

ACF II was purified by a three-step chromatography procedure of anion-exchange chromatography, gel permeation chromatography and cation-exchange chromatography[9] and is shown to be homogeneous by SDS-PAGE and MALDI-TOF mass spectrometry. Recently, we have reported that ACF II markedly inhibits the intrinsic coagulation pathway as indicated by prolongation of APTT.[15] APTT is prolonged to more than six-fold by ACF II at a dose of 5 mg/kg. The inhibitions of ACF II on the extrinsic coagulation pathway and thrombin activity have been measured in vivo by PT and TT assays, respectively. The PT is the time taken for the plasma to clot after the tissue factor is added to citrated plasma. It is used to assess the extrinsic coagulation system of the blood.[20] The TT is the time required for plasma fibrinogen to form fibrin after exogenous thrombin is added to citrated plasma. It is a measure of the rate of conversion of fibrinogen to fibrin and is generally used to assess fibrinogen deficiency and functional disturbances in fibrinogen. The PT and TT of rats are 14.7 ± 1.2 s and 40.6 ± 2.3 s (mean ± SD, $n = 5$), respectively, for the saline group (control). As shown in Figure 1A, intravenous administration of ACF II prolongs PT in a dose-dependent manner. PT is prolonged to more than two-fold by ACF II at a dose of 5 mg/kg. Prolongation of PT suggests the inhibition of the extrinsic pathway, while prolongation of APTT suggests the inhibition of the intrinsic and/or common pathway in blood coagulation.[21] The intrinsic and extrinsic pathways converge by the formation of FXa.[22] FX is a critical coagulation factor for both intrinsic and extrinsic pathways, while FIX is an intrinsic coagulation factor. ACF II is a FX-binding protein, therefore the binding of ACF II to FX/FXa results in the prolongation of both APTT and PT. Interestingly, comparison of the effects of ACF II on APTT and PT shows that ACF II extends APTT more strongly than PT, suggesting that besides the binding to FX/FXa, ACF II probably also binds to FIX like other FIX/X-bps,[12,13] and enhances the inhibition of the intrinsic pathway. As shown in Figure 1B, no obvious prolongation of TT has been observed by ACF II at the dose up to 5 mg/kg, suggesting that ACF II does not inhibit thrombin activity and fibrin polymerization.[20,23]

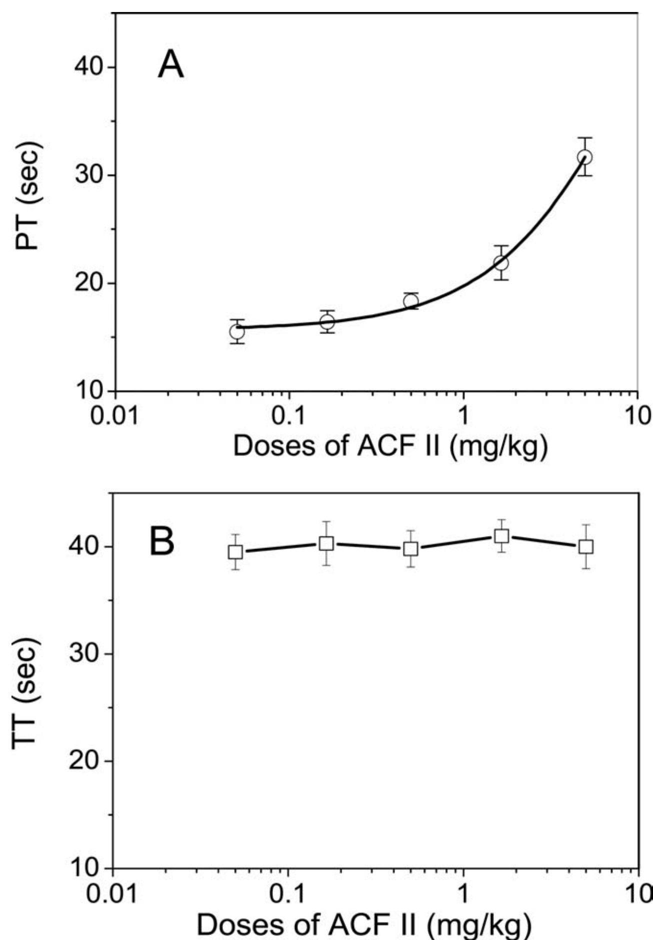


FIGURE 1 Effect of ACF II on PT and TT in vivo after i.v. administration of ACF II in anesthetized rats. ACF II was administered through the caudal vein in anaesthetized rats at selected doses. Blood samples were taken out 10 min after administration of ACF II, and then PT and TT were measured. Means± S.E.M., $n = 5$.

Binding of ACF II with FIX Assessed by PAGE

Native PAGE was used to examine whether ACF II binds to FIX in the presence of Ca^{2+}. As shown in Figure 2A, in the presence of 0.1 mM Ca^{2+}, the mixture of ACF II and FIX (molar ratio 1:1) produces a new band (Lane 3) and the bands corresponding to ACF II and FIX almost disappear, suggesting that ACF II should form a complex with FIX in the presence of 0.1 mM Ca^{2+}. When FIX was mixed with excessive ACF II (1 : 2 mol/mol) in the presence of 0.1 mM Ca^{2+}, two bands for ACF II and the complex of FIX and ACF

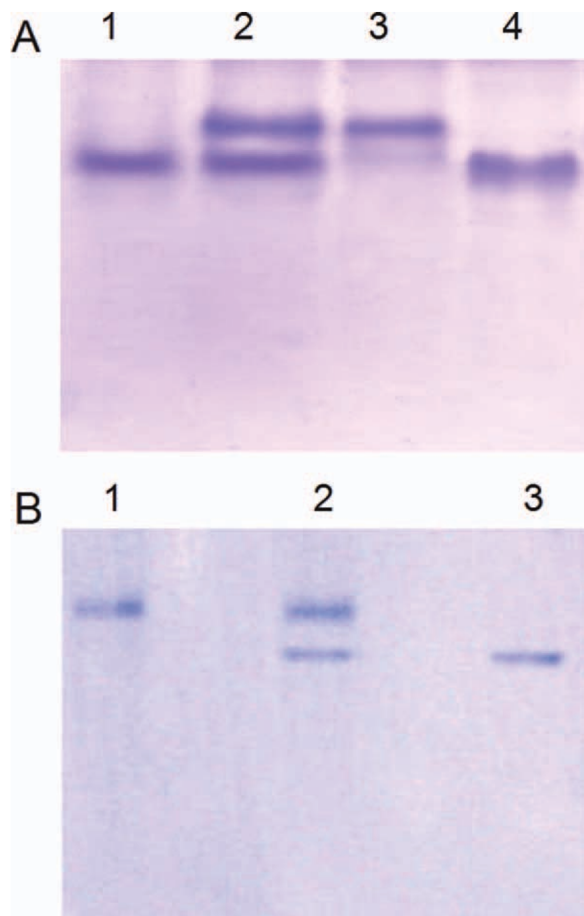


FIGURE 2 Native PAGE analysis of the binding of ACF II to FIX or BSA. (A) ACF II, FIX, and the mixture of ACF II and FIX were electrophoresed in the presence of 0.1 m*M* Ca(II). Lane 1, 3 μL ACF II; Lane 2, the mixture of 6 μL ACF II and 3 μL FIX; Lane 3, the mixture of 3 μL FIX and 3 μL ACF II; Lane 4, 3 μL FIX. (B) ACF II, BSA and the mixture of ACF II and BSA were electrophoresed in the presence of 0.1 m*M* Ca^{2+}. Lane 1, 3 μL ACF II; Lane 2, the mixture of 3 μL ACF II and 3 μL BSA; Lane 3, 3 μL BSA. The concentrations of ACF II, FIX and BSA are 15 μM.

II were observed on the gel (Lane 2). These results together indicate that ACF II and FIX forms a 1:1 complex in the presence of 0.1 m*M* Ca^{2+}. As shown in Figure 2B, in the presence of 0.1 m*M* Ca^{2+}, the mixture of ACF II and BSA (molar ratio 1:1) produces two bands (Lane 2), corresponding to the band of ACF II and the band of BSA, respectively, indicating that ACF II does not bind with BSA in the presence of 0.1 m*M* Ca^{2+}.

Binding of ACF II with FIX Assessed by SPR

We also used SPR spectroscopy to investigate the Ca^{2+}-induced binding between ACF II and FIX, using a CM5 chip to which apo-FIX was covalently attached. Figure 3A shows the association and dissociation curves for 3 μM apo-ACF II interacting with apo-FIX in the absence and presence of 0.1 m*M* Ca^{2+}. ACF II has been shown to bind to FIX in the presence of 0.1 m*M* Ca^{2+}. However, no obvious binding between apo-ACF II and apo-FIX has been observed in the absence of Ca^{2+}, indicating that ACF II binds with FIX in a Ca^{2+}-dependent manner. Figure 3B shows the specific binding between ACF II and FIX in the presence of 0.1 m*M* Ca^{2+}. The kinetic parameters, k_{on}, k_{off}, K_A, and K_D, were obtained for the interaction by a fit of the data to a 1:1 Langmuir model. The values of k_{on}, k_{off}, $K_{A,}$ and K_D are $(4.9 \pm 0.3)\times10^4$ M^{-1} s^{-1}, $(2.9 \pm 0.2)\times10^{-2}$ s^{-1}, $(1.7 \pm 0.2)\times10^6$ M^{-1}, and $(5.9 \pm 0.5) \times 10^{-7}$ M, respectively. The low K_D value indicates that ACF II binds FIX with high affinity in the presence of 0.1 m*M* Ca^{2+}. As shown in Figure 3C, no obvious binding between BSA and FIX has been observed in the presence of Ca^{2+}. These results together indicate that FIX specifically binds to ACF II.

To analyze the dependence on the Ca^{2+} concentration of the binding of ACF II to FIX, we studied the interaction between ACF II to FIX in the presence of Ca^{2+} of various concentrations. Figure 4A shows the association and dissociation curves for 3 μM ACF II interacting with FIX in the presence of increasing concentrations of Ca^{2+}. The surface charge density oscillation of the CM5 chip markedly increases with the increase of Ca^{2+} at low concentrations of Ca^{2+} (<0.10 m*M*) and obviously decreases with the increase of Ca^{2+} at high concentrations of Ca^{2+} (>0.10 m*M*) (Figure 4B). This result indicates that the binding of ACF II to FIX is dependent on the concentration of Ca^{2+} and the maximal binding of ACF II to FIX occurs at about 0.10 m*M* Ca^{2+}. However, Ca^{2+} at high concentrations (>0.10 m*M*) inhibits the binding between ACF II to FIX.

Effect of Ca^{2+} on the CD Spectrum of FIX

CD analysis was carried out to study the effect of Ca^{2+} on the secondary structure of FIX. As shown in Figure 5, the CD spectrum of FIX displays one positive peak at 195 nm and one negative peak at 207 nm with two small shoulders at 217 nm and 225 nm. Removal of Ca^{2+} from FIX by EDTA slightly decreases the positive peak at 195 nm and the negative peak at 207 nm. The secondary structure contents have been estimated by the LINCOMB software.[19] FIX contains 12% of α-helix, 28% of antiparallel β-sheet, 16% of β-turn/parallel β-sheet and 44% of other structures. The secondary structure contents of activated human FIX (FIXa) on the basis of the X-ray structure are 11% for α-helix and 30% for antiparallel β-sheet.[24] The α-helix and antiparallel β-sheet contents of FIX are very similar to that of FIXa, respectively, suggesting that FIX has a backbone structure similar to that

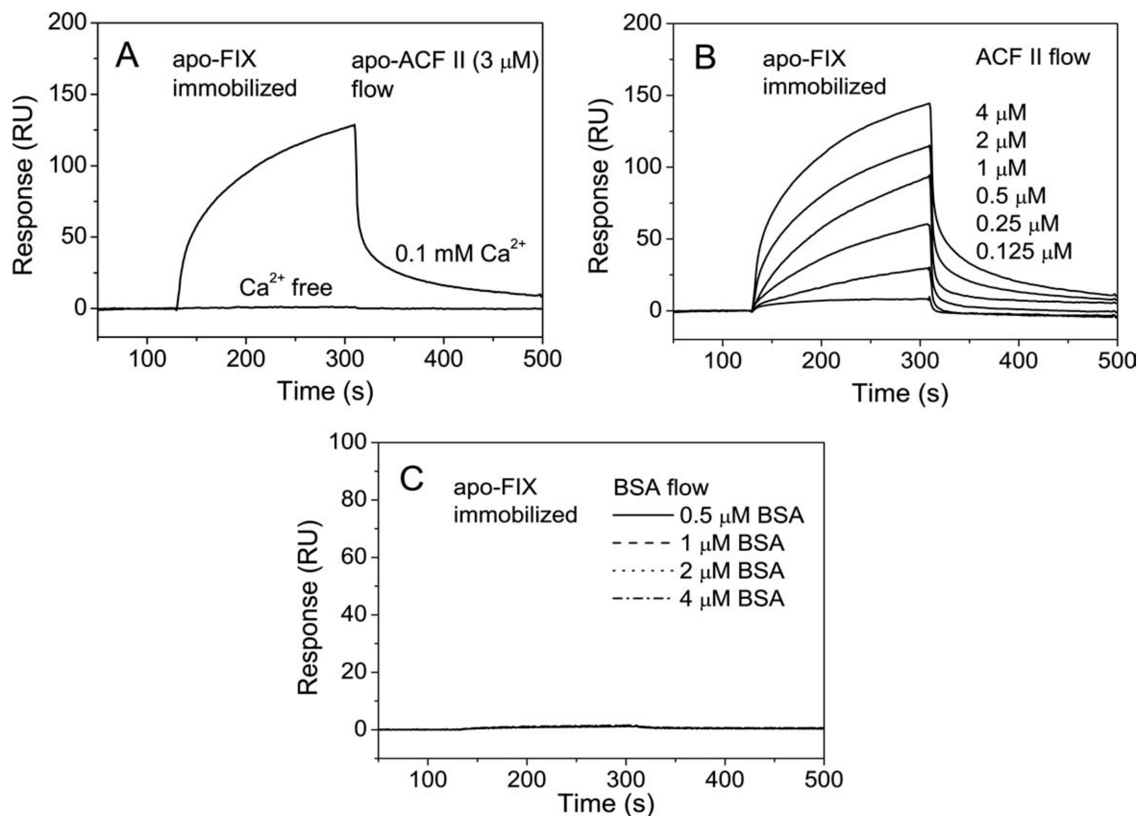


FIGURE 3 SPR kinetic analysis of Ca(II)-induced binding of FIX with ACF II or BSA. A: 3 *μM* apo-ACF II in 0.02*M* Tris-HCl (pH 7.4) in the presence and absence of 0.1 m*M* Ca(II) was injected over an immobilized apo-FIX surface for 2 min, and dissociation was monitored for 3 min. B: ACF II in 0.02*M* Tris-HCl (pH 7.4) containing 0.1 m*M* Ca(II) was injected over an apo-FIX-immobilized surface at concentrations of 0.125, 0.25, 0.5, 1, 2, 4 *μM* for 2 min, and dissociation was monitored for 3 min. (C) BSA in 0.02*M* Tris-HCl (pH 7.4) containing 0.1 m*M* Ca(II) was injected over an apo-FIX-immobilized surface at concentrations of 0.5, 1, 2, 4 *μ*M for 2 min, and dissociation was monitored for 3 min. A 1:1 Langmuir model was used to fit the data.

of FIXa. Apo-FIX contains 10% of α-helix, 31% of antiparallel *β*-sheet, 16% of *β*-turn/parallel *β*-sheet, and 43% of other structures. The removal of Ca^{2+} from FIX results in a slight decrease in the α-helix content and a slight increase in the antiparallel *β*-sheet, indicating that Ca^{2+} has a slight effect on the secondary structure of FIX.

DISCUSSION

IX/X-bps from various snake venoms have different binding affinities for FIX and FX.[8,9,25,26] According to their binding targets, these proteins can be divided into three groups: FIX binding proteins (IX-bps), FX binding proteins (X-bps), and IX/X-bps. All IX/X-bps bind with the Gla domain of FIX or FX in a Ca^{2+}-dependent manner. Previously, ACF II from the venom of *Agkistrodon acutus* has been identified as a Ca^{2+}-dependent X-bp.[14] Present results firstly show that ACF II also binds FIX in a Ca^{2+}-dependent manner. Therefore, ACF II is an IX/X-bp that prolongs the clotting time in vivo through its binding with both FIX and FX.

By using SPR technique, the K_D value between ACF II and FIX in the presence of 0.1 mM Ca^{2+} has been determined to be $(5.9 \pm 0.5) \times 10^{-7}$ M, which is close to that between ACF II and FXa $[(6.0 \pm 0.9)\times10^{-7}$ M] [15]. This result suggests that ACF II has a similar binding-affinity to FIX and FX. The binding of ACF II with FX/FXa results in the inhibition of both intrinsic and extrinsic coagulation pathways and the prolongation of both APTT and PT, while binding of ACF II with FIX results in the inhibition of the intrinsic coagulation pathway and the prolongation of APTT. As mentioned early, APTT is prolonged to more than six-fold by ACF II at a dose of 5 mg/kg.[15] Figure 1A shows that PT is prolonged to more than two-fold by ACF II at a dose of 5 mg/kg. ACF II extends APTT more strongly than PT, indicating that the binding of ACF II with FIX may play a dominant role in the anticoagulation of ACF II in vivo.

FIX is Ca^{2+}-binding protein with multiple Ca^{2+}-binding sites in its Gla domain.[27,28] The Gla domain is responsible for Ca^{2+}-dependent phospholipid membrane binding. Ca^{2+} ions are required to maintain in vivo function of FIX Gla domain during blood coagulation. Ca^{2+} has a slight effect on the secondary structure of FIX (Figure 5). Ca^{2+} may be required to maintain native conformation of FIX Gla domain. The binding of Ca^{2+} ions to FIX Gla domain and subsequent conformational rearrangement may be essential for its recognition of ACF II. In addition to the function of Ca^{2+} ions to maintain native conformation of FIX Gla domains, Ca^{2+} ions have been found to form a bridge between habu IX-bp from habu snake venom and FIX Gla domain as well as between the phosphatidylserine of membrane and FIX Gla domain.[27] Although we cannot infer whether the interaction of FIX with ACF II also involves Ca^{2+} ion bridging between them from the present data, it is certain that Ca^{2+} ions are required to maintain in vivo function of FIX Gla domain for its recognition of ACF II.

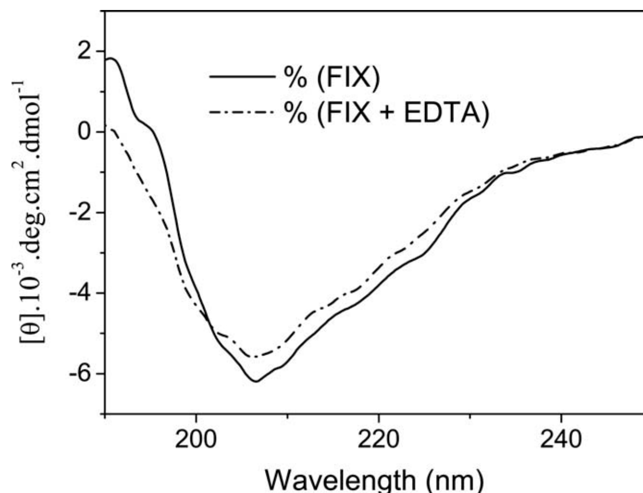


FIGURE 5 Effect of Ca(II) on the circular dichroism spectrum of FIX. The far-UV circular dichroism spectra of 10 μM FIX in 0.02 M Tris-HCl buffer (pH 7.4) at 25°C in the presence and absence of 1 mM EDTA.

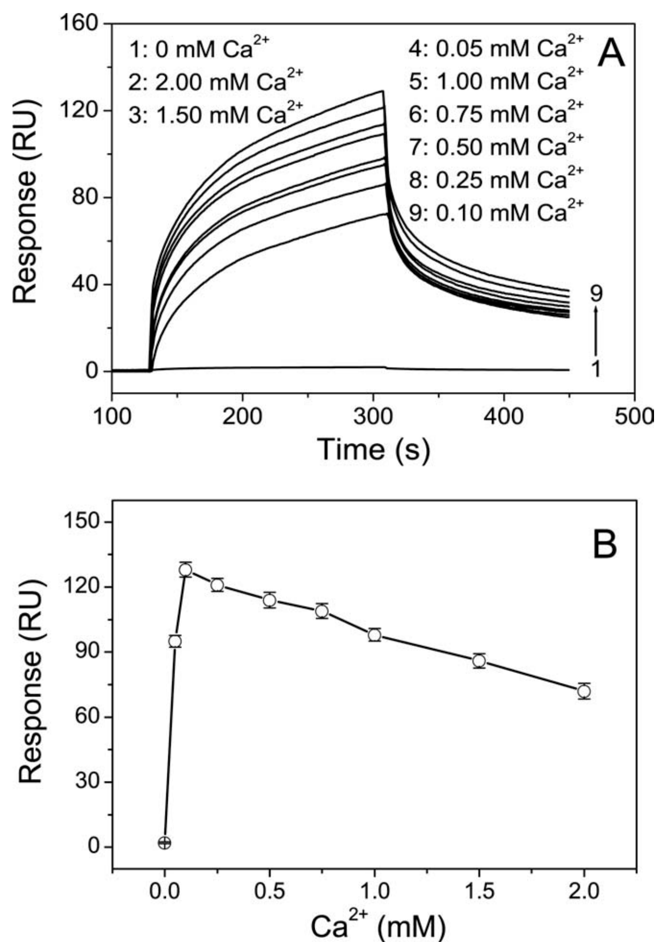


FIGURE 4 SPR analysis of the effect of the Ca(II) concentration on the binding of ACF II to FIX. A: 3 μM ACF II in 0.02M Tris-HCl (pH 7.4) containing 0, 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00 mM Ca(II) was injected over an apo-FIX-immobilized surface for 2 min, and dissociation was monitored for 3 min. B: The effect of Ca(II) concentration on the surface charge density oscillation of the CM5 chip at the end of ACF II injection. Each point represents the average of triplicate determinations.

As shown in Figure 4, Ca^{2+} at low concentrations (<0.10 mM) can induce the binding of ACF II to FIX. However, Ca^{2+} at high concentrations (>0.10 mM) inhibits the binding between ACF II to FIX. To our knowledge, Ca^{2+} has never been found to inhibit the binding between IX/X-bps and FIX or FX. Therefore, Ca^{2+} has been found, for the first time, to function as a switch for the binding between ACF II and FIX. Further investigation is necessary to elucidate the complex role of Ca^{2+} in the anticoagulation of ACF II.

Based on the results of this study it can be concluded that ACF II not only binds FX but also binds FIX in a Ca^{2+}-dependent manner. The most important and new finding of this study is revealing that Ca^{2+} has been found, for the first time, to function as a switch for the binding between ACF II and FIX. The next important finding of this study is revealing that the binding of ACF II with FIX plays a dominant role in the anticoagulation of ACF II in vivo. These findings will improve our understanding of the anticoagulant mechanism of ACF II as well as the role of Ca^{2+} in the binding of ACF II with FIX.

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