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Anticoagulant Mechanism of Factor IX/factor X-binding Protein Isolated from the Venom of *Trimeresurus flavoviridis*

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Anticoagulant mechanism of the coagulation factor IX/factor X-binding protein (IX/X-bp) isolated from the venom of *Trimeresurus flavoviridis* was investigated. IX/X-bp had no effect on the amidase activity of factor Xa measured with a synthetic peptide substrate Boc-Leu-Gly-Arg-*p*NA. Prothrombin activation by factor Xa without cofactors, such as factor Va and phospholipids, was only slightly influenced by IX/X-bp. However, prothrombin activation by factor Xa in the presence of factor Va resulted in IX/X-bp inhibiting the increase of k_{cat} of thrombin formation through inhibition of interaction between factor Xa and factor Va. IX/X-bp also inhibited the decrease of K_m for thrombin formation through interaction with phospholipids. Thus, IX/X-bp appears to act as an anticoagulant protein by inhibiting the interaction between factor Xa and its cofactors in the prothrombinase complex by binding to the Gla domain of factor Xa.

Key words: anticoagulant protein, coagulation factor IX, factor X, IX/X-bp, prothrombinase.

Abbreviations: IX/X-bp, blood coagulation factor IX/factor X-binding protein isolated from the venom of *T. flavoviridis*; IX-bp, factor IX-binding protein; EGF, Epidermal growth factor; Gla, γ-carboxyglutamic acid.

Factor IX/factor X-binding protein (IX/X-bp) is an anticoagulant protein isolated from the venom of *Trimeresurus flavoviridis* (habu snake) (*1*). Both factors IX and X are serine protease zymogens composed of a γ-carboxyglutamic acid (Gla)-containing domain, two epidermal growth factor (EGF) domains, an activation peptide, and a serine protease region. Previous studies revealed that IX/X-bp binds to the Gla domain of coagulation factors IX and X in the presence of Ca^{2+} ions in a 1 : 1 stoichiometry but not to that of prothrombin (*1*, *2*). IX/X-bp is a heterodimer consisting of two chains with primary structures that are 15–25% similar to those of mammalian C-type lectins, such as mannose-binding protein, asialoglycoprotein receptors and lithostathine (*3*). X-ray crystallographic studies showed that a 'domain swapping' structure exists between the two chains of IX/X-bp (*4*) and that the concave surface of these two chains interacts with the isolated Gla domain peptide of coagulation factors IX and X (*5*, *6*). To date, several IX/X-bp-like proteins have been found; IX-bp from *T. flavoviridis* bind factor IX/IXa, but not factor X/Xa, and X-bp from *Deinagkistrodon acutus* specifically bind only factor X/Xa (*7*, *8*).

The blood coagulation system involves sequential proteolytic reactions that activate the zymogen and form a complex between the activated enzyme and its cofactors, which then activates other zymogens. The prothrombinase complex, in which prothrombin is converted to thrombin by a serine protease factor Xa, is composed of factor Xa, phospholipids, Ca^{2+} ions and the activated protein cofactor factor Va. In the conversion of prothrombin to thrombin through factor Xa catalysis, the effects of prothrombinase cofactors on kinetics have been well investigated (*9*). It has been reported that relative rates of prothrombin activation by factor Xa are increased 2.3-fold by Ca^{2+} ions; 26.7-fold by Ca^{2+} ions and phospholipids; 433-fold by Ca^{2+} ions and factor Va, and over 330,000-fold by the combination of all three cofactors: Ca^{2+} ions, phospholipids and factor Va (*10*). While the binding of the Gla domain of factor Xa to the phospholipid membrane surface in the presence of Ca^{2+} ions diminishes K_m for prothrombin activation, the binding of factor Xa with factor Va to the phospholipid membrane dramatically increases V_{max}. Moreover, IX/X-bp dose-dependently prolongs the kaolin-induced partial thromboplastin time and the factor Xa-induced clotting time (*1*). In the present study, we investigated the binding properties of IX/X-bp and IX-bp, a IX/X-bp homologue that binds selectively to factor IX, by SPR and attempted to elucidate the molecular mechanism of the anticoagulant activity of IX/X-bp by kinetic analysis of prothrombin activation. Here we demonstrated that IX/X-bp is unique anticoagulants, which block the formation of prothrombinase complex with no inhibitory effect on active site of the enzyme. The results of present study provide insight into the anticoagulant mechanism of IX/X-bp and its related proteins and may also assist in the design of novel anticoagulant molecules.

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MATERIALS AND METHODS

Materials—IX/X-bp was purified as described elsewhere (*1*). Bovine factor IX, factor X and prothrombin were isolated from bovine plasma by a previously published method (*11*). Factor Xa was prepared as previously described (*12*). Phospholipid vesicles were prepared according to the method of Krishnaswamy *et al*. (*13*) using dioleoyl-phosphatidyl choline and phosphatidyl serine (3:1, w/w). Factor V was purified from bovine plasma by the method of Nesheim *et al*. (*14*), and factor Va was prepared by the method of Esmon (*15*). Boc-Leu-Gly-Arg-*p*NA and Boc-Val-Pro-Arg-*p*NA were purchased from Seikagaku coporation (Tokyo, Japan).

Binding Kinetics of IX/X-bp and IX-bp to Immobilized Factors IX and X—The interaction between anticoagulant proteins and immobilized factors IX and X was investigated using Biacore 3000 biosensor instrument (Biacore, Uppsala, Sweden). Factors IX and X (both 200 nM) were immobilized on a CM5 sensor chip with an amine coupling kit. Anticoagulant proteins were diluted with 10 mM HEPES-buffered saline containing 0.005% Surfactant P20 and 5 mM $CaCl_2$ and injected into a flowcell at a flow rate of 20 μl/min. The binding affinity (K_d) was determined using BIAevaluation Software ver. 4.0.

Measurement of Amidase Activity of Factor Xa—Factor Xa was incubated at 37°C in 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl, 5 mM $CaCl_2$ and 0.5 mg/ml BSA with or without a 5-fold molar excess of IX/X-bp. The sample was added to Boc-Leu-Gly-Arg-*p*NA at the final concentrations of 0.04–0.1 mM and the synthetic substrate was hydrolyzed by factor Xa in 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl and 0.5 mg/ml BSA at 37°C. Production of *p*-nitroaniline was continuously monitored at 405 nm.

Measurement of Kinetic Constants—The kinetic constants of the formation of α-thrombin was measured according to Rosing *et al*. (*10*) using Boc-Val-Pro-Arg-*p*NA as a substrate for thrombin. As IX/X-bp does not bind thrombin (*1*), the effects of IX/X-bp on reconstituted prothrombinase and related complexes could be measured in this system. Briefly, factor Xa alone or in the presence of the cofactors (phospholipids and factor Va) was preincubated for 5 min at 37°C in 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl, 5 mM $CaCl_2$ and 0.5 mg/ml BSA. The enzymatic reaction was started by the addition of prothrombin. After different time intervals, samples were taken and added to a cuvette (thermostated at 37°C) containing 0.1 mM Boc-Val-Pro-Arg-*p*NA. Production of *p*-nitroaniline was monitored at 405 nm. We carried out at least 3–5 independent experiments for all combinations and each figure represents typical result.

RESULTS AND DISCUSSION

Binding Properties of IX/X-bp and IX-bp to Immobilized Factors IX and X—To clarify the inhibitory mechanism of snake venom anticoagulants, we first analysed the binding properties of IX/X-bp and IX-bp to factors IX and X using Biacore. The binding sensorgram and parameters are shown in Fig. 1 and Table 1, respectively.

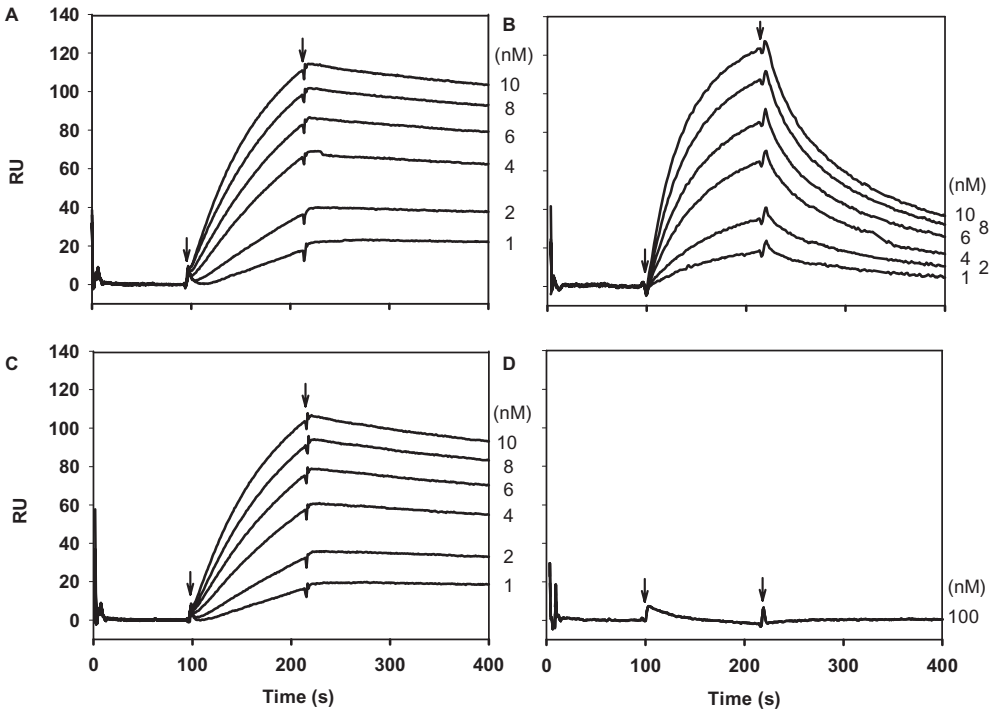

Fig. 1. **Binding properties of anticoagulant proteins to factor IX and factor X.** Binding of IX/X-bp (upper panel) and IX-bp (lower panel) to factor IX (A and C) and factor X (B and D) immobilized on a CM5 sensor chip were investigated. IX/X-bp and IX-bp were diluted with 10 mM HEPES-buffered saline containing 5 mM Ca^{2+} ions and 0.005% Surfactant P20 at concentrations of 1, 2, 4, 6, 8 and 10 nM (A, B and C). In (D), the concentration of IX-bp was 100 nM. RU, resonance units. Arrows indicate start and end points of the injection.

IX/X-bp and IX-bp directly bound to factor IX with K_ds of $\sim 10^{-10}$ M, which was completely dependent on the physiological Ca^{2+}-ion concentration (data not shown). In contrast, only IX/X-bp bound to factor X with a K_d of $\sim 10^{-8}$ M but IX-bp did not. The affinity results obtained by Biacore are comparable with our previous results using ELISA (*2*, *7*). Recently, another group claimed that IX-bp binds not only factor IX, but factor X with essentially equal affinities (*16*). Our previous work shows that IX-bp specifically inhibits factor IXa-induced plasma clotting, but not factor Xa-induced (*7*); indicating that IX-bp could be a specific inhibitor of factor IXa, consistent with our binding analysis. In the opposing report, the interaction among them was evaluated using immobilized IX-bp, in contrast to our present study (using immobilized factors IX and X); therefore this difference may be caused by the difference of experimental procedure.

No Influence of IX/X-bp on the Amidase Activity of Factor Xa—The influence of IX/X-bp on the amidase activity of factor Xa was investigated with the peptide substrate Boc-Leu-Gly-Arg-*p*NA. As shown in Fig. 2, a 5-fold molar excess of IX/X-bp had no effect on Boc-Leu-Gly-Arg-*p*NA hydrolase activity of 2.2×10^{-8} M factor Xa. These results indicate that the binding of IX/X-bp to the Gla domain of factor Xa does not influence the enzymatic activity of the active site of factor Xa against small peptide substrate such as Boc-Leu-Gly-Arg-*p*NA. Therefore, the influence of the cofactors, factor Va and phospholipids, to factor Xa may be involved in the anticoagulant activity of IX/X-bp.

Table 1. **Kinetic parameters for binding of anticoagulant proteins to immobilized F.IX and F.X.**

	K_a ($M^{-1} \cdot s^{-1}$)	K_{diss} (s^{-1})	K_d (M)
IX/X-bp			
F.IX	$(8.5 \pm 4.7) \times 10^4$	$(2.6 \pm 1.0) \times 10^{-5}$	$(3.4 \pm 0.5) \times 10^{-10}$
F.X	$(1.2 \pm 0.5) \times 10^4$	$(4.6 \pm 2.3) \times 10^{-4}$	$(3.0 \pm 0.6) \times 10^{-8}$
IX-bp			
F.IX	$(2.0 \pm 1.0) \times 10^4$	$(1.4 \pm 0.7) \times 10^{-5}$	$(6.6 \pm 2.0) \times 10^{-10}$
F.X	n.s.b.	n.s.b.	n.s.b.

n.s.b., not specific binding.
The data are means ± SE ($N = 8$).

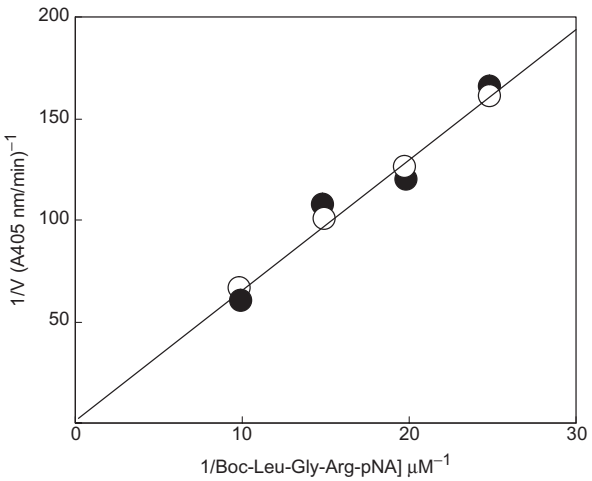

Fig. 2. **Effect of IX/X-bp on the amidase activity of factor Xa.** Factor Xa (2.2×10^{-8} M) with (filled circle) or without (open circle) 5-fold molar excess of IX/X-bp was incubated for 5 min at 37°C in 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl, 5 mM $CaCl_2$ and 0.5 mg/ml BSA. The reaction was started by the addition of the synthetic peptide substrate, Boc-Leu-Gly-Arg-*p*NA, at the final concentrations of 0.04–0.1 mM. The absorbance at 405 nm was continuously recorded and 1/v was plotted against 1/[Boc-Leu-Gly-Arg-*p*NA] to give a K_m of 0.57 mM.

Effects of IX/X-bp on the Kinetics of Prothrombin Activation—The kinetics of the conversion of prothrombin to thrombin by factor Xa has been determined in the absence and presence of the cofactors, factor Va and phospholipids (Table 2, Figs 3–6). These results are consistent with those reported by Rosing *et al*. (*10*). We next investigated the effects of IX/X-bp on the kinetics of prothrombin activation by factor Xa (Table 2, Figs 3–6). Prothrombin activation by 1.1×10^{-7} M factor Xa without cofactors did not change K_m and k_{cat} in the presence of IX/X-bp (Fig. 3). Factor Xa activated prothrombin, as in the case of the small peptide substrate, even in the presence of 1,000-fold molar excess IX/X-bp (Supplementary Fig. 1). These results indicate that IX/X-bp has no influence on the factor Xa active site and its prothrombin-binding site(s).

As a 2.54-fold increase in K_m for prothrombin and a 58% decrease in k_{cat} by IX/X-bp were observed in the presence of factor Va, IX/X-bp decreased the k_{cat}/K_m to 16% of that without IX/X-bp (Fig. 4). Moreover, prothrombin activation by factor Xa in the presence of phospholipids resulted in a 16-fold increase in K_m for prothrombin and a 83% decrease in k_{cat}/K_m by IX/X-bp (Fig. 5). In the complete prothrombinase complex, IX/X-bp decreased the k_{cat}/K_m to 32% of the control (Fig. 6).

The considerable increase in K_m for prothrombin by IX/X-bp in the presence of phospholipids can be explained by the X-ray crystallographic study data that indicates IX/X-bp interrupts the binding of factor Xa to the phospholipid membrane surface and the formation of the prothrombinase complex. The X-ray crystallographic study of the complex between the factor X Gla domain peptide and X-bp, a IX/X-bp homologue isolated from the venom of *D. acutus*, revealed that the anticoagulant protein traps the Gla domain peptide in its concave surface formed by the two, A and B, chains (*5*). The hydrophobic residues in the loop of B chain of X-bp (Arg112-Met113-Ile114) interact with the hydrophobic residues in the N-terminal loop, Phe4, Leu5 and Val8, of the factor X Gla domain peptide (*5*). X-bp also binds to the negatively charged surface of the factor X Gla domain, Gla25-Gla32, by a cluster of positively charged residues through nine salt bridges. Moreover, Phe4, Leu5 and Val8 of the factor Xa Gla domain (so-called ω-loop) are the predicted binding sites of phospholipids. Therefore, in the presence of IX/X-bp, factor Xa may not bind to the phospholipid membrane surface due to the putative phospholipid-binding sites Phe4, Leu5 and Val8 that are blocked by IX/X-bp. This may explain the effects of IX/X-bp observed in the present study (Fig. 5), in which IX/X-bp interrupts the cofactor(s)-enzyme-substrate complex formation between phospholipids, factor Xa and prothrombin, and dramatically increases K_m for prothrombin. Actually the triplet sequence is conserved in B chains of both IX/X-bp and

Table 2. **Effect of IX/X-bp to kinetic constants of thrombin formation with various prothrombin.**

		IX/X-bp (mol/mol Xa)	K_m (μM)	K_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \times \mu M^{-1}$)	Activity (%)
(i)	Xa	-	43	0.33	0.008	100
	+ IX/X-bp	10	45	0.26	0.006	75
(ii)	Xa • Va	-	13	125	9.9	100
	+ IX/X-bp	100	33	52	1.6	16
(iii)	Xa • PL	-	0.51	2.6	5.1	100
	+ IX/X-bp	10	8.17	7.12	0.9	17
(iv)	Xa • PL • Va	-	0.076	4,900	64,500	100
	+ IX/X-bp	100,000	0.13	2,700	20,800	32

It was summarized on the Table from Figs 3 to 6. Prothrombin-activating mixture (i) Xa (1.1×10^{-7} M) alone, (ii) Xa (6.6×10^{-10} M) Va (4 U/ml), (iii) Xa (4.4×10^{-9} M) PL (10 μM), (iv) Xa (2.2×10^{-12} M) PL (2 μM) Va (0.8 U/ml) with or without IX/X-bp.

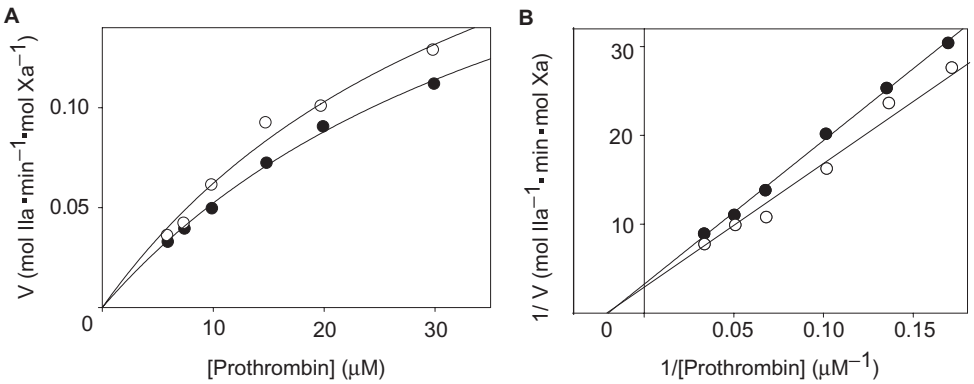

Fig. 3. **Prothrombin activation by factor Xa.** (A) A υ versus [Prothrombin] plot and (B) Lineweaver-Burk plot. Prothrombin (6–30 μM) was activated by factor Xa (1.1×10^{-7} M) in the absence (open circle) or presence (filled circle) of IX/X-bp (with 10-fold molar excess of factor Xa). Thrombin formation was measured using a synthetic peptide substrate as described in 'MATERIALS AND METHODS' section. Representative data are shown.

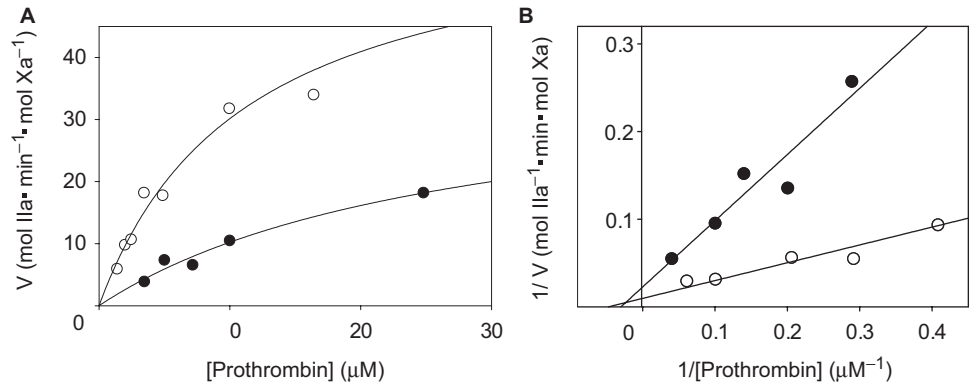

Fig. 4. **Prothrombin activation by factor Xa in the presence of factor Va.** (A) A υ versus [Prothrombin] plot and (B) Lineweaver-Burk plot. Prothrombin (2.6–26 μM) was activated by factor Xa (6.6×10^{-10} M) with factor Va (4 U/ml) in the absence (open circle) or presence (filled circle) of IX/X-bp (with 100-fold molar excess of factor Xa). Thrombin formation was measured using a synthetic peptide substrate as described in 'MATERIALS AND METHODS' section. Representative data are shown.

IX-bp (Arg112-Met113-Met114). Crystal structure studies of Gla domain complex indicate that A chain would be determinant for the specificity to Gla domain (*5*, *6*, *8*), however, we could not find out the responsible sequence.

IX/X-bp decreased k_{cat}/K_m in the prothrombin activation by factor Xa with factor Va (Fig. 4) and therefore the enzymatic activity of factor Xa enhanced by factor Va was repressed by IX/X-bp. As it has been reported that the factor Xa Gla domain and EGF1 domain do not interact directly with factor Va (*17*), the binding of IX/X-bp to the Gla domain of factor Xa does not directly influence complex formation between factor Va and factor Xa, but in fact may hinder prothrombin approaching the factor Xa active site due to a conformational change of factor Xa or some other steric hindrance.

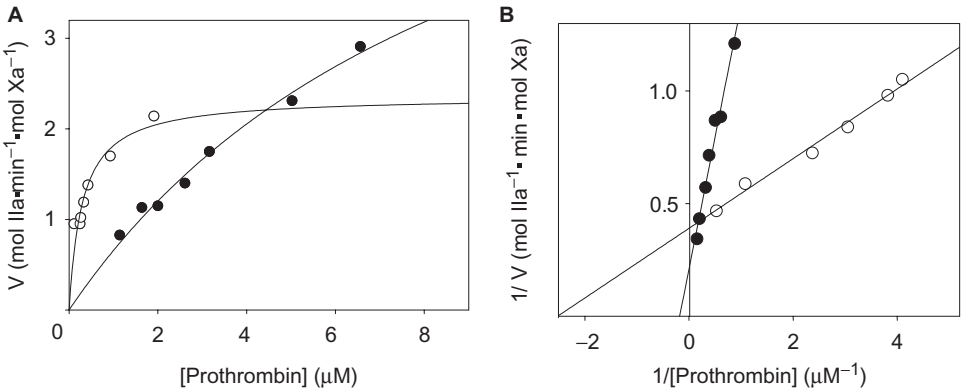

Fig. 5. **Prothrombin activation by factor Xa in the presence of phospholipids.** (A) A υ versus [Prothrombin] plot and (B) Lineweaver-Burk plot. Prothrombin (1–6.9 μM) was activated by factor Xa (4.4×10^{-9} M) with phospholipids (10 μM) in the absence (open circle) or presence (filled circle) of IX/X-bp (with 10-fold molar excess of factor Xa). Thrombin formation was measured using a synthetic peptide substrate as described in 'MATERIALS AND METHODS' section. Representative data are shown.

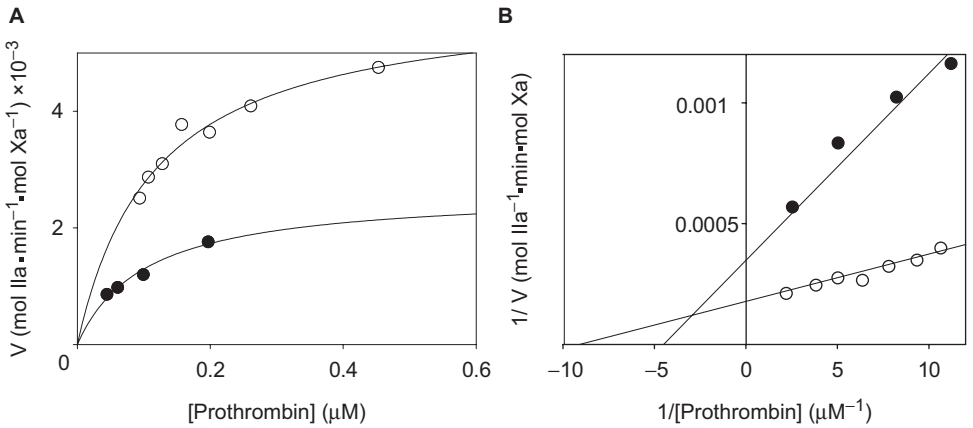

Fig. 6. **Prothrombin activation by factor Xa in the presence of phospholipids and factor Va.** (A) A υ versus [Prothrombin] plot and (B) Lineweaver-Burk plot. Prothrombin (0.05–1.0 μM) was activated by factor Xa (2.2×10^{-12} M) with phospholipids (2 μM) and factor Va (0.8 U/ml) in the absence (open circle) or presence (filled circle) of IX/X-bp (with 100,000-fold molar excess of factor Xa). Thrombin formation was measured using a synthetic peptide substrate as described in 'MATERIALS AND METHODS' section. Representative data are shown.

The less pronounced effect of IX/X-bp on the complete prothrombinase complex is likely due to a lower concentration of factor Xa (2.2×10^{-12} M) that was used than K_d between factor Xa and IX/X-bp (Table 2, Fig. 6) to measure K_m and k_{cat}. At such low concentrations of factor Xa, the complex of factor Xa and IX/X-bp may dissociate, while at higher concentrations, the activation rate of prothrombin is too fast to determine the kinetics. In fact, prothrombin activation by reconstituted prothrombinase complex using relatively high concentration of factor Xa (2×10^{-10} M factor Xa, 2×10^{-10} M factor Va, and 0.2 μg/ml phospholipids) was almost completely inhibited by 200-fold molar excess of IX/X-bp, although only slight inhibition could be observed on factor Xa, even in 1,000-fold excess of IX/X-bp (Supplementary Fig. 1).

In our preliminary study, the kinetics of the activation of factor X by factor VIIa was also not influenced by IX/X-bp in the absence of cofactors (data not shown). However, IX/X-bp increased K_m for factor X activation by factor VIIa in the presence of phospholipids, and decreased V_{max} in the presence of tissue factor (data not shown), indicating that enzyme cofactor(s) such as phospholipids, tissue factor and factor Va are necessary for the expression of IX/X-bp anticoagulant activity.

In the present study, we investigated the binding affinity between factors IX and X and IX/X-bp and IX-bp using Biacore and determined the anticoagulant mechanism of IX/X-bp on prothrombin activation by factor Xa by means of kinetics. We conclude that IX/X-bp acts as an anticoagulant protein by the inhibition of the complex formation between factor Xa and its cofactors in the prothrombinase by binding to the Gla domain of factor Xa. Although numerous numbers of anticoagulant proteins/peptides have been found in several organisms, such as snakes, ticks and leeches (*18*, *19*). Our findings show that IX/X-bp and its related proteins are unique anticoagulants, which do not affect enzymatic activity of coagulation proteases. Our results would help to clarify the molecular mechanism of these proteins, and also would be useful in the design of novel molecules for anticoagulant therapy.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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