

## BATROXOBIN BINDS FIBRIN WITH HIGHER AFFINITY AND PROMOTES CLOT EXPANSION TO A GREATER EXTENT THAN THROMBIN

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Running title: Mechanism of batroxobin binding to fibrinogen and fibrin

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**Background:** Snake venom protease batroxobin clots fibrinogen in a manner distinct from thrombin.

**Results:** Batroxobin binds fibrin(ogen) with higher affinity than thrombin and promotes greater clot expansion.

**Conclusion:** Batroxobin's distinctive interaction with fibrin(ogen) may contribute to its unique pattern of fibrinopeptide release.

**Significance:** Clinically, batroxobin is used as a defibrinogenating agent, but its capacity to promote clot expansion may promote microvascular thrombosis.

### SUMMARY

Batroxobin is a thrombin-like serine protease from the venom of *Bothrops atrox moojeni* that clots fibrinogen. In contrast to thrombin, which releases fibrinopeptide A and B from the NH<sub>2</sub> terminal domains of the A $\alpha$ - and B $\beta$ - chains of fibrinogen, respectively, batroxobin only releases fibrinopeptide A. Because the mechanism responsible for these differences is unknown, we compared the interactions of batroxobin and thrombin with the predominant  $\gamma_A/\gamma_A$  isoform of fibrin(ogen) and the  $\gamma_A/\gamma'$  variant with an extended  $\gamma$ -chain. Thrombin binds to the  $\gamma'$ -chain and forms a higher affinity interaction with

$\gamma_A/\gamma'$ -fibrin(ogen) than  $\gamma_A/\gamma_A$ -fibrin(ogen). In contrast, batroxobin binds both fibrin(ogen) isoforms with similar high affinity (K<sub>d</sub> values of about 0.5  $\mu$ M) even though it does not interact with the  $\gamma'$ -chain. The batroxobin binding sites on fibrin(ogen) only partially overlap with those of thrombin because thrombin attenuates, but does not abrogate, the interaction of  $\gamma_A/\gamma_A$ -fibrinogen with batroxobin. Furthermore, although both thrombin and batroxobin bind to the central E region of fibrinogen with a K<sub>d</sub> value of 2-5  $\mu$ M, the  $\alpha$ 17-53 and B $\beta$ 1-42 regions bind thrombin, but not batroxobin. Once bound to fibrin, the capacity of batroxobin to promote fibrin accretion is 18-fold greater than that of thrombin; a finding that may explain the microvascular thrombosis that complicates envenomation by *Bothrops atrox moojeni*. Therefore, batroxobin binds fibrin(ogen) in a manner distinct from thrombin, which may contribute to its higher affinity interaction, selective fibrinopeptide A release, and prothrombotic properties.

Fibrinogen is important for optimal primary and secondary hemostasis because of its critical role in platelet aggregation and fibrin clot formation (1). Fibrinogen is a dimeric glycoprotein composed of two pairs of

$\alpha$ -,  $\beta$ - and  $\gamma$ -chains connected by numerous disulfide bonds (2). The  $\text{NH}_2$ -termini of the six chains form the central E domain, which is connected by the coiled-coil regions to the peripheral D-domains formed by the  $\text{COOH}$ -termini (2). There are two circulating isoforms of fibrinogen that differ with respect to their  $\gamma$ -chains. The predominant fibrinogen isoform is a homodimer that contains two  $\gamma_A$ -chains consisting of 411 residues and is designated  $\gamma_A/\gamma_A$ -fibrinogen. About 10-15% of circulating fibrinogen is heterodimeric, containing one  $\gamma_A$ -chain and one variant  $\gamma'$ -chain that possesses a 16-residue anionic extension at its  $\text{COOH}$ -terminus. This minor fibrinogen population is designated  $\gamma_A/\gamma'$ -fibrinogen (3-5). Epidemiological studies suggest that elevated levels of circulating  $\gamma_A/\gamma'$ -fibrinogen are associated with an increased risk of cardiovascular disease (6,7).

Thrombin is the protease that converts fibrinogen to insoluble fibrin by releasing fibrinopeptides (Fp)<sup>1</sup> A and B from the  $\text{NH}_2$ -termini of the  $\alpha$ - and  $\beta$ -chains, respectively (8). Fibrin polymerization occurs when the newly exposed  $\text{NH}_2$ -termini on one fibrin monomer bind to pre-existing, complementary sites on the D-domains of adjacent fibrin monomers (2,8,9). During this process, some thrombin remains bound to the fibrin clot (10). Thrombin possesses two anion binding sites that flank the active site and mediate substrate binding and catalysis (11,12). Exosite 1 mediates thrombin binding to the  $\alpha$ - and  $\beta$ -chains in the central E-region of fibrin(ogen), whereas exosite 2 is responsible for thrombin's interaction with the unique  $\text{COOH}$ -terminus of the  $\gamma'$ -chain of a second fibrin(ogen) molecule (13,14). Consequently, thrombin binds  $\gamma_A/\gamma_A$ -fibrin solely via exosite 1 with a  $K_d$  value of 2 to 4  $\mu\text{M}$ . In contrast, both exosites are engaged when thrombin binds to  $\gamma_A/\gamma'$ -fibrin(ogen), which results in a ~20-fold higher affinity interaction than that with  $\gamma_A/\gamma_A$ -fibrin ( $K_d$  value of 80 to 200 nM) (15,16). Because fibrin-bound thrombin retains activity and is protected from inhibition by antithrombin and heparin cofactor II, the fibrin clot serves as a reservoir of thrombin that promotes thrombus expansion by locally activating platelets and factor (f) XI,

fVIII and fV (10,17). The higher affinity, bivalent interaction of thrombin with  $\gamma_A/\gamma'$ -fibrin affords thrombin more protection from fluid-phase inhibitors than the univalent interaction of thrombin with  $\gamma_A/\gamma_A$ -fibrin (18).

Batroxobin is a serine protease isolated from *Bothrops atrox moojeni* venom that clots fibrinogen (19). In contrast to thrombin, fibrinogen is the sole substrate for batroxobin, and batroxobin only releases FpA and is not inhibited by antithrombin or heparin cofactor II (20). Because of these properties, batroxobin is often used in clinical laboratories to determine whether prolonged thrombin clotting times are the result of heparin contamination or abnormal fibrinogen molecules (21). In addition, batroxobin has been used clinically for prevention and treatment of thrombosis because of its capacity to lower circulating fibrinogen levels (22-25).

Although it is well established that batroxobin induces defibrinogenation, the interaction of batroxobin with fibrinogen has not been studied nor is it known whether, like thrombin, batroxobin binds to fibrin and triggers thrombus expansion. The potential procoagulant activity of batroxobin is important because patients envenomated by *Bothrops atrox moojeni* often experience thrombotic complications (26,27). To address these questions, we (a) characterized the binding of batroxobin to  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrinogen to determine whether its fibrinogen binding sites overlap with those of thrombin because both enzymes release FpA; (b) assessed the binding of batroxobin to  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrin clots to determine whether the protease remains bound to the product following catalysis; and (c) measured the procoagulant activity of fibrin-bound batroxobin to determine whether like fibrin-bound thrombin, batroxobin promotes clot expansion.

## EXPERIMENTAL PROCEDURES

### Materials

*Reagents*—Human  $\alpha$ -thrombin, fXIII and plasminogen-free fibrinogen were from

Enzyme Research Laboratories (South Bend, IN). Fibrinogen was rendered fXIII-free and  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrinogen were separated by fractionation on DEAE-Sepharose (GE Healthcare) as previously described (18,28). Batroxobin from the venom of *Bothrops atrox moojeni* was from Pentapharm (Basel, Switzerland). A 20-amino acid analog of the COOH-terminus of the  $\gamma'$ -chain of fibrinogen with a Cys at the NH<sub>2</sub>-terminus, termed  $\gamma'$ -peptide (CVRPEHPAETEDSLYPEDDL), and a  $\gamma'$ -peptide-directed IgG from sheep were prepared by Bachem Bioscience, Inc. (King of Prussia, PA). The two Tyr residues of the  $\gamma'$ -peptide were modified with phosphate groups to mimic the sulfated Tyr residues found in the native  $\gamma'$ -chain and to increase the affinity for thrombin (29). The  $\gamma'$ -peptide-directed IgG was subjected to affinity chromatography using immobilized  $\gamma'$ -peptide as described previously (30). The  $\alpha$ 17-53-peptide (CGPRVVERHQSAAKDSDWPFASDEDW NYKAPSGCRM) with an NH<sub>2</sub>-terminal Cys residue was synthesized by Life(pro)tein (Plainfield, New Jersey). Chromogenic substrates H-D-Phe-Pip-Arg-*p*-nitroaniline (S-2238) and pyroGlu-Pro-Arg-nitroaniline (CS-21(66)) were from Chromogenix (Milano, Italy) and Aniaria (Neuville-sur-Oise, France), respectively. D-Phe-Pro-Arg-chloromethyl ketone (FPR) and D-Tyr-Pro-Arg-chloromethyl ketone (YPR) were from Calbiochem. Full-length hirudin was from Dade-Behring (Marburg, Germany). Citrated human plasma was prepared as described (31). Unless otherwise specified, other reagents were from Sigma-Aldrich.

*Preparation of fibrinogen derivatives and fragments*—Fibrinogen derivatives and fragments were isolated from  $\gamma_A/\gamma_A$ -fibrinogen to eliminate the contribution of the  $\gamma'$ -chain. DesB $\beta$ 1-42 fibrinogen was prepared by incubating  $\gamma_A/\gamma_A$ -fibrinogen with protease III fraction from *C. atrox* venom and purified using ethanol precipitation as described (16). Fragments D and E were generated by plasmin digestion and purified as previously reported (30). The NH<sub>2</sub>-terminal disulfide knot (NDSK) was obtained with cyanogen bromide treatment and purified as described (32).

*Modification of proteins and peptides*—Batroxobin and thrombin were radiolabeled by reaction with <sup>125</sup>I-YPR, prepared using Na<sup>125</sup>I (McMaster University Nuclear Reactor, Hamilton, ON) and Iodo-beads (Pierce, Rockford, IL) as described (18,30). <sup>125</sup>I-YPR-batroxobin and <sup>125</sup>I-YPR-thrombin concentrations were 1-2 and 6-7  $\mu$ M, respectively, as determined by absorbance at 280 nm, and had specific radioactivities of ~500,000-700,000 cpm/ $\mu$ g. Unfractionated fibrinogen (0.5 mg in phosphate buffered saline) was radiolabeled with 1 mCi Na<sup>125</sup>I using 1 Iodo-bead for 5 min at 23°C. The sample was then subjected to gel filtration on a PD-10 column (GE Healthcare). The labeled fibrinogen was over 90% clottable and had a specific radioactivity of 925,000 cpm/ $\mu$ g. FPR-batroxobin, FPR-thrombin, and biotin conjugated  $\gamma'$ - and  $\alpha$ 17-53-peptides were prepared as described (33).

## **Methods**

*Characterization of thrombin and batroxobin*—The integrity of thrombin and batroxobin was assessed by SDS-PAGE analysis on 4-15% polyacrylamide gradient gels (Bio-Rad, Mississauga, Ontario) under non-reducing conditions. Thrombin and batroxobin migrated as single bands with expected apparent molecular weights of 37,000 and 32,000, respectively (Fig. 1). The identity of batroxobin was confirmed by amino acid sequence analysis. Briefly, batroxobin was resolved by SDS-PAGE, protein bands were transferred to a PVDF membrane (Bio-Rad, Mississauga, Ontario), and identified by Ponceau Red staining. The bands of interest were submitted for amino acid sequence analysis (Hospital for Sick Children Advanced Protein Technology Centre, Toronto, ON). In addition, SDS-PAGE analysis of batroxobin-treated fibrinogen revealed bands corresponding solely to  $\alpha$ -, B $\beta$ - and  $\gamma$ -chains, indicating the absence of contaminating proteases in the batroxobin preparation (data not shown). The specificity of batroxobin was confirmed by comparing its capacity to cleave FpA and/or FpB from fibrinogen with that of thrombin. FpA and

FpB release were quantified by HPLC using synthetic FpA (Bachem) and FpB (Life(pro)tein) as internal standards (34), and the identities of released FpA and FpB were confirmed by mass spectrometry (Bioanalytical and Mass Spectrometry Laboratory, McMaster University). As expected, batroxobin only released FpA from fibrinogen, whereas thrombin released both FpA and FpB (data not shown); findings in agreement with previously published results (35).

*Surface plasmon resonance (SPR)*—The interaction of FPR-batroxobin and FPR-thrombin with immobilized  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrin(ogen), biotinylated- $\gamma'$ - and  $\alpha 17$ -53-peptides, NDSK and fragments D or E was assessed by SPR on a BIAcore 1000 (GE Healthcare) as described (18,30,33), but with some modifications. Briefly, fibrinogen and fibrinogen fragments were covalently linked to separate flow cells of a carboxymethylated dextran CM5 biosensor chip at a flow rate of 5  $\mu$ l/min using an amine coupling kit (GE Healthcare). Proteins were immobilized in 10 mM acetate buffer at varying pH values to maximize adsorption.  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrinogen and fragment D were immobilized at pH 5.5 to ~6000-8000 response units (RU). Fragment E and NDSK were immobilized at pH 4.5 and 5.0, respectively, to ~6000-8000 RU. For fibrin studies, immobilized  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrinogen were converted to fibrin by three successive 60 min injections of 100 nM  $\alpha$ -thrombin (30,33). To prepare streptavidin-conjugated CM5 cells, 0.4 mg/ml streptavidin was immobilized at pH 4.5. Biotinylated  $\gamma'$ -peptide and  $\alpha 17$ -53 were adsorbed onto separate streptavidin-containing cells to 200-600 RU, whereas 0.4 mg/ml ovalbumin was immobilized at pH 4.5 to ~6000-8000 RU in control flow cells. Remaining reactive amine groups were neutralized with 1 M ethanolamine. All SPR procedures were done in 10 mM Hepes-NaOH, 150 mM NaCl at pH 7.4 (HBS) containing 0.005% Tween 20 and 2 mM  $\text{CaCl}_2$  (HBS-Tw-Ca). Flow cells were regenerated with 0.5 M NaCl between runs. To measure the affinity of proteases for immobilized fibrinogen, fibrin and fibrinogen fragments, aliquots of FPR-

batroxobin or FPR-thrombin (0-40  $\mu$ M) were injected at a flow rate of 30  $\mu$ l/min. In reciprocal experiments, FPR-batroxobin and FPR-thrombin were immobilized at pH 4.5 to ~4000-6000 RU, and the binding of fibrinogen to the adsorbed proteins was assessed. The sensorgram tracings were obtained from the instrument software and all experiments were performed at least twice.

To determine the extent to which the batroxobin and thrombin binding sites on fibrinogen overlap, the binding of  $\gamma_A/\gamma_A$ -fibrinogen to immobilized FPR-batroxobin was measured as previously described in the absence or presence of FPR-thrombin up to 40  $\mu$ M. As a positive control, the experiment was repeated in the presence of 80  $\mu$ M hirudin; as a negative control, ovalbumin (0-40  $\mu$ M) was used in place of FPR-thrombin. The amount of  $\gamma_A/\gamma_A$ -fibrinogen bound to adsorbed batroxobin in the presence of FPR-thrombin was normalized to that determined in the absence of competitor. All competition experiments were performed three times.

*SPR data analysis*— $K_d$  values for one-site binding were determined by kinetic analysis of on- and off-rates of batroxobin or thrombin binding to immobilized ligands using Scrubber2 version 2.0a (Bio-Logic Software Co., Campbell, Australia) as described (33). To calculate two-site binding of thrombin to  $\gamma_A/\gamma'$ -fibrin(ogen), the amount of analyte bound at equilibrium ( $R_{eq}$ ) was plotted against input protease concentrations and analyzed by non-linear regression analysis of a two-site binding equation (Table Curve, Jandel Scientific, San Rafael, CA).

*Binding of batroxobin and thrombin to fibrin clots*—In a series of micro-centrifuge tubes, up to 30  $\mu$ M of  $\gamma_A/\gamma_A$ -,  $\gamma_A/\gamma'$ -, or desB $\beta$ 1-42  $\gamma_A/\gamma_A$ -fibrinogen was clotted with 10 nM thrombin in the presence of 20-40 nM  $^{125}$ I-YPR-batroxobin or  $^{125}$ I-YPR-thrombin in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) containing 0.005% Tween 20 and 2 mM  $\text{CaCl}_2$  (TBS-Tw-Ca) (33). After 60 min incubation at 23°C, clots were compacted by centrifugation at 14000 x g for 4 min. Fibrin-bound  $^{125}$ I-protease was quantified by subtracting the radioactivity in the clot supernatant from that obtained in controls

prepared without fibrinogen. Plots of bound  $^{125}\text{I}$ -protease *versus* fibrin concentration were analyzed by non-linear regression of a rectangular hyperbola to determine the composite  $K_d$ . Experiments were performed twice in duplicate.

*Effect of competitors on the binding of  $^{125}\text{I}$ -YPR-batroxobin or  $^{125}\text{I}$ -YPR-thrombin to fibrin*—Affinity purified  $\gamma'$ -peptide-directed IgG (30) was used to assess the importance of the  $\gamma'$ -region in protease binding to fibrin clots. After preincubating 1  $\mu\text{M}$   $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrinogen with  $\gamma'$ -peptide-directed IgG (0-8  $\mu\text{M}$ ) for 60 min at 23°C, 40 nM  $^{125}\text{I}$ -YPR-thrombin or  $^{125}\text{I}$ -YPR-batroxobin was added, and clotting was initiated with 10nM thrombin. The fraction of  $^{125}\text{I}$ -YPR-protease bound to the clot was then calculated as described above.

To determine whether batroxobin and thrombin have overlapping binding sites, clots were formed with 1  $\mu\text{M}$   $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrinogen, 10 nM thrombin, 20-40 nM  $^{125}\text{I}$ -YPR-batroxobin or  $^{125}\text{I}$ -YPR-thrombin and 0-25  $\mu\text{M}$  FPR-thrombin in TBS-Tw-Ca. Studies with  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrinogen were performed in the absence or presence of 0-8  $\mu\text{M}$   $\gamma'$ -peptide-directed IgG to explore the influence of the COOH-terminus of the  $\gamma'$ -chain on protease binding. Fibrin-bound  $^{125}\text{I}$ -protease was quantified as described above. Experiments were performed twice in duplicate.

*Batroxobin and thrombin diffusion from preformed fibrin clots*—The rate of  $^{125}\text{I}$ -protease diffusion from clots formed from  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrinogen was determined as described (30,33). Briefly, 120  $\mu\text{l}$  fibrin clots were formed around plastic loops (Bac-Loop, Thermo-Fisher Scientific, Waltham, MA) in microcentrifuge tubes by adding 100 nM thrombin to 9  $\mu\text{M}$   $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrinogen, 30 nM fXIII, and 25 nM  $^{125}\text{I}$ -YPR-batroxobin or -thrombin. After incubation at 37°C for 45 min, clots were removed and immersed in TBS-Ca or 2 M NaCl containing 5 mM EDTA. At intervals, the fraction of clot-bound  $^{125}\text{I}$ -YPR-protease was quantified and time courses were fit to a two-phase exponential decay curve (Table Curve, Jandel scientific, San Rafael, CA). Experiments were repeated three times.

*Fibrin clot accretion*—The capacity of fibrin-bound batroxobin or thrombin to promote clot accretion was assessed by counting clots for radioactivity after incubation in plasma containing 500 cpm  $^{125}\text{I}$ -fibrinogen. Briefly, clots were formed around plastic loops with concentrations of batroxobin or thrombin that yielded comparable clot times. Thus, 8.3  $\mu\text{M}$   $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrinogen in HBS-Tw-Ca containing 30 nM fXIII was incubated with 76.5 units/ml batroxobin or 45 nM thrombin for 30 min at 37°C. Clots were then incubated in citrated human plasma supplemented with ~800,000 cpm/ml of  $^{125}\text{I}$ -fibrinogen for up to 3.3 h at 23°C. At intervals, clots were removed from the plasma, washed with HBS-Tw, and counted for radioactivity. Experiments were performed three times, each in triplicate.

*Statistical Analysis*—Results are presented as the mean  $\pm$  standard deviation. The extent of clot accretion over time induced by batroxobin and thrombin were compared by two-way repeated measures ANOVA using the Greenhouse Geisser correction, whereas t-tests were used for other analyses. In all cases,  $p < 0.05$  was considered statistically significant.

## RESULTS

*Binding of batroxobin and thrombin to  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrinogen*—Although the interaction of thrombin with  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrinogen is well characterized, binding of batroxobin to fibrinogen has not been investigated. SPR was used to quantify binding, and Req values from the sensorgrams are plotted against input protease concentrations. As previously reported, the amount of FPR-thrombin bound to  $\gamma_A/\gamma'$ -fibrinogen is about 2-fold greater than that bound to  $\gamma_A/\gamma_A$ -fibrinogen ( $p < 0.05$ ) (Fig. 2A). In contrast, similar amounts of FPR-batroxobin bind to both isoforms of fibrinogen (Fig. 2B). Sensorgrams showing real-time binding to  $\gamma_A/\gamma_A$ -fibrinogen reveal rapid association and dissociation phases for both proteases. However, the off-rate of FPR-batroxobin is about 16-fold slower than that of FPR-thrombin ( $p < 0.005$ ), which suggests that batroxobin binds  $\gamma_A/\gamma_A$ -fibrinogen with higher

affinity than thrombin (Fig. 2A and B, *insets*). Kinetic analyses of the on- and off-rates reveal that FPR-thrombin binds  $\gamma_A/\gamma_A$ -fibrinogen via a single site ( $K_d$  of  $2.3 \pm 0.3 \mu\text{M}$ ), whereas thrombin binds  $\gamma_A/\gamma'$ -fibrinogen via high and low affinity sites ( $K_d$  values of  $0.05 \pm 0.02$  and  $1.5 \pm 0.1 \mu\text{M}$ ); findings in agreement with previous studies (16,18). In contrast, FPR-batroxobin binds both  $\gamma_A/\gamma_A$  and  $\gamma_A/\gamma'$ -fibrinogen via a single high affinity site ( $K_d$  values of  $0.6 \pm 0.1$  and  $0.5 \pm 0.04 \mu\text{M}$ , respectively,  $p=0.9$ ). As illustrated in Table I, the affinities of batroxobin for both isoforms of fibrinogen are 3- to 4-fold higher than that of thrombin for  $\gamma_A/\gamma_A$ -fibrinogen and resemble the high affinity interaction of thrombin with  $\gamma_A/\gamma'$ -fibrinogen. Similar results are obtained in reciprocal experiments, where the binding of fibrinogen to immobilized FPR-batroxobin or FPR-thrombin is assessed (data not shown).

The  $\gamma'$ -chain, which distinguishes  $\gamma_A/\gamma'$ -fibrinogen from  $\gamma_A/\gamma_A$ -fibrinogen, affords thrombin a high affinity binding site. Because batroxobin binds  $\gamma_A/\gamma_A$  and  $\gamma_A/\gamma'$ -fibrinogen with similar affinities, it is unlikely to bind to the  $\gamma'$ -chain. To verify this, SPR was used to compare the affinity of batroxobin for immobilized  $\gamma'$ -peptide with that of thrombin. Whereas thrombin binds  $\gamma'$ -peptide with a  $K_d$  value of  $1.1 \pm 0.1 \mu\text{M}$ , batroxobin does not bind (Table I); findings that suggest that the  $\gamma'$ -region on  $\gamma_A/\gamma'$ -fibrinogen is important for thrombin, but not batroxobin, binding.

*Interaction of batroxobin and thrombin with  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrin*—Thrombin binds fibrinogen and fibrin with similar affinities. To determine whether the same is true for batroxobin, SPR was used to compare the affinity of batroxobin for immobilized  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrin with that of thrombin (30,33). Like thrombin, batroxobin binds both isoforms of fibrin with affinities similar to those for fibrinogen (Table I); findings that suggest that batroxobin binds fibrin as well as fibrinogen.

To confirm the SPR results, the binding of  $^{125}\text{I}$ -YPR-proteases to fibrin clots was assessed.  $^{125}\text{I}$ -YPR-thrombin binds  $\gamma_A/\gamma_A$ -fibrin clots with a  $K_d$  of  $2.5 \pm 0.3 \mu\text{M}$ , whereas it binds  $\gamma_A/\gamma'$ -fibrin clots with a composite  $K_d$  of  $230 \pm 70.0 \text{ nM}$  (Fig. 3A) and 1.5-fold more thrombin binds  $\gamma_A/\gamma'$ -fibrin clots

than  $\gamma_A/\gamma_A$ -fibrin clots; findings in agreement with previous work (33). In contrast,  $^{125}\text{I}$ -YPR-batroxobin binds  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrin clots with  $K_d$  values of  $268 \pm 18.5$  and  $409 \pm 48.4 \text{ nM}$  ( $p=0.18$ ), respectively (Fig. 3B) and similar amounts of batroxobin bind to clots formed from either isoform of fibrinogen. Consistent with their distinct modes of binding to  $\gamma_A/\gamma'$ -fibrin, the  $\gamma'$ -peptide-directed IgG has no effect on  $^{125}\text{I}$ -YPR-batroxobin binding to  $\gamma_A/\gamma'$ -fibrin clots, but decreases the binding of  $^{125}\text{I}$ -YPR-thrombin to  $\gamma_A/\gamma'$ -fibrin clots by  $\sim 70\%$  at  $8 \mu\text{M}$  (Fig. 4). In contrast, the  $\gamma'$ -peptide-directed IgG has only non-specific effects on  $^{125}\text{I}$ -YPR-batroxobin and  $^{125}\text{I}$ -YPR-thrombin binding to  $\gamma_A/\gamma_A$ -fibrin clots (Fig. 4, *inset*). Thus, the findings with fibrin clots are similar to those with immobilized fibrin and demonstrate that batroxobin does not bind to the COOH terminus of the  $\gamma'$ -chain.

*Comparison of the rates of batroxobin and thrombin diffusion from  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrin clots*—To further characterize batroxobin binding to fibrin, we compared the rates of dissociation of batroxobin and thrombin from preformed  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrin clots. Consistent with previously reported results, the rate of  $^{125}\text{I}$ -YPR-thrombin dissociation from  $\gamma_A/\gamma'$ -fibrin clots is 9-fold slower than that from  $\gamma_A/\gamma_A$ -fibrin clots ( $p<0.005$ ) (Fig. 5A); a difference that reflects the bivalent interaction of thrombin with  $\gamma_A/\gamma'$ -fibrin and its univalent interaction with  $\gamma_A/\gamma_A$ -fibrin (18). In contrast, the rates of  $^{125}\text{I}$ -YPR-batroxobin diffusion from  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrin clots are similar and comparable with the rate of  $^{125}\text{I}$ -YPR-thrombin diffusion from  $\gamma_A/\gamma'$ -fibrin clots (Fig. 5B). Thus, even though batroxobin does not interact with the  $\gamma'$ -chain, it binds both isoforms of fibrin with affinities similar to the high affinity, bivalent interaction of thrombin with  $\gamma_A/\gamma'$ -fibrin. As a control, rates of diffusion were determined in the presence of 2 M NaCl. Under these conditions, the rates of diffusion of batroxobin from both  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrin clots are 3- to 4-fold slower than those of thrombin ( $p<0.05$ ) (Fig. 5). This finding raises the possibility that ionic interactions are less important for batroxobin binding to fibrin than they are for thrombin.

*Thrombin competition of batroxobin*

*binding to fibrin(ogen)*—SPR was used to determine whether thrombin and batroxobin have overlapping binding sites. The extent to which increasing concentrations of FPR-thrombin attenuate  $\gamma_A/\gamma_A$ -fibrinogen (2.5  $\mu$ M) binding to immobilized FPR-batroxobin was assessed. FPR-thrombin attenuates  $\gamma_A/\gamma_A$ -fibrinogen binding to adsorbed FPR-batroxobin in a concentration-dependent fashion with an  $IC_{50}$  of  $2.5 \pm 0.5 \mu$ M. At 40  $\mu$ M, FPR-thrombin reduces fibrinogen binding by 39% (Fig. 6A). Because hirudin binds exosite 1 on thrombin (36), we hypothesized that hirudin would attenuate the capacity of FPR-thrombin to compete with adsorbed FPR-batroxobin for fibrinogen binding. As expected, hirudin reduces competition by FPR-thrombin by 1.3- to 1.7-fold. This is not a non-specific protein effect because, even at 40  $\mu$ M, ovalbumin has no effect on  $\gamma_A/\gamma_A$ -fibrinogen binding to immobilized FPR-batroxobin (data not shown). The finding that saturating concentrations of FPR-thrombin reduce  $\gamma_A/\gamma_A$ -fibrinogen binding to FPR-batroxobin by a maximum of 39% suggests that the batroxobin binding sites on fibrinogen partially overlap with those of thrombin.

We next examined the effect of increasing concentrations of FPR-thrombin on  $^{125}$ I-YPR-batroxobin or  $^{125}$ I-YPR-thrombin binding to  $\gamma_A/\gamma_A$ -fibrin clots. At 25  $\mu$ M, FPR-thrombin reduces  $^{125}$ I-YPR-thrombin binding by ~60%. In contrast, 25  $\mu$ M FPR-thrombin reduces  $^{125}$ I-YPR-batroxobin binding by 40%; a value consistent with the SPR results with fibrinogen (Fig. 6B). Collectively, these data suggest that the batroxobin binding sites on fibrin(ogen) partially overlap with those of thrombin.

*Localization of the batroxobin binding sites on fibrinogen*—To begin to localize the batroxobin binding sites on fibrinogen, the interaction of batroxobin with immobilized fibrinogen fragments was examined using SPR and compared with that of thrombin. The fibrinogen derivatives include fragment X, which lacks the  $\alpha$ C-domains, fragment E and NDSK, which consist of the  $NH_2$ -termini of all six chains, and fragment D, which contains the  $COOH$ -terminal portions of the three chains. Fragments were generated from  $\gamma_A/\gamma_A$ -

fibrinogen to avoid possible contribution of the  $\gamma'$ -chain to thrombin binding. FPR-batroxobin and FPR-thrombin bind to fragment E and NDSK ( $K_d$  values of 3 to 5  $\mu$ M), but not to fragment D (Table I). Thrombin binds fragment E and NDSK with affinities comparable with that of intact  $\gamma_A/\gamma_A$ -fibrinogen, suggesting that most of the sequences necessary for thrombin binding to fibrinogen are localized to the E-domain. Like thrombin, batroxobin binds fragment E and NDSK, suggesting that the protease primarily binds to the central region of fibrinogen. However, because batroxobin binds the central E-region with affinities 4- to 8-fold lower than that for intact  $\gamma_A/\gamma_A$ -fibrinogen, the batroxobin binding sites may extend beyond the E-domain.

To further distinguish the batroxobin and thrombin binding sites on fibrin(ogen), their interaction with  $\alpha$ 17-53 was compared because this sequence contains a thrombin binding site (37). Biotinylated  $\alpha$ 17-53 peptide was adsorbed onto a streptavidin-containing cell and the binding of FPR-batroxobin and FPR-thrombin was assessed by SPR. Whereas FPR-thrombin binds  $\alpha$ 17-53 peptide ( $K_d$  value of  $2.2 \pm 1.0 \mu$ M), FPR-batroxobin displays minimal binding ( $K_d > 25 \mu$ M), suggesting that the  $NH_2$ -terminus of the  $\alpha$ -chain is only important for thrombin binding (Table I).

To determine the role of the  $NH_2$ -terminus of the  $\beta$ -chain, the binding of  $^{125}$ I-YPR-batroxobin and  $^{125}$ I-YPR-thrombin to clots generated from intact or desB $\beta$ 1-42  $\gamma_A/\gamma_A$ -fibrinogen was examined.  $^{125}$ I-YPR-batroxobin binds intact and desB $\beta$ 1-42  $\gamma_A/\gamma_A$ -fibrin with similar affinities ( $K_d$  values of  $0.27 \pm 0.02$  and  $0.24 \pm 0.1 \mu$ M, respectively). In contrast, the affinity of thrombin for desB $\beta$ 1-42  $\gamma_A/\gamma_A$ -fibrin is 2.6-fold lower than that for intact  $\gamma_A/\gamma_A$ -fibrin ( $K_d$  values of  $6.5 \pm 1.5$  and  $2.5 \pm 0.3 \mu$ M, respectively;  $p=0.07$ ); a finding in agreement with previous work (16). These results further support the contention that the batroxobin binding sites on fibrin(ogen) are distinct from those of thrombin.

*Comparison of the capacities of fibrin-bound batroxobin and thrombin to promote clot accretion*—To determine whether, like fibrin-bound thrombin, fibrin-bound

batroxobin also promotes clot accretion,  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrinogen were clotted with batroxobin or thrombin and the clots were counted for radioactivity after incubation in citrated plasma containing  $^{125}\text{I}$ -labeled fibrinogen. Both fibrin-bound batroxobin and thrombin trigger progressive fibrin accretion over time (Fig. 7). The extent of fibrin accretion onto  $\gamma_A/\gamma_A$ -fibrin clots prepared with thrombin is 1.5-2.0 fold greater than that onto  $\gamma_A/\gamma'$ -fibrin clots (*inset*,  $p < 0.001$ ). Therefore, thrombin bound to  $\gamma_A/\gamma_A$ -fibrin clots promotes more fibrin accretion than thrombin bound to  $\gamma_A/\gamma'$ -fibrin clots. In contrast, clot accretion induced by batroxobin is similar regardless of whether batroxobin is bound to  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrin clots ( $p = 0.18$ ). It is notable that clot accretion induced by fibrin-bound batroxobin is ~18-fold greater than that induced by fibrin-bound thrombin ( $p < 0.001$ ); indicating that the procoagulant activity of fibrin-bound batroxobin is greater than that of fibrin-bound thrombin, likely reflecting the fact that batroxobin is not inhibited by antithrombin or heparin cofactor II (20).

## DISCUSSION

Although the thrombin-like activity of batroxobin is well known, the interaction of batroxobin with fibrinogen has not been studied. To address this, we compared the interaction of batroxobin with  $\gamma_A/\gamma_A$ - and  $\gamma_A/\gamma'$ -fibrin(ogen) with that of thrombin. Batroxobin forms a high affinity interaction with both isoforms of fibrin(ogen), which resembles the high affinity thrombin interaction with  $\gamma_A/\gamma'$ -fibrin(ogen). The batroxobin binding sites on fibrin(ogen) only partially overlap with those of thrombin because thrombin incompletely displaces batroxobin from fibrinogen and fibrin clots. In keeping with the concept that the batroxobin binding sites on fibrin(ogen) are distinct from those of thrombin, batroxobin binds NDSK and fragment E with lower affinity than intact  $\gamma_A/\gamma_A$ -fibrinogen, whereas the affinity of thrombin for these fragments is similar to that for fibrinogen, suggesting that the batroxobin binding sites on fibrinogen extend beyond the E-domain. Furthermore, the  $\alpha 17$ -53 and B $\beta$ 1-

42 regions in the E-domain are important for thrombin binding, but not for batroxobin binding. In addition, fibrin-bound batroxobin retains catalytic activity and is a more potent stimulus for fibrin accretion than fibrin-bound thrombin.

Structural and functional studies have elucidated the structural determinants of thrombin's interaction with fibrinogen (12,38-41). In the absence of a crystal structure for batroxobin, structural appraisal relies on sequence comparison and modeling. Based on primary sequence alignments, batroxobin exhibits 25% and 37% sequence identity to thrombin and trypsin, respectively. Notably, batroxobin lacks the 60- and  $\gamma$ -loops and the  $\text{Na}^+$  binding site; domains that endow thrombin with its specificity for a wide repertoire of macromolecular substrates (37,41). The absence of these domains may explain why fibrinogen is the sole substrate of batroxobin (20). Without the 60- and  $\gamma$ -loops and the  $\text{Na}^+$  binding site, the interaction of batroxobin with fibrinogen is likely to depend on other residues in the vicinity of the active site of batroxobin (42,43).

The exosites flanking the active site of thrombin also are critical determinants of its interaction with fibrin(ogen) and other substrates (11,33,41). Exosite 1 on thrombin contains hydrophobic and basic regions that mediate substrate and cofactor binding (39,40). In particular, Tyr76 of thrombin provides the anchor for a hydrophobic patch surrounded by basic residues that interacts with a complementary region on fibrinogen (39,40). Although only 50% of the corresponding residues are retained, a homologous fibrinogen recognition exosite in batroxobin has been proposed (43,44). The observation that the interaction of batroxobin with  $\gamma_A/\gamma_A$ -fibrin clots is less readily disrupted by high salt than that of thrombin raises the possibility that hydrophobic interactions may be more important for batroxobin binding to the fibrinogen recognition exosite than they are for thrombin binding. Although both proteases have similar affinities for fibrinogen, batroxobin does not bind other thrombin exosite 1-mediated ligands, such as hirudin or HD1 aptamer (data not shown). These findings



support the concept that exosite specificity contributes to the distinct functional properties of batroxobin and thrombin.

Although sequence alignments reveal conserved exosite regions consisting of hydrophobic and basic residues, snake venom proteases possess fewer basic residues in the region corresponding to exosite 1 (44-46). This is demonstrated by modeling the putative exosite residues in batroxobin onto the crystal structure of *Trimeresurus stejnejeri* venom plasminogen activator (TSV-PA), which has 64% sequence identity to batroxobin (42). The spatial organization of these residues reveals a putative exosite that resembles exosite 1 on thrombin and may contribute to the interaction of batroxobin with fibrin(ogen) (46,47) (Fig. 8A). Although the existence of a second exosite on batroxobin cannot be excluded, there is little surface homology with thrombin in the exosite 2 region; and a thrombin variant with mutations in exosite 2 displays normal fibrin binding (16).

In addition to the structural differences between the proteases, binding site differences on fibrinogen may also affect specificity. The importance of the  $\beta$ -chain to binding is demonstrated by the observation that FpA release from fibrinogen Naples I, a variant with a point mutation at residue 68 in the B $\beta$ -chain, is normal with batroxobin, but impaired with thrombin (48). The crystal structure of thrombin bound to fragment E provides further insight into the differences between the batroxobin and thrombin binding sites on fibrinogen (40). Thrombin exosite 1 interacts with a patch populated by residues from both the  $\alpha$ - and  $\beta$ -chains, including  $\alpha$ F35,  $\alpha$ D38,  $\beta$ A68,  $\beta$ D69, and  $\beta$ D71. Likewise, the  $\alpha$ -chain segment DSDWPF ( $\alpha$ 30-35), which lies proximal to where FpA resides, contains a similar combination of acidic and hydrophobic residues that may support batroxobin binding (Fig. 8B). However, our observation that the affinity of thrombin, but not batroxobin, for  $\alpha$ 17-53 is comparable with its affinity for intact fibrinogen suggests that a tertiary structure consisting of multiple fibrinogen chains also may be necessary to optimally position batroxobin for FpA cleavage. These

results suggest that the proteases have related, but unique, modes of interaction with fibrinogen.

The distinct batroxobin binding sites in the E-domain of fibrinogen may explain why batroxobin only induces FpA release. Current thinking is that fibrin generation and fibrin monomer assembly in solution occur in two distinct steps. In the first step, thrombin releases FpA, thereby generating desAA-fibrin monomers that associate to form protofibrils (2). This is followed by a second step whereby FpB is released, resulting in lateral growth of the fibrin protofibrils; a process dependent on desAA-fibrin polymerization (2,49). Thus, the generation of desAA-fibrin facilitates FpB release either by rendering the B $\beta$ Arg14-Gly15 bond more susceptible to thrombin hydrolysis or by positioning FpB in close proximity to the active site of thrombin (8,9,50). A conformational change in the vicinity of the B $\beta$ Arg14-Gly15 bond cannot be the sole explanation for FpB release because, like thrombin, batroxobin also generates desAA-fibrin polymers, yet it does not induce FpB release (20,35). Whereas thrombin cleaves the B $\beta$ Arg14-Gly15 bond on B $\beta$ 1-42-peptide, thereby releasing FpB, batroxobin does not, which suggests that unlike thrombin, batroxobin does not bind to the B $\beta$ 1-42 region (data not shown). In support of this concept, the affinity of batroxobin for intact and desB $\beta$ 1-42  $\gamma_A/\gamma_A$ -fibrinogen is the same, whereas the affinity of thrombin for  $\gamma_A/\gamma_A$ -fibrinogen is reduced by 2.6-fold when the B $\beta$ 1-42 sequence is removed. Therefore, these data suggest that thrombin interaction with the B $\beta$ 1-42 region is an important determinant of its capacity to release FpB; because batroxobin fails to bind to this region, it does not induce FpB release.

Batroxobin is a potent defibrinogenating enzyme; a property that has been exploited in studies of batroxobin for prevention or treatment of thrombosis. Batroxobin binds fibrin(ogen) with high affinity and fibrin-bound batroxobin is more potent than fibrin-bound thrombin at triggering fibrin accretion, likely because batroxobin is not inhibited by antithrombin and heparin cofactor II (20). Therefore, the procoagulant activity of fibrin-

bound batroxobin may contribute to the microvascular thrombosis observed in patients envenomated with *Bothrops atrox moojeni* (26,27). The defibrinogenating effects of batroxobin and other venom-derived thrombin-like serine proteases underlie their therapeutic potential. Our results suggest that treatment with these agents may be complicated by microvascular thrombosis, which may explain the disappointing results of studies that explored the use of fibrinogen-depleting snake venom enzymes for treatment of acute ischemic stroke (51,52).

In summary, we have characterized the interaction of batroxobin with fibrin(ogen) and have shown how it differs from that of thrombin. These differences likely reflect the fact that batroxobin is missing the surface loops and allosteric sites that modulate thrombin's interaction with fibrin(ogen) (37,41). The structural features on batroxobin that mediate the high affinity interaction with fibrin(ogen) remain to be elucidated.

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## FOOTNOTES

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## <sup>1</sup>ABBREVIATIONS

f	factor
Fp	fibrinopeptide
FPR	Phe-Pro-Arg-chloromethyl ketone
HBS	Hepes buffered saline
NDSK	NH <sub>2</sub> -terminal disulfide knot
Req	amount of analyte bound at equilibrium
RU	response units
SPR	surface plasmon resonance
TBS	Tris buffered saline
TSV-PA	<i>Trimeresurus stejnegeri</i> venom plasminogen activator
Tw	Tween
YPR	Tyr-Pro-Arg-chloromethyl ketone
$\gamma'$ -peptide	synthetic analog of the COOH-terminal portion of fibrinogen $\gamma'$ -chain

## FIGURE LEGENDS

Figure 1. **Integrity of thrombin and batroxobin as assessed by SDS-PAGE analysis.** Thrombin (*lane 2*) and batroxobin (*lane 4*) were subjected to SDS-PAGE analysis on a 4-15% polyacrylamide gradient gel under non-reducing conditions. The molecular weights of the mobility markers are shown on the left (*lane 1*).

Figure 2. **SPR analysis of the interaction of FPR-batroxobin and FPR-thrombin with immobilized  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrinogen.**  $\gamma_A/\gamma_A$ - (●) and  $\gamma_A/\gamma'$ -fibrinogen (▲) were adsorbed on individual flow cells to ~ 6000-8000 RU. Increasing concentrations (0-15  $\mu$ M) of FPR-thrombin (Panel A) or FPR-batroxobin (Panel B) were successively injected into flow cells for 2 min, followed by a 4 min wash to monitor dissociation. The amount of protease bound at equilibrium (Req) after background correction is plotted against the input FPR-protease concentration. The *insets* show representative sensorgrams for the interaction of FPR-thrombin (Panel A) and FPR-batroxobin (Panel B) with  $\gamma_A/\gamma_A$ -fibrinogen in concentrations up to 15 and 6.4  $\mu$ M, respectively. Data points represent the mean  $\pm$  SD of 2 to 3 experiments, while the *lines* represent nonlinear regression analyses.

Figure 3. **Binding of  $^{125}\text{I}$ -YPR-batroxobin and  $^{125}\text{I}$ -YPR-thrombin to  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrin clots.** 20 nM  $^{125}\text{I}$ -YPR-thrombin (Panel A) or 40 nM  $^{125}\text{I}$ -YPR-batroxobin (Panel B) was added to microcentrifuge tubes containing 0-8  $\mu\text{M}$   $\gamma_A/\gamma_A$ - (●) or  $\gamma_A/\gamma'$ -fibrinogen (▲) and 10 nM  $\alpha$ -thrombin was used to initiate clotting. After incubation for 60 min, clots were pelleted by centrifugation and free  $^{125}\text{I}$ -YPR-protease in the supernatant was used to calculate the bound fraction. Data are plotted as the percentage of  $^{125}\text{I}$ -YPR-protease bound to fibrin clots *versus* the fibrinogen concentration. Data points represent the mean  $\pm$  SD of 2 experiments, each performed in duplicate, while the *lines* represent nonlinear regression analyses.

Figure 4. **Effect of  $\gamma'$ -peptide-directed IgG on  $^{125}\text{I}$ -YPR-batroxobin and  $^{125}\text{I}$ -YPR-thrombin binding to  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrin clots.** The binding of 40 nM  $^{125}\text{I}$ -YPR-thrombin (●/▲) or  $^{125}\text{I}$ -YPR-batroxobin (○/△) to clots formed from 1  $\mu\text{M}$   $\gamma_A/\gamma_A$ - (●) or  $\gamma_A/\gamma'$ -fibrinogen (▲) was assessed in the absence or presence of  $\gamma'$ -peptide-directed IgG up to 8  $\mu\text{M}$ . Clots were generated with thrombin, pelleted and clot-bound  $^{125}\text{I}$ -YPR-protease was determined. The percentage of  $^{125}\text{I}$ -YPR-protease bound in the absence of antibody is plotted *versus* antibody concentration. The main figure illustrates the percentage of  $^{125}\text{I}$ -YPR-protease bound to  $\gamma_A/\gamma'$ -fibrin clots, whereas the *inset* shows the percentage of  $^{125}\text{I}$ -YPR-protease bound to  $\gamma_A/\gamma_A$ -fibrin clots. Symbols represent the mean  $\pm$  SD of two experiments, each performed in duplicate, while the *lines* represent nonlinear regression analyses of the data.

Figure 5. **Diffusion of  $^{125}\text{I}$ -YPR-batroxobin and  $^{125}\text{I}$ -YPR-thrombin from  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrin clots.** Clots were formed around plastic loops by incubating 9  $\mu\text{M}$   $\gamma_A/\gamma_A$ - (●/○) or  $\gamma_A/\gamma'$ -fibrinogen (■/□) with 100 nM thrombin and 30 nM fXIII in the presence of 20 nM  $^{125}\text{I}$ -YPR-thrombin (Panel A) or 40 nM  $^{125}\text{I}$ -YPR-batroxobin (Panel B), and then immersed in solutions containing TBS-Ca (●/■), or 2M NaCl and 5 mM EDTA (○/□). At the indicated times points, 0.5 ml aliquots of the bathing solutions were removed and counted for radioactivity to quantify bound  $^{125}\text{I}$ -YPR-protease. Data are plotted as the percentage of clot-bound  $^{125}\text{I}$ -YPR-protease *versus* time and lines represent nonlinear regression analyses. Data points represent the mean  $\pm$  SD of 3 separate determinations.

Figure 6. **Effect of FPR-thrombin on batroxobin binding to  $\gamma_A/\gamma_A$ -fibrinogen or  $\gamma_A/\gamma_A$ -fibrin clots.** Panel A. The effect of FPR-thrombin on  $\gamma_A/\gamma_A$ -fibrinogen binding to immobilized FPR-batroxobin was determined using SPR.  $\gamma_A/\gamma_A$ -fibrinogen (2.5  $\mu\text{M}$ ) was incubated with FPR-thrombin concentrations up to 40  $\mu\text{M}$  in the absence (●) or presence (■) of 80  $\mu\text{M}$  hirudin and the mixture was then injected into flow cells containing immobilized FPR-batroxobin. Binding in the presence of competitors was normalized relative to that determined in their absence. Data are plotted as the percentage of  $\gamma_A/\gamma_A$ -fibrinogen bound *versus* the FPR-thrombin concentration. Panel B. Binding of  $^{125}\text{I}$ -YPR-batroxobin (■) and  $^{125}\text{I}$ -YPR-thrombin (●) to  $\gamma_A/\gamma_A$ -fibrin clots was measured in the absence or presence of FPR-thrombin concentrations up to 25  $\mu\text{M}$ . Clots were formed by incubating 2 or 0.06  $\mu\text{M}$   $\gamma_A/\gamma_A$ - fibrinogen with 20 nM  $^{125}\text{I}$ -YPR-thrombin or 40 nM  $^{125}\text{I}$ -YPR-batroxobin, respectively, and 10 nM thrombin. After 60 min, clots were counted for radioactivity to quantify bound  $^{125}\text{I}$ -YPR-protease. Data are plotted as the percentage of  $^{125}\text{I}$ -YPR-protease bound *versus* the FPR-thrombin concentration. Data points represent the mean  $\pm$  SD of 3 to 4 experiments while the *lines* represent nonlinear regression analyses.

Figure 7. **Clot accretion induced by batroxobin or thrombin bound to  $\gamma_A/\gamma_A$ - or  $\gamma_A/\gamma'$ -fibrin clots.** Clots were formed around plastic loops by incubating 8.3 $\mu\text{M}$   $\gamma_A/\gamma_A$ -fibrinogen (●/○) or  $\gamma_A/\gamma'$ -fibrinogen (■/□) with 76.5 units/ml of batroxobin (●/■) or 45 nM thrombin (○/□) in the presence of 2 mM  $\text{CaCl}_2$ , 30 nM fXIII and 500 cpm of  $^{125}\text{I}$ -fibrinogen for 30 min at 37°C. After determining radioactivity, clots were incubated in 1 ml citrated human plasma containing ~800,000 cpm/ml of  $^{125}\text{I}$ -fibrinogen. At intervals, clots were washed twice with 1 ml HBS and



again counted for radioactivity so that fibrin accretion could be determined. The *inset* provides an expanded view of clot accretion induced by thrombin. Data points represent the mean  $\pm$  SD of 3 experiments, each performed in triplicate, while the *lines* represent nonlinear regression analyses. (\*  $p < 0.001$ ).

**Figure 8. Models of binding sites on batroxobin and fibrinogen.** Panel A. The crystal structure of TSV-PA (Protein Data Bank accession number 1BQY) in complex with Glu-Gly-Arg chloromethyl ketone (green) is shown in space-filling format using Pymol. The image is rotated clockwise approximately  $90^\circ$  from standard view with the active site Ser195 (magenta) facing left. Hydrophobic (yellow) and basic (blue) residues in batroxobin that constitute a putative exosite are mapped onto the TSV-PA structure. Residues are identified using chymotrypsin numbering system. Panel B. The crystal structure of the  $\text{NH}_2$ -termini of fibrinogen  $\alpha$ - and  $\beta$ -chains forming the thrombin binding site (Protein Data Bank accession number 2A45) is shown in space-filling format. Both the  $\gamma$ -chains and the reciprocal  $\alpha$ - and  $\beta$ -chains are omitted for clarity. The  $\alpha$ - and  $\beta$ -chain surfaces are white and grey, respectively. Thrombin binding residues  $\alpha$ -chain F35, D38 and  $\beta$ -chain A68, P69, D70 and A71 are shown within a solid black oval with hydrophobic residues in yellow and acidic residues in red. Residues comprising the putative batroxobin binding site include  $\alpha$ -chain D30, S31, D32, W33, P34, and F35 (hatched black oval). The site where the unmapped  $\alpha$ 1-26 segment exits the  $\alpha$ -chain at A27 indicates the vicinity of FpA.

**TABLE**

TABLE I. Dissociation constants for the binding of FPR-thrombin and FPR-batroxobin to fibrinogen, fibrin, and related fragments

The affinities of FPR-thrombin or FPR-batroxobin for immobilized fibrin(ogen), fibrinogen fragments or peptides was determined using SPR.  $K_d$  values were determined by kinetic analysis of the binding sensorgrams. The interaction of thrombin with  $\gamma_A/\gamma'$ -fibrin(ogen) was analyzed using a two-site binding model. Each value represents the mean  $\pm$  SD of two to three experiments. NB represents no binding.

	FPR-thrombin		FPR-batroxobin
	$K_{d1}$ ( $\mu$ M)	$K_{d2}$ ( $\mu$ M)	$K_d$ ( $\mu$ M)
$\gamma_A/\gamma_A$ -fibrinogen	$2.3 \pm 0.3$		$0.6 \pm 0.1$
$\gamma_A/\gamma'$ -fibrinogen	$1.5 \pm 0.1$	$0.05 \pm 0.02$	$0.5 \pm 0.04$
$\gamma_A/\gamma_A$ -fibrin	$2.0 \pm 0.9$		$0.5 \pm 0.9$
$\gamma_A/\gamma'$ -fibrin	$2.1 \pm 0.4$	$0.1 \pm 0.06$	$0.5 \pm 0.9$
Fragment D	NB		NB
Fragment E	$3.2 \pm 1.5$		$2.1 \pm 0.5$
NDSK	$3.0 \pm 1.0$		$4.5 \pm 1.7$
$\gamma'$ -peptide	$1.1 \pm 0.1$		NB
$\alpha$ 17-53-peptide	$2.2 \pm 1.0$		>25

Figure 1

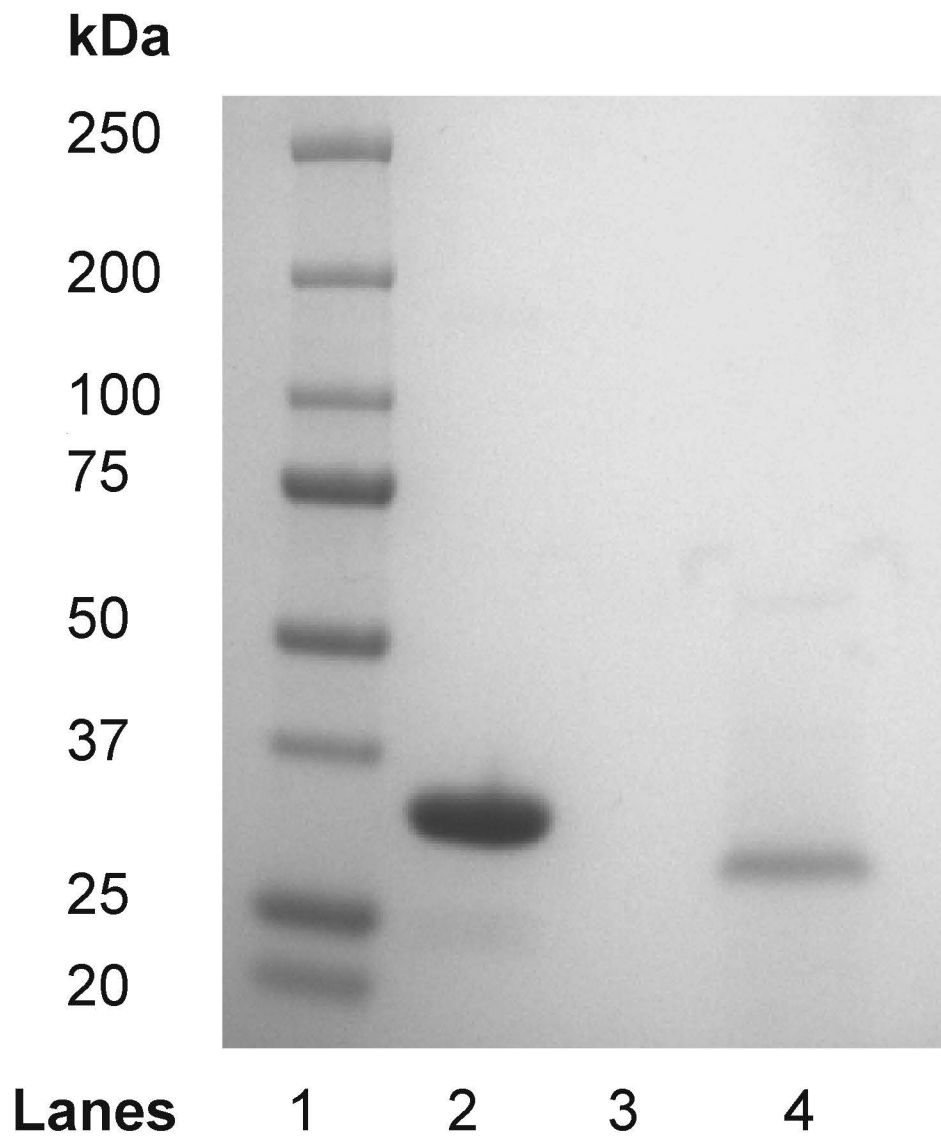


Figure 2

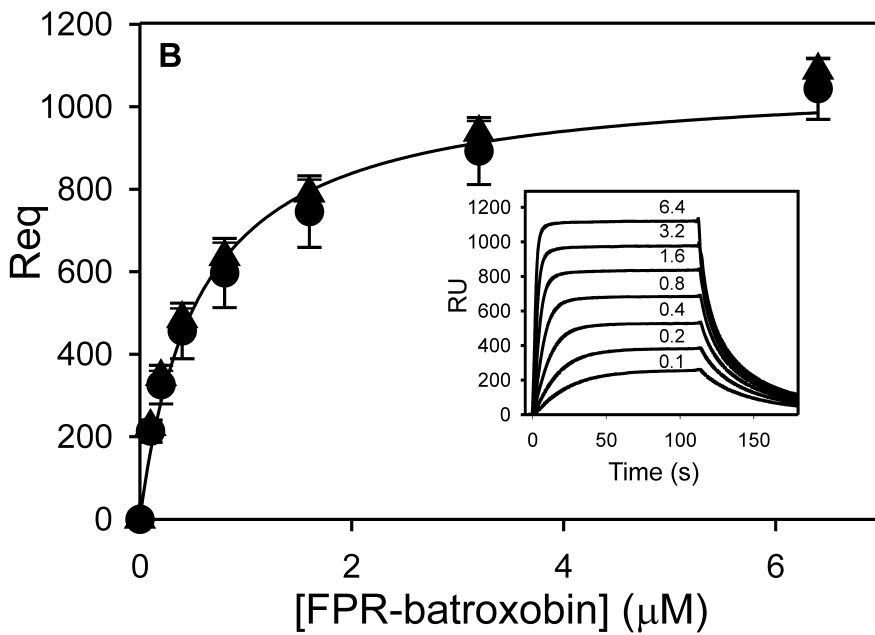
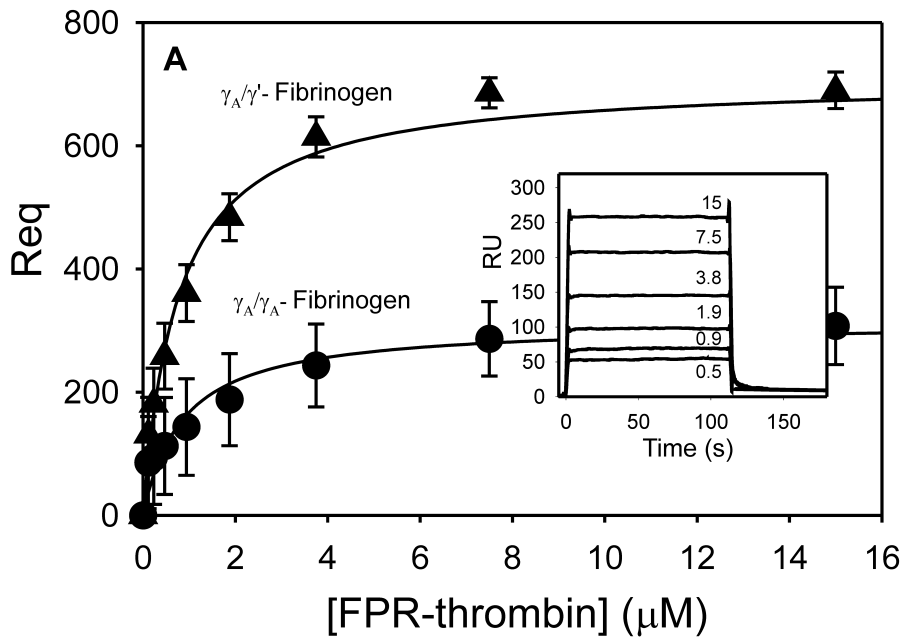


Figure 3

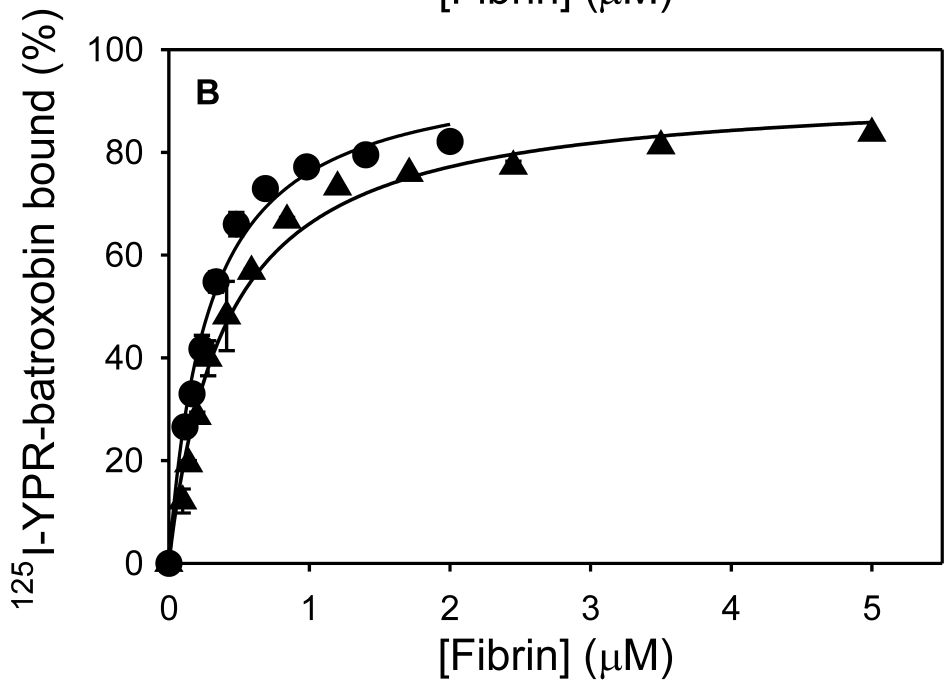
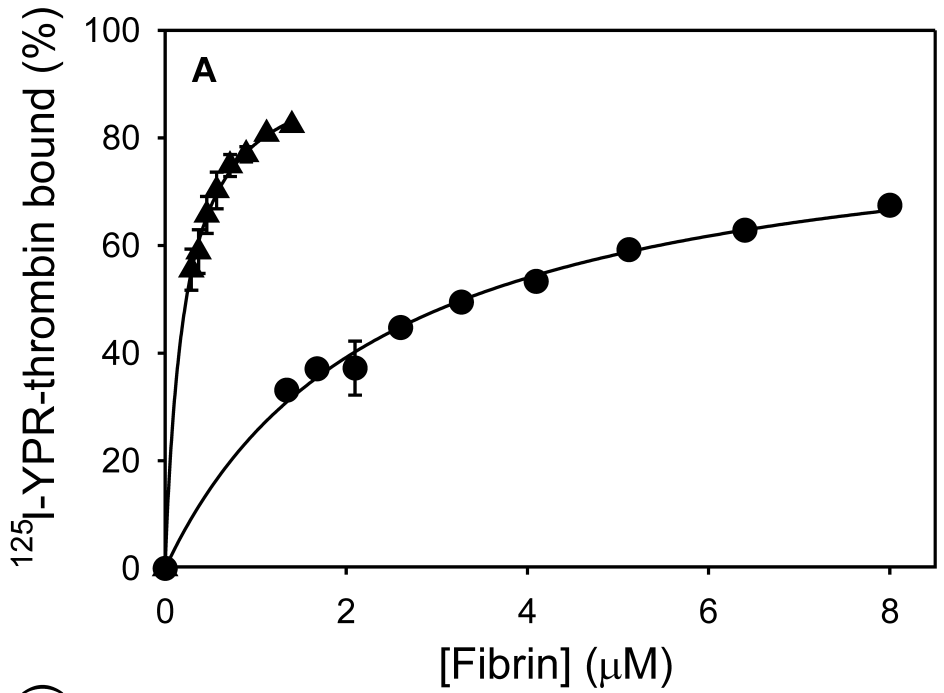


Figure 4

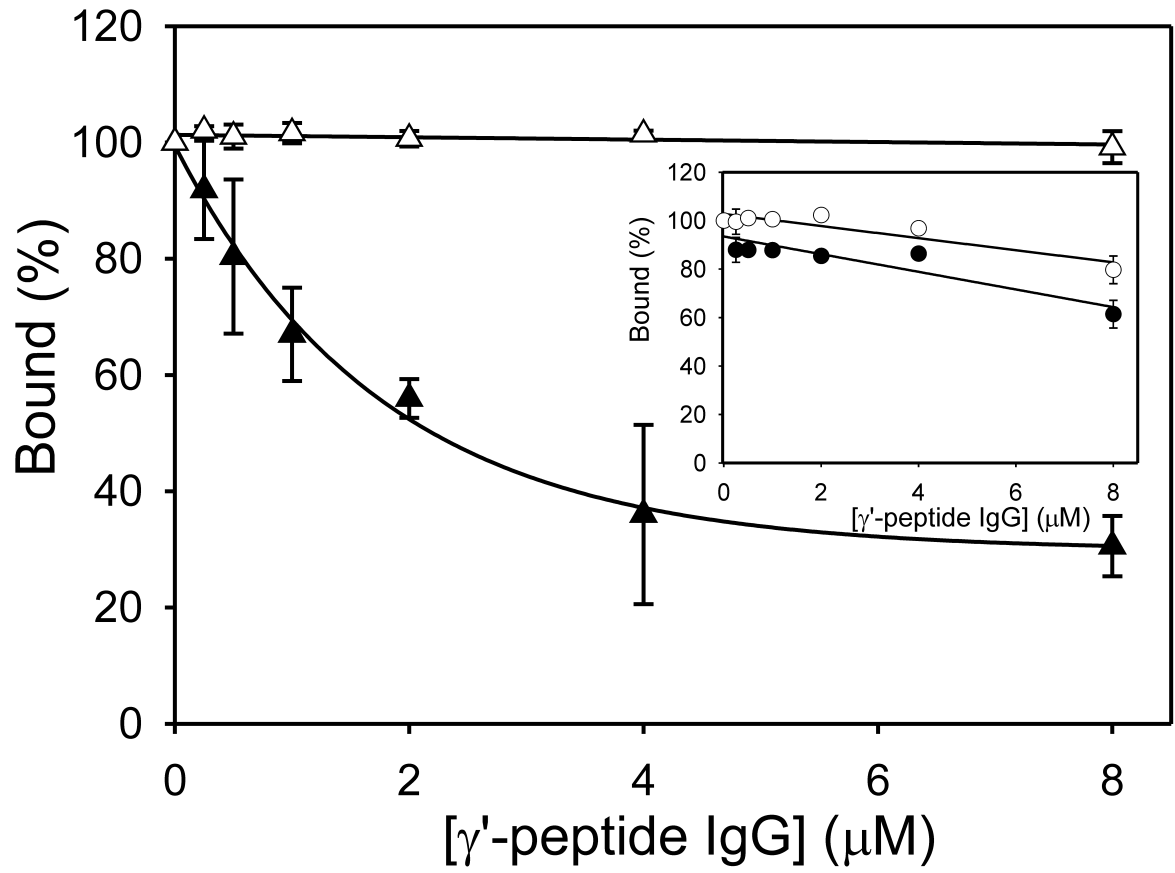


Figure 5

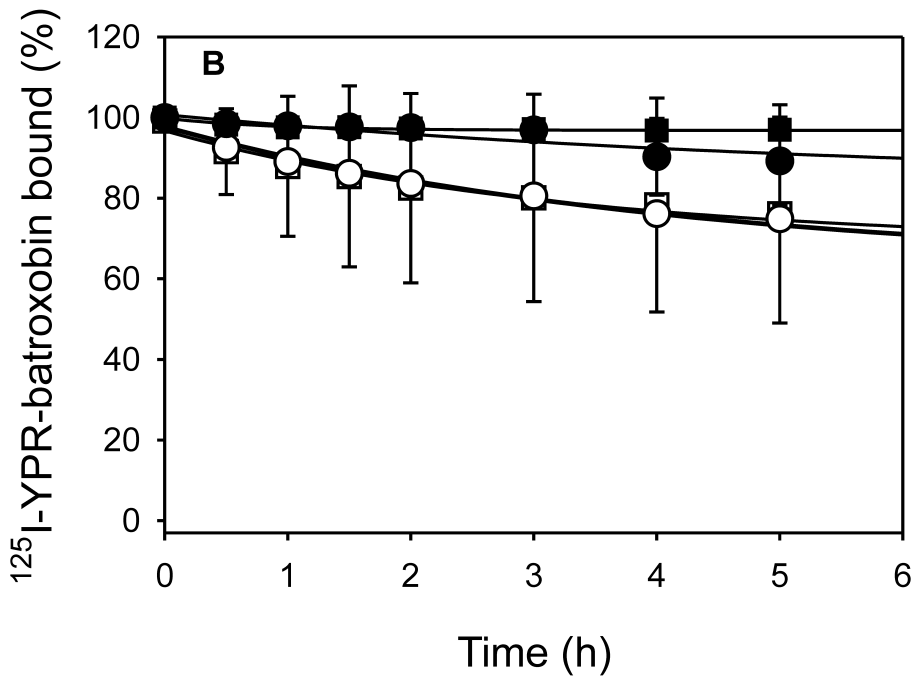
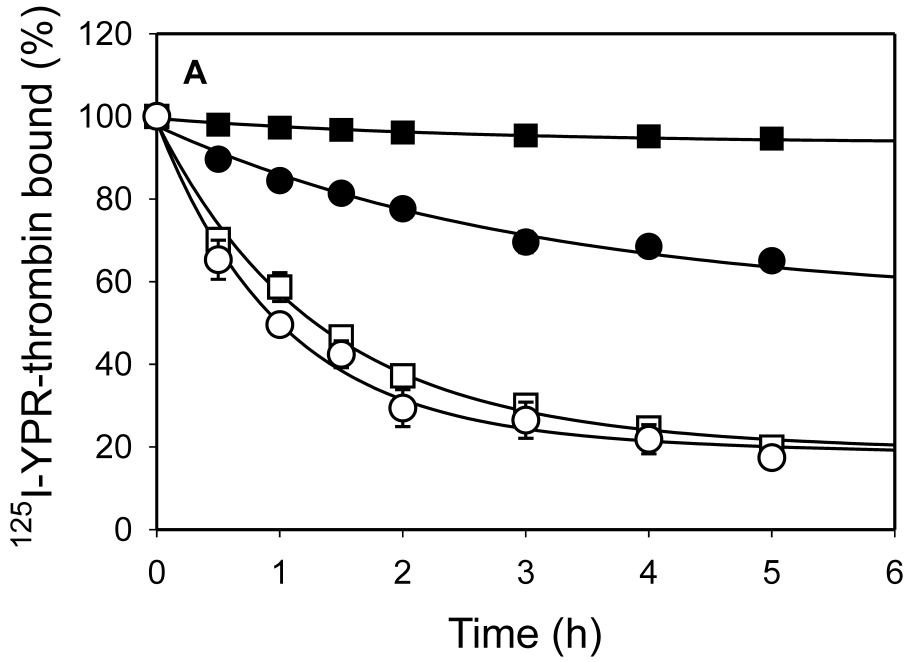


Figure 6

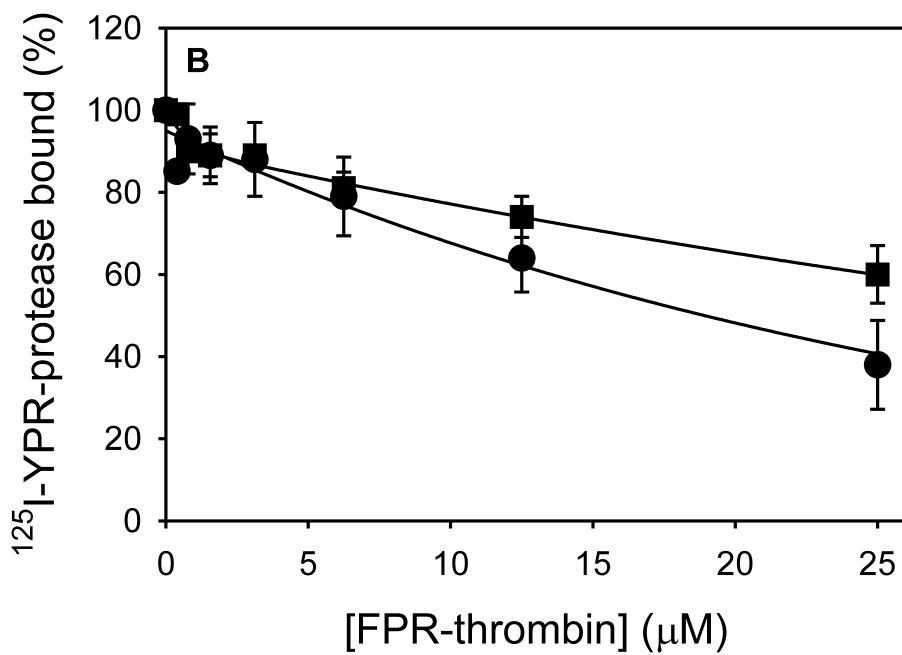
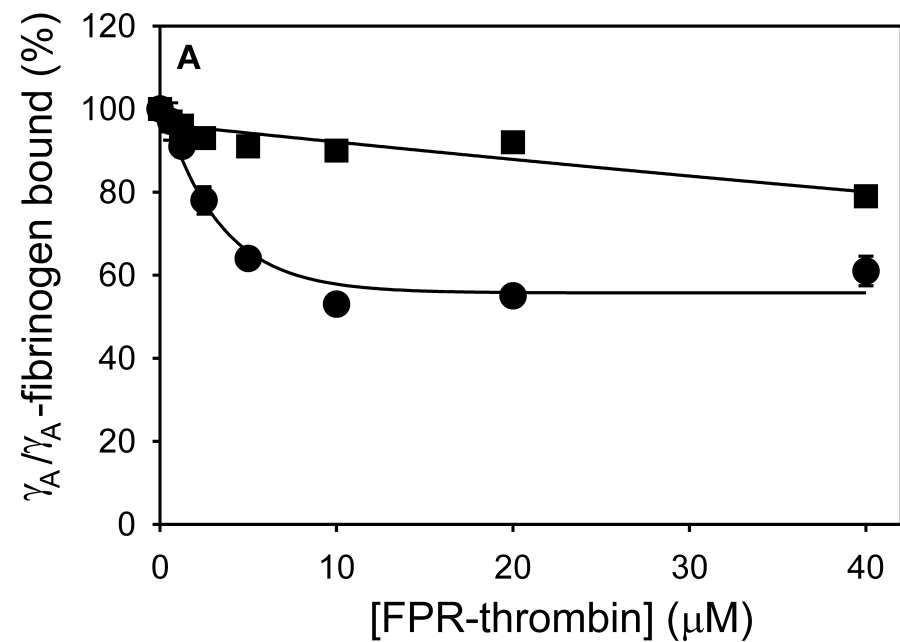




Figure 7

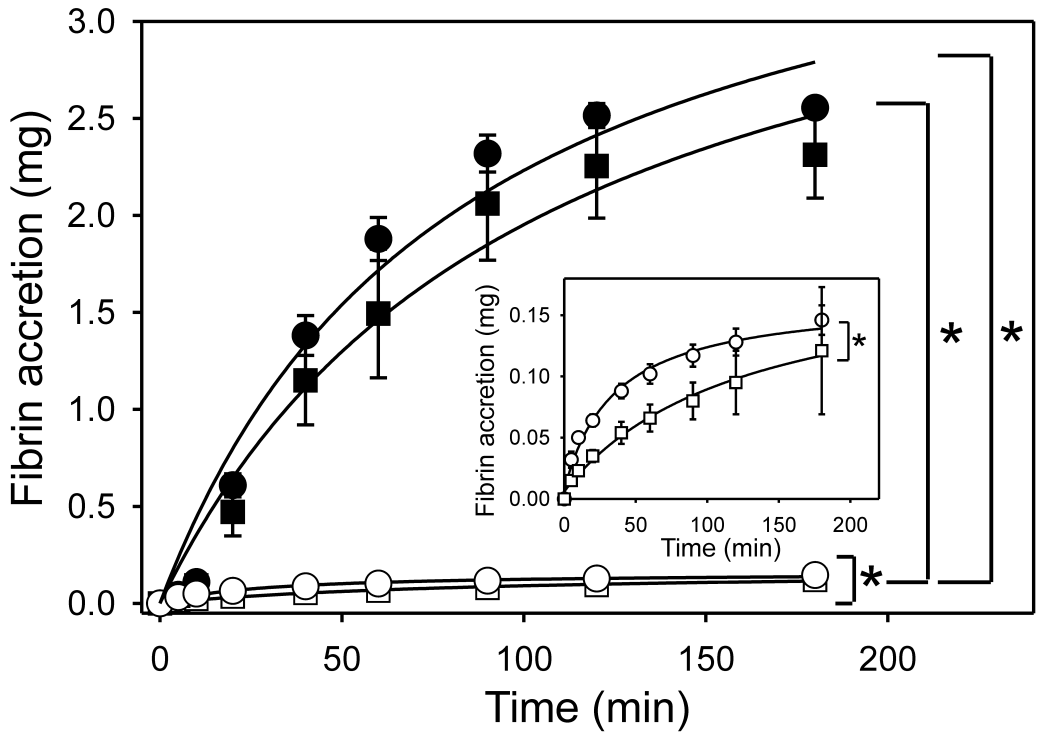
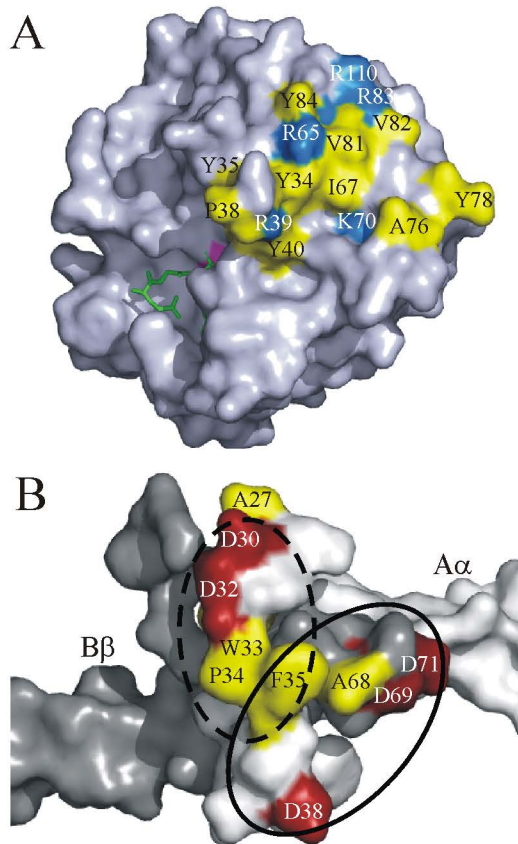


Figure 8



**Batroxobin binds fibrin with higher affinity and promotes clot expansion to a greater extent than thrombin**

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