# Bothrojaracin: A Potent Two-Site-Directed Thrombin Inhibitor†

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ABSTRACT: The thrombin inhibitor, bothrojaracin [Zingali, R. B., Jandrot-Perrus, M., Guillin, M. C., & Bon, C. (1993) *Biochemistry 32*, 10794−10802], is a 27 kDa protein isolated from the venom of *Bothrops jararaca* that blocks several thrombin functions, including fibrinogen clotting, platelet activation, and fibrin and thrombomodulin binding, but does not interact with the catalytic site. In the present report, we show that the high affinity binding of α-thrombin to immobilized bothrojaracin ($K_d$ = 0.6 nM) is inhibited by the C-terminal peptide of hirudin and that the γ-cleavage within exosite 1 reduces the affinity of bothrojaracin for thrombin ($K_d$ = 0.3 μM), indicating that bothrojaracin binding to exosite 1 is a major determinant of the thrombin−bothrojaracin interaction. In addition, we show that bothrojaracin decreases the rate of inhibition of α- and γ-thrombin by the antithrombin III−heparin complex. Competition of bothrojaracin with heparin or prothrombin fragment 2 for binding to thrombin indicates that bothrojaracin not only binds exosite 1 but also binds exosite 2 or in close proximity. Bothrojaracin binds to the thrombin precursor, prothrombin. This interaction is calcium-independent and is prevented by heparin, suggesting that it is mediated by exosite 2. Bothrojaracin inhibits platelet activation induced by clot-bound thrombin and slowly dissociates thrombin from the fibrin clots. Altogether, our results indicate that the high affinity of bothrojaracin for thrombin is supported by a double-site interaction and results in an efficient inhibition of both soluble and clot-bound thrombin.

At sites of vascular injury, thrombin is the primary mediator of platelet activation, promotes the conversion of fibrinogen to clottable fibrin, and self-amplifies its own production through both the activation of factor V and factor VIII and platelet recruitment. In addition, thrombin has stimulative effects on a variety of cell types and is a potent mitogenic and angiogenic agent (Fenton, 1991). The crystal structure of human α-thrombin bound to specific inhibitors (Grutter et al., 1990; Rydel et al., 1991; Bode et al., 1992) revealed that prominent structural features of the thrombin molecule are the location of the catalytic triad within a deep canyon-like active site cleft and the presence of two extended surfaces that are mainly composed of positively charged residues and are referred to as exosite 1 and exosite 2. Exosite 1 is required for thrombin binding to several thrombin substrates (fibrinogen, thrombin receptor, and heparin cofactor II) or ligands (thrombomodulin and glycoprotein 1b) [see Guillin et al. (1995) for a review]. Exosite 2, which is located close to the carboxy-terminal B chain helix, is involved in heparin and prothrombin fragment 2 binding (Arni et al., 1993; Gan et al., 1994; Sheehan & Sadler, 1994; Ye et al., 1994). Heparin, by forming a complex with both antithrombin III and thrombin, greatly accelerates the rate of inactivation of thrombin by the serpin. However, the heparin−antithrombin III complex does not inactivate thrombin that is bound to a fibrin clot (Hogg & Jackson, 1989; Weitz et al., 1990).

The limited efficacy of heparin in preventing arterial thrombosis has stimulated the development of specific thrombin inhibitors that can directly inhibit both circulating and clot-bound thrombin. Site-directed thrombin inhibitors that are currently under evaluation include a variety of proteins, peptides, chemical analogs, and oligonucleotides that differ in their mechanism of interaction with thrombin (Maraganore, 1993; Tapparelli et al., 1993: Paborsky et al., 1993). Small compounds such as the boroarginine tripeptides or argatroban interact only with the active site. Hirudins and hirulogs bind to both the active site and exosite 1. The C-terminal hirudin peptide, hirugen, interacts only with exosite 1 (Rydel et al., 1990, Grutter et al., 1990), whereas a thrombin aptamer binds both exosite 1 and exosite 2 on two different molecules (Padmanabhan et al., 1993). The selectivity and potency of the various thrombin inhibitors are highly dependent upon these different mechanisms of interaction (Harker, 1995).

We recently identified bothrojaracin, a new anticoagulant protein isolated from the venom of *Bothrops jararaca* (Zingali et al., 1993). Bothrojaracin is a 27 kDa protein composed of two polypeptidic chains of 13 and 15 kDa. It presents different isoforms with an isoelectric point ranging from 4 to 4.5. These characteristics, as well as the N-terminal sequences of the two chains, established its structural similarity with the C-type lectins (Ozeki et al., 1994). Bothrojaracin forms a noncovalent equimolecular complex with α-thrombin, in which the active site remains accessible to small peptide substrates. However, bothro

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jaracin is a potent inhibitor of thrombin-clotting activity, thrombin-induced platelet activation, as well as thrombin binding to thrombomodulin and hirudin. These observations suggested that bothrojaracin interacted with thrombin exosite 1. In the present study, we confirm this hypothesis and show that, in addition, bothrojaracin also interacts with thrombin exosite 2. The two-exosite-targeted inhibitor binds tightly with thrombin, inactivates both fibrin-bound and soluble thrombin, and efficiently, slowly displaces clot-bound thrombin to the soluble phase.

## MATERIALS AND METHODS

*Purified Proteins*. Human prothrombin, α-thrombin, and γ-thrombin were purified as previously described (Bezeaud et al., 1984, 1985). Prothrombin fragment 2 was obtained from the prothrombin activation mixture, in the heparin−Sepharose flow-through fraction, and further purified by gel filtration using a Sephadex G-75 superfine column (Pharmacia, Uppsala, Sweden). α-Thrombin was iodinated using the solid-phase reagent lodogen and $Na^{125}I$ (Jandrot-Perrus et al., 1988). Antithrombin III (AT)[1] was purified according to McKay (1981), and the trace amounts of heparin present in the preparation were removed by fast flow chromatography on DEAE-Sepharose (Bezeaud et al., 1985). Bovine factor IXa and human factor X were generously provided by Dr. A. Bezeaud. Human purified fibrinogen was from Kabi Vitrum (Stockholm, Sweden), and the C-terminal segment of hirudin 54−65, sulfated on Tyr 63, was from Bachem Feinchemikalien AG (Bubendorf, Switzerland).

The method previously described for purification of bothrojaracin (Zingali et al., 1993) was modified by the use of an affinity chromatography column. The lyophilized crude venom was a pool of several *B. jararaca* venoms purchased from Instituto Butantan (Sao-Paulo, SP, Brazil). The first step of purification consisted of a gel filtration on a Sephacryl S-200 (Pharmacia) column. The fractions containing the thrombin-inhibitory activity were pooled. After treatment with 8 mM EDTA and 5 mM PMSF, the pool was applied to a column of PPACK-inactivated thrombin covalently linked to Sepharose 4B, equilibrated with 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl. Bothrojaracin was eluted with 10 mM HCl and 500 mM NaCl (pH 2) immediately neutralized with 1/100 (v/v) of 1 M Tris (pH 12). The concentration of bothrojaracin was determined as previously reported (Zingali et al., 1993). The protein displayed a single band in nonreducing SDS−PAGE. Several bands, corresponding to the different isoforms present in the preparation, were observed by nondenaturing gel electrophoresis. The purified preparations of bothrojaracin used throughout this study were devoid of botrocetin, protease, or phospholipase activity. The inhibitory effect of these preparations of bothrojaracin on thrombin-induced coagulation of purified fibrinogen was comparable to that reported for one purified isoform (Zingali et al., 1993), suggesting that the different isoforms inhibit thrombin similarly.

*Solid-Phase Assay for Thrombin and Prothrombin Binding to Bothrojaracin*. Microwell plates (Immulon I, Dynatech, Guyancourt, France) were coated with 0.5 μg of bothrojaracin in 15 mM $Na_2CO_3$ and 35 mM $NaHCO_3$ at pH 9.5 for 4 h at room temperature. After saturation with 5 mg/mL bovine serum albumin (BSA, fraction V, Sigma, St. Louis, MO) and washing, the wells were incubated with human α-thrombin, γ-thrombin, or prothrombin, in 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl containing 0.05% (v/v) Tween 20 and 1 mg/mL BSA. After 18 h at 4 °C, the wells were washed. Bound α- or γ-thrombin was detected by measuring the rate of hydrolysis of S-2238 (Biogenic, Montpellier, France) on a microwell plate reader (iEMS Labsystems, Helsinki, Finland). Bound prothrombin was detected using a polyclonal antibody to prothrombin coupled to peroxidase (Stago, Asnières, France) and *o*-phenylenediamine dihydrochloride (Sigma). Blanks were obtained by omitting coating with bothrojaracin. Nonspecific binding was determined by incubating the ligand in the presence of a 100-fold molar excess of bothrojaracin.

*Nondenaturing Gel Electrophoresis*. Complex formation between bothrojaracin and various proteins (thrombin, prothrombin, factor IXa, and factor X) was analyzed by polyacrylamide gel electrophoresis in nondenaturing conditions. After preincubation for 2 min in 20 mM Tris-HCl (pH 7.5) with or without 5 mM $CaCl_2$, the mixtures were applied to 12% acrylamide gels in 365 mM Tris-HCl (pH 8.8). The migration buffer consisted of 88 mM L-alanine and 25 mM Tris-HCl at pH 8.8.

*Thrombin Inhibition by AT in the Absence or Presence of Heparin*. Human α-thrombin [10 nM in 10 mM Tris-HCl (pH 7.8), 10 mM Hepes, and 100 mM NaCl containing 1 mg/mL PEG 8000] was preincubated with bothrojaracin or buffer for 2 min at 37 °C. Heparin-free AT (100 nM), or 25 nM AT in the presence of 1.5 mu/mL heparin, was added to the samples, and the residual activity of thrombin was measured by S-2238 hydrolysis at various time intervals. The same experiment was performed with γ-thrombin (10 nM in the same buffer) in the presence of 40 nM AT and 2.4 mu/mL heparin.

*Platelet Stimulation by Clot-Bound Thrombin*. Clot-bound thrombin was tested for its ability to induce platelet aggregation and secretion. Fibrin clots were prepared by incubating 300 μL of purified fibrinogen [2 mg/mL in 10 mM imidazole (pH 7.5), 150 mM NaCl, and 10 mM $CaCl_2$ containing 1 mg/mL PEG 8000] with 30 nM α-thrombin. After 2 h at 37 °C, the clots were extensively washed in the same buffer that was changed seven times over a period of 24 h. That no unbound thrombin remained with the clot was checked by the absence of the platelet-stimulating activity in the last washing buffer. Human platelets, isolated and labeled with [$^{14}$C]-5-HT (Amersham) as previously described (Jandrot-Perrus et al., 1991), were resuspended in Tyrode-Hepes buffer (pH 7.4) containing 35 mg/mL BSA. Fibrin clots, freshly washed with the platelet resuspension buffer, were added to the platelet suspension, in the presence or absence of thrombin inhibitors. Platelet aggregation was monitored at 37 °C under stirring (1100 rpm) using a Chronolog aggregometer (Coultronics). For the measurement of platelet secretion, aggregation was stopped after 5 min of incubation by adding 0.2 volume of 100 mM ice-cold EDTA, followed by a 1 min centrifugation at 12000*g*. Supernatants were assayed for [$^{14}$C]-5-HT by liquid scintillation counting.

[1] Abbreviations: PPACK, D-Phe-Pro-Arg-chloromethyl ketone; AT, antithrombin III; BSA, bovine serum albumin; S-2238, H-D-Phe-Pip-Arg-*p*-nitroanilide hydrochloride; PEG, polyethylene glycol; $IC_{50}$, concentration of inhibitor reducing thrombin activity by 50%; H54−65, sulfated C-terminal peptide of hirudin; 5-HT, 5-hydroxytryptamine.

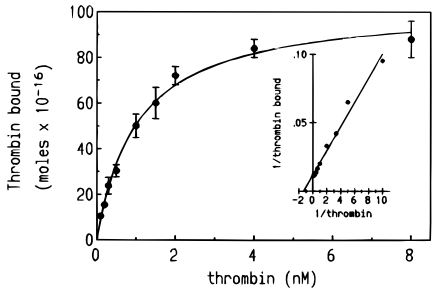


FIGURE 1: Binding of α-thrombin to immobilized bothrojaracin. α-Thrombin [0.1 to 8 nM in 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl, containing 0.05% Tween 20 and 1 mg/mL BSA] was added to microwell plates coated with bothrojaracin (0.5 $\mu$g/well). After 1 night at 4 °C and washing, thrombin bound to the wells was detected by S-2238 hydrolysis [0.2 mM in 20 mM Tris-HCl (pH 7.8), 10 mM Hepes, and 150 mM NaCl]. The amount of thrombin bound to bothrojaracin, calculated using a thrombin titration curve, is plotted as a function of the thrombin concentration added to the wells. A double-reciprocal plot of the results is presented in the inset. Results are the mean of duplicate points from five independent experiments ± 1 standard deviation (SD).

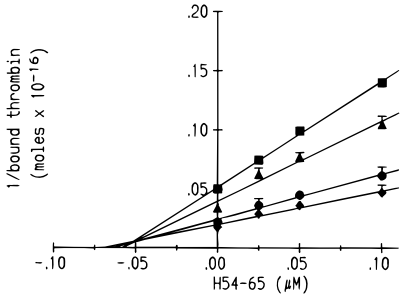


FIGURE 2: Inhibition of thrombin binding to bothrojaracin by the C-terminal hirudin peptide H54−65. Thrombin binding to bothrojaracin was measured as in Figure 1 but in the presence of hirudin 54−65. Dixon plots represent the binding of thrombin to bothrojaracin as a function of hirudin 54−65 concentration, for various concentrations of thrombin: (◆) 1.5 nm, (●) 1.0 nM, (▲) 0.5 nM, and (■) 0.3 nM. Results are the mean of duplicate points from three independent experiments ± 1 SD.

*Mobilization of Clot-Bound Thrombin*. Fibrin clots were prepared by incubating 150 $\mu$L of purified fibrinogen [2 mg/mL in 10 mM imidazole (pH 7.5), 150 mM NaCl, and 10 mM $CaCl_2$ containing 1 mg/mL PEG 8000] with 5 nM $^{125}$I-labeled α-thrombin. After 2 h at 37 °C, the clots were extensively washed as described above. The radioactivity accumulated in the solution of the last washing buffer was less than 2% of the initial radioactivity. The clots were then incubated with 200 $\mu$L of buffer or buffer containing either bothrojaracin, hirudin, sulfated hirudin 54−65 (Bachem Feinchemikalien AG, Bubendorf, Switzerland), or AT−heparin, as indicated in the text. The radioactivity contained in the clot and in the incubation medium was measured in order to quantify the amount of thrombin displaced from the clot.

## RESULTS AND DISCUSSION

*(1) Two Distinct Regions of Thrombin are Involved in the Interaction with Bothrojaracin*. In a previous study (Zingali et al., 1993), we have shown that bothrojaracin blocks several thrombin functions by forming a noncovalent stoichiometric complex with the enzyme. The inhibition constants ($IC_{50}$ and $K_i$) that we measured indicated a high-affinity interaction. In the present study, a solid-phase assay has been developed in order to determine the dissociation constant ($K_d$) of the thrombin−bothrojaracin complex. Specific binding of α-thrombin to immobilized bothrojaracin determined in microwell plates was concentration-dependent and saturable (Figure 1). Specific binding was indicated from the complete inhibition of the reaction when an excess of bothrojaracin (100 nM) was added in α-thrombin samples. The double-reciprocal plot of the amount of α-thrombin bound to bothrojaracin as a function of the concentration of thrombin defined a $K_d$ of 0.62 ± 0.2 nM.

We have previously shown (Zingali et al., 1993) that bothrojaracin prevented α-thrombin interaction with platelets, fibrin(ogen), thrombomodulin, and hirudin, although it did not prevent α-thrombin-catalyzed hydrolysis of the chromogenic substrate S-2238. An identical effect has been reported with the carboxy-terminal peptide of hirudin, H54−65 (Naski et al., 1990; Jakubowski & Maraganore, 1990) which is known to bind to exosite 1 (Grütter et al., 1990). Consistent with this observation, we show here that the hirudin peptide H54−65 prevents α-thrombin binding to immobilized bothrojaracin. Inhibition is competitive, with a $K_i$ of 50 ± 16 nM (Figure 2). γ-Thrombin is characterized by the disruption of exosite 1. However, the controlled proteolysis of α-thrombin by trypsin producing γ-thrombin does not result in the disruption of the overall structure of the molecule that is still active on small substrates (Guillin & Bezeaud, 1992; Rydel et al., 1994). γ-Thrombin bound to bothrojaracin, with a $K_d$ value of 0.30 ± 0.07 $\mu$M, 3 orders of magnitude higher than that of α-thrombin for bothrojaracin. Altogether, these results clearly indicate that thrombin structures involved in exosite 1 or within its close proximity are implicated in the interaction of α-thrombin with bothrojaracin.

However, although the trypsin-catalyzed γ-cleavages within the thrombin molecule resulted in a significant decrease in the affinity for bothrojaracin, γ-thrombin retained the ability to bind the inhibitor, suggesting that additional binding site(s), distinct from exosite 1, could be involved in thrombin interaction with bothrojaracin. A cluster of positively charged residues is located in the C-terminal region of the molecule. This region interacts with heparin, as recently confirmed by mutagenesis (Gan et al., 1994; Sheehan & Sadler, 1994), and has been designated the "heparin-binding site" or "exosite 2" (Bode et al., 1992). Interaction of thrombin exosite 2 with heparin contributes to the formation of a ternary complex bridging AT, thrombin, and the polysaccharide, which results in the acceleration of thrombin inhibition by AT (Olson & Shore, 1982). In order to determine if thrombin exosite 2 could be involved in the enzyme interaction with bothrojaracin, we examined the effect of bothrojaracin on thrombin interaction with heparin. We first observed that, in the absence of heparin, bothrojaracin did not modify the rate of thrombin inhibition by AT, 50% of α-thrombin (10 nM) activity being neutralized by AT (100 nM) in 9.2 and 9.5 min, in the absence and presence of 100 nM bothrojaracin, respectively. In contrast, in the presence of heparin, the rate of α-thrombin inhibition by AT was significantly decreased by the addition of bothrojaracin (Figure 3A). The inhibition was dependent on the concentration of bothrojaracin and heparin. The observation that bothrojaracin interfered with the inhibition of thrombin by AT in the presence of heparin could result either from a direct interaction between bothrojaracin and

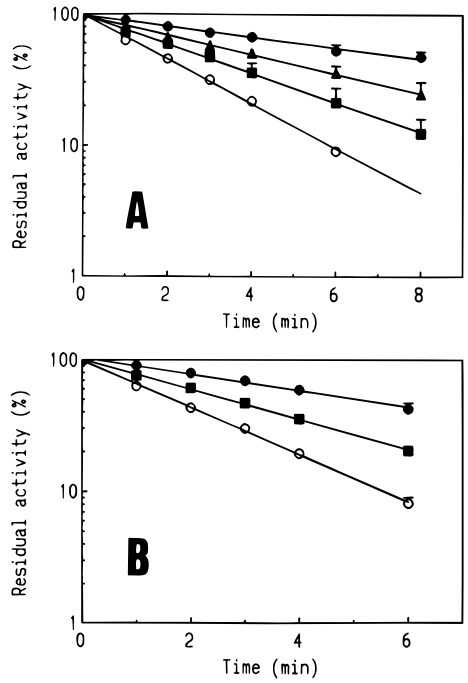


FIGURE 3: Bothrojaracin reduces thrombin inhibition by antithrombin in the presence of heparin. (A) α-Thrombin [10 nM in 10 mM Tris-HCl, 10 mM Hepes (pH 7.8), 100 mM NaCl, and 0.1% PEG] was incubated for 2 min at 37 °C in the presence of buffer (○) or bothrojaracin: (■) 2.5 nM, (▲) 5 nM, and (●) 10 nM. After addition of 25 nM AT and 1.5 mu/mL heparin, aliquots were taken at timed intervals and the residual activity of α-thrombin was measured using S-2238 (0.1 mM in the same buffer). (B) γ-Thrombin [10 nM in 10 mM Tris-HCl, 10 mM Hepes (pH 7.8), 150 mM NaCl, and 1 mg/mL PEG] was incubated for 2 min at 37 °C in the presence of buffer (○) or bothrojaracin: (■) 50 nM and (●) 200 nM. After addition of 40 nM AT and 2.4 mu/mL heparin, the residual activity of γ-thrombin was measured using S-2238. Results are the mean of three experiments ± 1 SD.

heparin or from a competition between bothrojaracin and heparin for binding to thrombin. The first hypothesis was examined using affinity columns. After incubation with heparin−Sepharose, more than 95% of the anticoagulant activity of bothrojaracin was found in the flow-through fraction, indicating that heparin−Sepharose did not retain any bothrojaracin activity, whereas thrombin was fully retained on identical columns. This observation ruled out the hypothesis of a direct interaction between bothrojaracin and heparin. The hypothesis of a competition between heparin and bothrojaracin for binding to thrombin was then examined using the solid-phase assay. Heparin at concentrations as high as 10 μM failed to inhibit the binding of α-thrombin to bothrojaracin. We considered the possibility that, in this solid-phase assay, the inability of heparin to inhibit α-thrombin binding to bothrojaracin was related to the strong interaction between α-thrombin exosite 1 and bothrojaracin. As the γ-thrombin derivative retains the ability to bind heparin, corresponding to the fact that the core of the exosite 2 remains unmodifed (Hofsteenge et al., 1988; Rydel et al., 1994), it provides a model to examine the effect of bothrojaracin on thrombin−heparin interaction independently of bothrojaracin interaction through exosite 1. The rate of γ-thrombin inhibition by AT in the presence of heparin was efficiently reduced by bothrojaracin (Figure 3B). In addition, the binding of γ-thrombin to immobilized bothrojaracin was inhibited in the presence of heparin. Binding of γ-thrombin (150 nM) to bothrojaracin was inhibited by 54 and 100% in

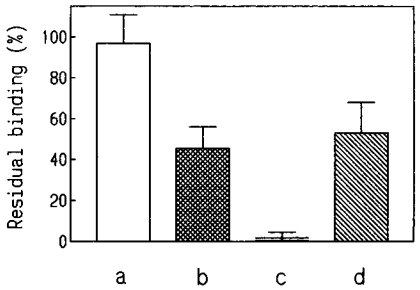


FIGURE 4: Inhibition of the binding of γ-thrombin to bothrojaracin by heparin or prothrombin fragment 2. The binding of γ-thrombin [150 nM in 20 mM Tris-HCl (pH 7.4) containing 0.05% v/v Tween 20 and 1 mg/mL BSA] to microwell plates coated with bothrojaracin was performed as in Figure 1. Results are expressed as the residual binding of γ-thrombin to bothrojaracin in the presence of (a) 2 μM hirudin 54−65, (b) 40 u/mL heparin, (c) 100 u/mL heparin, and (d) 2 μM prothrombin fragment 2. Results are the mean of duplicate points from three experiments ± 1 SD.

the presence of 40 and 100 u/mL heparin, respectively, whereas it was not affected by the exosite 1 ligand, H54−65 (Figure 4). As prothrombin fragment 2 also binds to the heparin-binding site of thrombin (Arni et al., 1993), we examined the effect of fragment 2 on thrombin binding to bothrojaracin. As above, γ-thrombin was used in this assay to avoid the interaction of exosite 1 with bothrojaracin. In control experiments, the interaction of fragment 2 with α-thrombin and also with γ-thrombin was observed by nondenaturing gel electrophoresis (data not shown). In the solid-phase assay, fragment 2 efficiently inhibited the binding of γ-thrombin to bothrojaracin (Figure 4). These observations demonstrate that the two exosite 2 ligands, heparin and fragment 2, interfere with thrombin binding to bothrojaracin, indicating that thrombin structures within exosite 2, or in close proximity, are involved in the enzyme interaction with bothrojaracin.

Altogether, our investigations indicated that bothrojaracin interacts with both exosite 1 and exosite 2. The inhibition of α-thrombin binding to bothrojaracin was fully achieved with the C-terminal peptide of hirudin, whereas it was not observed with heparin, the effect being observed only on γ-thrombin. This suggests that exosite 1 plays a major role in thrombin−bothrojaracin interaction. Nevertheless, the interaction of bothrojaracin with exosite 2 clearly differentiates this inhibitor from those derived from the C-terminal segment of hirudin that neither interact with this site nor alter its function (Rydel et al., 1991; Naski et al., 1990).

We next examined the ability of bothrojaracin to interact with the thrombin precursor, prothrombin. Using the solid-phase assay, we observed that (i) prothrombin bound to immobilized bothrojaracin, in a concentration-dependent and saturable manner, with a $K_d$ of 31 ± 14 nM (data not shown) and (ii) prothrombin inhibited the binding of α-thrombin (0.5 nM) to bothrojaracin in a concentration-dependent manner (Figure 5). Inhibition reached a plateau at 50% for prothrombin concentrations above 250 nM, half-maximal inhibition being obtained with 70 nM. Prothrombin interaction with bothrojaracin was further demonstrated by electrophoresis in nondenaturing conditions. As shown in Figure 6, the preparations of bothrojaracin, purified by affinity chromatography on a PPACK−thrombin−Sepharose column, presented multiple bands corresponding to the different isoforms. After incubation with α-thrombin, all these bands disappeared and a diffuse band corresponding to complexes with

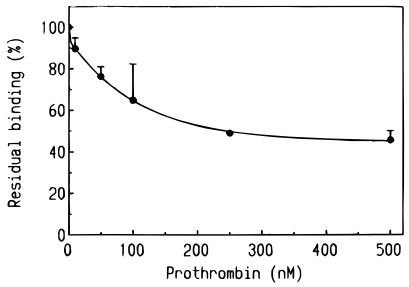


FIGURE 5: Inhibition of thrombin binding to bothrojaracin by prothrombin. The binding of thrombin (0.5 nM) to bothrojaracin was measured as in Figure 1 but in the presence of prothrombin. The plot represents the residual binding of thrombin as a function of the concentration of prothrombin. Results are the mean of duplicate points from three independent experiments ± 1 SD.

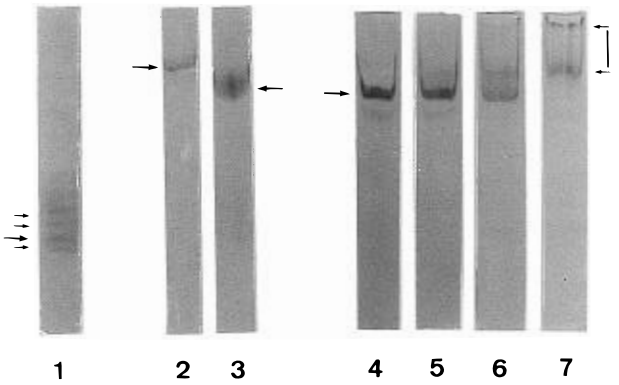


FIGURE 6: Nondenaturing gel electrophoresis. After incubation of bothrojaracin at 37 °C for 2 min with purified proteins, nondenaturing PAGE (12% acrylamide) was performed. Samples were as follows: lane 1, 3.5 $\mu$M bothrojaracin; lane 2, 2.6 $\mu$M thrombin; lane 3, 2.6 $\mu$M thrombin after incubation with 3.5 $\mu$M bothrojaracin; lane 4, 2 $\mu$M prothrombin; and lanes 5−7, prothrombin incubated with 0.5 $\mu$M (5), 1 $\mu$M (6), and 2 $\mu$M (7) bothrojaracin. Results are from one representative experiment out of three.

α-thrombin was observed, indicating that all the isoforms bound to α-thrombin. After incubation with human prothrombin, bands corresponding to bothrojaracin also disappeared and a smear delimited by a doublet was observed, confirming that bothrojaracin binds to the thrombin precursor. The opposite direction of the shift in prothrombin mobility compared to that in thrombin mobility could be related to a lesser degree of compaction and a greater size of the prothrombin−bothrojaracin complex in relation at least in part to the mass of prothrombin. An experiment was performed with a constant amount of prothrombin and increasing amounts of bothrojaracin (Figure 6). Results indicate that prothrombin was fully complexed to bothrojaracin for a molar ratio of 1/1. The heterogeneity of the migration is thus not related to the formation of complexes with different stoichiometry but rather results from the heterogeneity in bothrojaracin preparation, only one band being observed when one purified isoform is used (data not shown). Another member of the C-type animal lectin family, IX/X-bp, which is also present in the venom of *B. jararaca* (Sekiya et al., 1993), has been reported to form a complex with both the zymogen and the activated form of two clotting factors, factors IX and X (Atoda et al., 1994). However, despite structural similarities between bothrojaracin and IX/X-bp, the binding specificity of the two proteins is quite different. IX/X-bp does not bind to prothrombin (Atoda & Morita, 1989), and we did not observe bothrojaracin binding to factor IX or factor X in nondenaturing gel electrophoresis (not shown). In addition, the binding of IX/X-bp to factors IX and X is absolutely dependent on $Ca^{2+}$ ions (Sekiya et al., 1993; Atoda et al., 1994), whereas that of bothrojaracin to prothrombin or thrombin is $Ca^{2+}$-independent. Thus, at the difference of IX/X-bp which has been proposed to bind on Gla domains of factor IX and X (Atoda et al., 1994), it is very unlikely that bothrojaracin binds to the N-terminal Gla domain of prothrombin. Exosite 1 is not exposed at the surface of the prothrombin molecule (Liu et al., 1991) and cannot account for prothrombin interaction with bothrojaracin, as confirmed by the lack of any effect of the hirudin peptide H54−65 on prothrombin binding to immobilized bothrojaracin. In contrast, heparin inhibited prothrombin binding to immobilized bothrojaracin, in a dose-dependent manner, 50% inhibition of prothrombin (50 nM) binding being obtained with 5 u/mL heparin. This observation indicated that prothrombin binding to bothrojaracin is most likely mediated by exosite 2 or structures in close proximity. Nevertheless, the affinity of prothrombin for bothrojaracin is better than that of $\gamma$-thrombin, suggesting either that additional structures on prothrombin might be involved in the interaction or that the conformation of exosite 2 in $\gamma$-thrombin is less favorable to the interaction with bothrojaracin.

*(2) Bothrojaracin Inhibits and Mobilizes Clot-Bound Thrombin.* During the clot formation, a fraction of thrombin remains associated with insoluble fibrin (Liu et al., 1979) through the interaction of exosite 1 with structures located on the fragment E of fibrin(ogen) (Bouton et al., 1993). As a consequence, although the clot behaves mainly as a trap for thrombin (Liu et al., 1979), it can also act as a reservoir for active thrombin able to catalyze the release of fibrinopeptide A from fibrinogen (Weitz et al., 1990). Thrombin, in this environment, is protected from inhibition by the heparin−AT complex (Weitz et al., 1990). Since bothrojaracin is a potent inhibitor of soluble thrombin and inhibits the binding of thrombin to fibrin (Zingali et al., 1993), we have investigated its effect on clot-bound thrombin.

Significant platelet aggregation and secretion were induced by the addition of clots to a suspension of washed platelets (Figure 7). The kinetics of the platelet responses differed from that obtained with soluble thrombin by the presence of a prolonged lag phase preceding the onset of aggregation. Platelet aggregation and secretion were completely inhibited by hirudin, indicating that thrombin was the agonist and excluding the possibility that, in our system, the interaction of insoluble fibrin with the platelet integrin GPIIb−IIIa (Kieffer & Phillips, 1990) played a major role in platelet activation. In addition, we did not observe any platelet activation when the buffer bathing the clots was tested, indicating that aggregation was due to clot-associated thrombin. Clot-induced platelet activation was inhibited by bothrojaracin in a concentration-dependent manner (Figure 7), 50% inhibition being obtained with 35 nM bothrojaracin. The C-terminal peptide of hirudin, H54−65, also inhibited platelet activation induced by clot-bound thrombin, although with a much lower efficacy when compared to that of bothrojaracin.

Since platelet activation resulting from the presence of clots in suspensions of washed platelets is a rapid reaction, one can assume that it is triggered by thrombin present at the surface of the clot. However, this represents only a

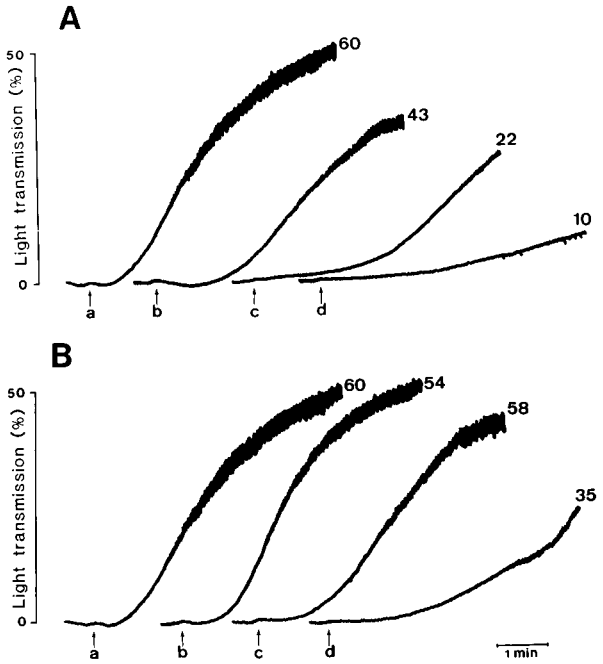


FIGURE 7: Inhibition of clot-bound thrombin. (A) Aggregation of washed human platelets was initiated in the presence of increasing concentrations of bothrojaracin, by the addition of clots formed with 300 $\mu$L of 2 mg/mL purified fibrinogen and 30 nM $\alpha$-thrombin and extensively washed. Bothrojaracin (a) 0, (b) 12 nM, (c) 50 nM, and (d) 100 nM] was added to the platelet suspension just before the addition of the thrombin-containing clots. The extent of [$^{14}$C]-serotonin secretion (percent) is indicated above each curve. (B) Same experiment as in panel A but in the presence of hirudin 54−65: (a) 0, (b) 1 $\mu$M, (c) 5 $\mu$M, and (d) 10 $\mu$M hirudin 54−65. Results are from one representative experiment out of three.

Table 1: Comparison of Clot-Bound Thrombin Mobilization Induced by Bothrojaracin and Other Thrombin Inhibitors

| | % of [$^{125}$I]thrombin displaced after 4 h of incubation |
|---|---|
| buffer | 8 ± 1 |
| 0.8 $\mu$M AT plus 50 mu/mL heparin | 19 ± 5 |
| 200 nM bothrojaracin | 30 ± 7 |
| 200 nM hirudin 54−65 | 22 ± 3 |
| 4 u/mL hirudin (40 nM) | 60 ± 5 |

fraction of the thrombin associated to the clot. We have thus studied the possibility that thrombin retained within the clot may be mobilized. After seven washing steps over a period of 24 h, 40 ± 3% of the labeled thrombin remained associated with clots formed from purified fibrinogen and [$^{125}$I]-thrombin. Upon further incubation in buffer, thrombin diffused slowly from the clot to the medium at a slow rate of 8 ± 1% in 4 h (Table 1). The heparin−AT complex slightly increased the displacement of thrombin. Displacement was more important with bothrojaracin, 30 ± 7% of the radioactivity initially associated to the clot being found in the medium after 4 h in the presence of 200 nM bothrojaracin. The mobilization of clot-bound thrombin by bothrojaracin was time- and concentration-dependent (Figure 8). In the same system, hirudin also mobilized thrombin rapidly and efficiently, in agreement with previous studies (Rubens et al., 1993). At equivalent molar concentrations (200 nM), bothrojaracin was more efficient than the C-terminal hirudin peptide H54−65 in displacing clot-bound thrombin to the soluble phase (Table 1). Thus, several factors appear to determine the ability of a thrombin inhibitor to release clot-bound thrombin. Firstly, the ability of the

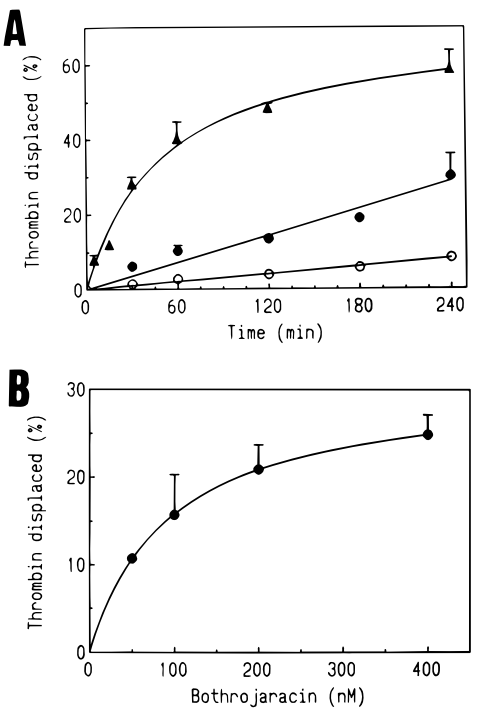


FIGURE 8: Mobilization of clot-bound thrombin. (A) Kinetics. Fibrin clots were formed by incubating human fibrinogen [2 mg/mL in 10 mM imidazole, 0.15 M NaCl, $CaCl_2$, (pH 7.5), and 0.1% PEG] in the presence of 5 nM $^{125}$I-labeled $\alpha$-thrombin and washed seven times over a period of 24 h. Clots were then incubated for different times in the presence of (○) buffer, (●) 200 nM bothrojaracin, or (▲) 4 u/mL hirudin. At timed intervals, the radioactivity associated to the clot and to the incubation medium was measured. The ratio of free [$^{125}$I]-thrombin versus free plus clot-bound [$^{125}$I]-thrombin × 100 is plotted as a function of the time of incubation. (B) Dose−response curve. Same experiment as above but the clots were incubated for 4 h with different concentrations of bothrojaracin. The amount of spontaneously released thrombin was subtracted from that released in the presence of bothrojaracin. Results are the mean of at least three independent experiments ± 1 SD.

inhibitor to interact with thrombin exosite 1 appears crucial. Secondly, the higher the affinity is, the better the release, as indicated by the decreasing order of efficacy: hirudin > bothrojaracin > hirudin 54−65. In addition, the size of the inhibitors seems to be a limiting factor, a smaller inhibitor having an easier access to the clot, as shown by the fact that bothrojaracin (27 kDa) is only 2.5 times more potent than hirudin 54−65 (1.5 kDa) although it has at least a 10-fold better affinity for thrombin.

The observations reported here indicate that bothrojaracin is a new type of thrombin inhibitor characterized by a high-affinity interaction with both exosite 1 and exosite 2. In addition, bothrojaracin might inhibit thrombin as early as it is formed due to its binding to prothrombin. Bothrojaracin is a potent inhibitor of fibrin−associated thrombin, being able to rapidly block the activity of the enzyme at the surface of the clot and to slowly induce its release from the clot. These observations underline the benefit for an inhibitor of thrombin to be multiple-site-directed as are hirudin and bothrojaracin.

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