Bothrojaracin, a New Thrombin Inhibitor Isolated from *Bothrops jararaca* Venom: Characterization and Mechanism of Thrombin Inhibition†

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ABSTRACT: A new thrombin inhibitor, bothrojaracin, has been identified and purified to homogeneity from the venom of *Bothrops jararaca*, the most common venomous snake of South America. Bothrojaracin has an isoelectric point of 4.2 and a molecular mass of 27 kDa and is made of two distinct polypeptide chains of 15 and 13 kDa, linked by disulfide bridges. Purified bothrojaracin is devoid of phospholipase A_2, amidolytic, or fibrino(geno)lytic activity. Bothrojaracin forms a noncovalent complex with α-thrombin, without changing its catalytic activity on small peptide substrates. Bothrojaracin behaves as a potent and specific antagonist of thrombin-induced platelet aggregation and secretion, characterized by an IC_{50} ranging from 1 to 20 nM depending on the α-thrombin concentration. Bothrojaracin prolongs fibrinogen clotting time, and this effect is related to a competitive inhibition of the binding of α-thrombin to fibrin(ogen) (K_i 15 nM). Binding of α-thrombin to thrombomodulin is inhibited up to 87% by bothrojaracin, and the rate of protein C activation by α-thrombin is also decreased. Bothrojaracin antagonizes the inhibition of thrombin amidolytic activity by hirudin. These results indicate that bothrojaracin acts as a very potent ligand of the exosite of α-thrombin.

Venoms from *Viperidae* snakes alter blood coagulation and platelet functions in a complex manner. Each venom contains several protein components which behave as pro- or anticoagulants or which induce or inhibit platelet aggregation (Brinkhous & Smith, 1988; Kini & Evans, 1990; Stocker, 1990; Longenecker, 1991). Most venoms from *Bothrops* species induced blood clotting. This activity has been attributed to a fibrinogen clotting enzyme, batroxobin (Funk *et al.*, 1971; Stocker & Egberg, 1973; Stocker & Barlow, 1976; Holleman & Weiss, 1976), and to metalloproteinases which can convert prothrombin into thrombin (Nahas *et al.*, 1964; Hofmann & Bon, 1987a; Rosing *et al.*, 1988) or activate factor X (Denson *et al.*, 1972; Furukawa & Hayashi, 1977; Hofmann & Bon, 1987b). Several proteins able to activate platelet aggregation have also been purified and characterized from venoms of *Bothrops* snakes. Among them, thrombocytin (Kirby *et al.*, 1979; Zingali *et al.*, 1990) is a serine protease which induces platelet aggregation by a mechanism which resembles that of thrombin (Holleman & Weiss, 1976; Niewiarowski *et al.*, 1979), while other components such as thrombolectin are lectins which agglutinate erythrocytes and induce aggregation (Gartner *et al.*, 1980). Another protein, botrocetin, from the venom of *Bothrops jararaca* (Brinkhous *et al.*, 1983) promotes platelet agglutination by forming a complex with von Willebrand factor, which in turn binds to the platelet membrane glycoprotein GPIb-IX (Read *et al.*, 1989).

Recently, snake venom components blocking platelet aggregation have attracted considerable attention because of their potential therapeutic use as antithrombotic agents. The first anti-aggregating venom compounds to be described were enzymes such as fibrinogenase and 5′-nucleotidase that interfere with the platelet activation mediators, fibrinogen and ADP, respectively (Boffa & Boffa, 1974; Ouyang & Huang, 1983, 1986). Snake venoms also contain peptides presenting an Arg-Gly-Asp (RGD) sequence, called desintegrins (Gould *et al.*, 1990), which act by inhibiting the interaction of fibrinogen with the platelet receptor glycoprotein GPIIb-IIIa, thus blocking platelet aggregation (Huang *et al.*, 1987; Savage *et al.*, 1990) and thrombus formation (Shebuski *et al.*, 1990). However, up to now, no inhibitory protein specifically interacting with thrombin has yet been described in snake venoms.

Thrombin is a serine protease which plays a central role in hemostasis. Thrombin not only converts fibrinogen into fibrin but also regulates its own production from prothrombin by activating other coagulation proteins (factors V, VIII, and XI, protein C, *etc.*). In addition, it is a potent cellular agonist. Thrombin is unique among proteinases in that it has at least one exosite, *i.e.*, a site different from the catalytic site, that increases the efficiency of the recognition of protein substrates and inhibitors (Fenton *et al.*, 1991). This exosite is involved in the binding of thrombin to fibrinogen, thrombomodulin, protein C, platelet receptor, and GPIb (Fenton *et al.*, 1988; Bezeaud & Guillin, 1988; Hofsteenge *et al.*, 1988; Vu *et al.*, 1991; Jandrot-Perrus *et al.*, 1992).

In this paper, we describe the purification and characterization of a new protein from *B. jararaca* venom, bothrojaracin, that specifically interacts with α-thrombin. This protein presents a structural similarity with botrocetin, although it has a distinct biological activity. We show that it forms a complex with thrombin and inhibits most activity of the

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enzyme. Several aspects of α-thrombin inhibition by bothrojaracin are reported, in particular, those related to the activation of platelet aggregation and to interactions with fibrinogen, fibrin, thrombomodulin, protein C, and hirudin. We show that bothrojaracin does not interact with the α-thrombin catalytic site but appears to act as an exosite-directed competitive inhibitor.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA),[1] prostaglandin E_1 (PGE_1) grade V, apyrase, hirudin, arachidonic acid, adenosine diphosphate (ADP), and phenylmethanesulfonyl fluoride (PMSF) were from Sigma (St. Louis, MO). Chromogenic substrates S-2238 (H-D-Phe-Pip-Arg-*p*-nitroanilide hydrochloride) and S-2366 (Glu-Pro-Arg-*p*-nitroanilide hydrochloride) and purified human fibrinogen were from Kabi Vitrum (Stockholm, Sweden). PAF was a gift from B. B. Vargaftig (Institut Pasteur, Paris, France). Ristocetin was from Diagnostica Stago (Asnières, France). Collagen was from Horn Chemie (Munich, Germany), PPACK from Calbiochem (San Diego, CA), and Iodogen from Pierce Chemical Co. (Rockford, IL). Na ^{125}I and [^{14}C]-5-HT were from Amersham (Les Ulis, France). Lecithin, phenol red, and β-mercaptoethanol were from Merck (Darmstad, Germany). Sephacryl S-200 and Mono-Q (HR 10/16) were obtained from Pharmacia (Uppsala, Sweden).

Venoms. Venom samples from *B. jararaca* were purchased from Instituto Butantan (São-Paulo, SP, Brazil) and Ventoxin (Gaitherburg, MD) in a desiccated form and from Latoxan (Rosans, France) and Miami Serpentarium (Salt Lake City, UT) as a lyophilized powder. They showed important differences in their effects on platelet aggregation: venom from Instituto Butantan actively induced platelet aggregation (EC_{50} of 50 μg/mL), while venom samples obtained from Miami Serpentarium and Latoxan inhibited the aggregation of platelets induced by bovine or human α-thrombin (IC_{50} 10 μg/mL); the venom sample purchased from Ventoxin was the less active in both effects. This indicated that these various venom samples contained different proportions of aggregation inducers and inhibitors. Since the purpose of this investigation was to identify components that prevent platelet aggregation, further experiments were performed with the sample of *B. jararaca* venom obtained from Latoxan, which appeared the most active.

Purified Coagulation Proteins. Human α-thrombin was purified as previously described (Bezeaud *et al.*, 1985). The α-thrombin preparations used throughout this study were 97% α-thrombin, 2% β-thrombin, and 1% γ-thrombin, as judged by SDS–PAGE, and their activity was 3000 ± 300 units/mg. Human γ-thrombin was prepared by controlled passage through a trypsin–Sepharose 4B column (Fenton *et al.*, 1977). α-Thrombin was iodinated using the solid-phase reagent Iodogen and Na^{125}I according to the procedure described by Jandrot-Perrus *et al.* (1988). Specific radioactivity was 50 kBq/μg of protein. ^{125}I-Labeled α-thrombin induced platelet aggregation and secretion as efficiently as unlabeled α-thrombin.

Rabbit lung thrombomodulin, bovine protein C, and human antithrombin III were isolated according to published procedures (Esmon *et al.*, 1982; Stenflo, 1976; Bezeaud *et al.*, 1985).

Protein Concentration Measurements and Enzymatic Assays. Protein concentration was determined by absorbance at 280 nm using an extinction coefficient, $E^{1\%}_{cm}$, of 10 and/or by the method of Bradford (1976), using ovalbumin as a standard.

Thrombin-like amidolytic activity was determined in microtitration plates with the chromogenic substrate S-2238. Briefly, S-2238 (80 μM) in 0.02 M Tris-HCl and 0.15 M NaCl, pH 8.0, was incubated for 5–20 min at 37 °C with different concentrations of venom fractions to be tested; absorbance at 405 nm was then read.

Phospholipase A_2 activity was determined in microtitration plates by the colorimetric method described by Lôbo de Araujo & Radvanyi (1987), using phenol red as a pH indicator.

Fibrinogenolytic activity was measured by incubating 1 volume of 50 mg/mL fibrinogen for 1 h at 37 °C with 1 volume of the sample to be tested. After addition of 13% ammonium sulfate and centrifugation at 13 000 rpm for 5 min, the absorbance of the supernatant at 280 nm was measured. Fibrinogenolysis was also determined following the hydrolysis of Aα, Bβ, and γ protein bands of fibrinogen (2 mg/mL) after a 20-h incubation at 37 °C by SDS–PAGE in reducing conditions.

PAGE and Isoelectric Focusing on Polyacrylamide Gels. Analytical PAGE, in the absence of detergent or in the presence of 2% SDS, and isoelectric focusing (pH 4.0–6.5) were performed with a Phast System (Pharmacia) according to the recommendations of the manufacturer. Molecular mass standards were phosphorylase B (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and α-lactoalbumin (14.4 kDa). Proteins were stained with Coomassie Brilliant Blue R-250.

Treatment with Glycosidase. Possible N- or O-glycosylation of bothrojaracin was examined by treating the protein at 37 °C for 18 h with *N*-glycanase and then for 7 h with *O*-glycanase. Acetylcholinesterase from *Torpedo* electric organ served as a positive control to verify the efficiency of deglycosylation by *N*-glycanase. The treated proteins were subsequently analyzed by SDS-PAGE under reducing (2% β-mercaptoethanol) and nonreducing conditions.

Polypeptide Sequence. Sequence determinations of proteins or peptides were performed by Edman degradation with an Applied Biosystem 470-A gas-phase sequencer. Phenylthiohydantoin amino acids were identified by on-line reverse-phase HPLC in an RP_{18} column, with an Applied Biosystem 120-A analyzer. The purified bothrojaracin was first submitted to SDS–PAGE and electrotransferred to an Immobilon membrane. The protein bands were cut off and subjected to Edman degradation using a standard program (03RPTH).

Platelet Preparation, Aggregation, Secretion, and Agglutination Assays. Initial assays have been performed on rabbit platelets and subsequently on human platelets. Rabbit platelets were prepared from whole blood obtained from a 3–4-kg male (HY/CR) and anticoagulated with 5 mM EDTA. Platelets were then separated from blood and washed as previously described (Ardlie *et al.*, 1970). Platelets were washed twice with calcium-free Tyrode's buffer, pH 6.5, containing 0.1% glucose, 0.25% gelatin, and 0.2 mM EGTA.

[1] Abbreviations: ADP, adenosine diphosphate; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; EC_{50}, concentration of inducer resulting in half-maximal platelet activation; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GP, glycoprotein; HPLC, high-performance liquid chromatography; 5-HT, 5-hydroxytryptamine; IC_{50}, concentration of inhibitor reducing thrombin activity by 50%; PAF, platelet activating factor; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); PGE_1, prostaglandin E_1; PMSF, phenylmethanesulfonyl fluoride; PPACK, D-Phe-Pro-Arg chloromethyl ketone; PRP, platelet-rich plasma; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

Table I: Specific Activity and Yield of Bothrojaracin at Different Stages of Purification

purification step	fraction	protein		inhibition of aggregation			purification factor
		mg	%	IC_{50} (μg/mL)	no. of IC_{50}	%	
	crude venom	2768	100	10	278 000	100	1
Sephacryl	S-IV	431	15.6	3	144 000	52	3.3
first Mono-Q	M-1	59	2.1	1.5	39 000	14	6.7
	M-2	27	1.0	0.6	55 000	20	16.7
	M-3	17	0.6	1.5	11 000	4.0	6.7
	M-4	11	0.4	0.8	19 000	6.8	12.5
	total	114	4.1		124 000	45	
second Mono-Q	bothrojaracin	5.6	0.2	0.1	56 000	20	100

The final suspension was in a modified Tyrode's buffer (pH 7.4) where EGTA was replaced by 2 mM $CaCl_2$ and 1 mM $MgCl_2$. Human platelets were prepared according to the published procedure (Jandrot-Perrus, 1988). Platelet-rich plasma was preincubated for 30 min at 37 °C with 0.6 mM [^{14}C]-5-HT. Apyrase (25 μg/mL) and 100 nM PGE_1 were added to the platelets. The platelets were then washed three times in washing buffer (pH 6.5) containing 100 nM PGE_1, 25 μg/mL apyrase, and 3.5 mg/mL BSA. Washed platelets were resuspended in Tyrode–Hepes buffer, pH 7.4, containing 3.5 mg/mL BSA. Aggregation was performed at 37 °C under stirring (1100 rpm) in a Chronolog aggregometer (Coultronics). Platelet aggregation was induced by the addition of agonists after preincubation with or without bothrojaracin as indicated in the text. For the measure of dense-granule secretion, aggregation was stopped at the designated times 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.

Platelet agglutination was performed with platelets fixed with paraformaldehyde, as described by Brinkhous and Read (1989). Fixed platelets in 84 mM imidazole buffer, pH 7.35, containing 0.154 M NaCl and 0.1% BSA were mixed with normal human plasma and ristocetin (0.5 mg/mL) or samples to be tested. The time for the formation of macroscopic platelet aggregates was recorded.

Iodination and Binding Assays of α-Thrombin and Bothrojaracin. Bothrojaracin was labeled with the Iodogen procedure. Iodination was performed for 8 min in a tube coated with 10 μg of Iodogen containing 50 μL of 20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 1.8 nmol of bothrojaracin, and 2.5 nmol of Na^{125}I (4 mCi/pmol). The ^{125}I-labeled bothrojaracin was separated from free Na^{125}I by gel filtration on a Sephadex G-25 column equilibrated with 20 mM Tris-HCl, pH 7.5. The incorporation was 0.82 mol of ^{125}I/mol of bothrojaracin. The labeled bothrojaracin retained 100% of its capacity to inhibit thrombin-induced platelet aggregation.

Washed rabbit platelets (400 000/μL) were incubated for 45 min at room temperature in a final volume of 400 μL with 10 mg/mL BSA and increasing amounts ^{125}I-labeled bothrojaracin. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled bothrojaracin.

Equilibrium binding assays of ^{125}I-labeled α-thrombin to rabbit washed platelets were essentially performed as described by Harmon and Jamieson (1985) under the conditions described above for the binding of bothrojaracin. In order to measure inhibition of ^{125}I-labeled α-thrombin binding, unlabeled bothrojaracin was added to the assays before addition of labeled α-thrombin.

α-Thrombin Amidolytic Activity. The hydrolysis of S-2238 by thrombin was monitored at 25 °C in 10 mM Tris-HCl, 10 mM Hepes, and 100 mM NaCl, pH 7.8, containing 0.1% PEG. Human α-thrombin (4 nM) which had been preincubated with 10 mM bothrojaracin or buffer for 3 min was added to S-2238 (2–8 μM).

α-Thrombin Clotting Activity. Fibrinogen clotting time was measured as described by Hofmann *et al.* (1983). Briefly, the clotting time of 0.2 mL of human fibrinogen (4 mg/mL) in Michaelis buffer was measured after addition of 0.2 mL of α-thrombin (0.5 NIH/mL final concentration) preincubated at 37 °C for 30 s with bothrojaracin or buffer.

α-Thrombin Binding to Fibrin. Fibrin monomers were prepared as previously described (Jandrot-Perrus *et al.*, 1992). Reaggregation of fibrin monomers from acetic acid solutions was initiated by diluting an aliquot of solubilized fibrin into 9 volumes of 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and 1% BSA in the presence of 2 nM ^{125}I-labeled α-thrombin and variable amounts of bothrojaracin. After centrifugation at 12000*g* for 10 min, free thrombin in the supernatants and thrombin associated to the fibrin polymer in the pellets were quantified by γ counting in a CG 4000 Kontron Intertechnique counter.

α-Thrombin Binding to Thrombomodulin. α-Thrombin binding to thrombomodulin was measured using a solid-phase assay (Hayashi *et al.*, 1990). Briefly, microwell plates (Immulon, Dynatech) were coated with 25 ng of rabbit thrombomodulin. After saturation with BSA, human α-thrombin, at various concentrations in 50 mM Tris-HCl and 50 mM NaCl, pH 7.4, containing 0.5% BSA and 0.05% Tween 20, was added to the wells in the presence of various amounts of bothrojaracin. After 90-min incubation and washing, α-thrombin bound to thrombomodulin was detected using S-2238. Blanks were obtained by omitting the coating with thrombomodulin or replacing α-thrombin by buffer.

α-Thrombin Interaction with Protein C. The rate of protein C activation was measured in the absence of thrombomodulin as previously described (Bezeaud & Guillin, 1988). Human α-thrombin (10 nM) was incubated at 37 °C with 0.12 μM protein C in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.5, and 1% BSA, in the presence of buffer or 50 nM bothrojaracin. At timed intervals, activation was quenched by addition of antithrombin III (3.8 μM) and heparin (0.5 unit/mL), and activated protein C was measured using S-2366.

α-Thrombin Inhibition by Hirudin. The inhibition of thrombin amidolytic activity by hirudin was measured in microtitration plates. α-Thrombin (0.5 nM) was incubated at 37 °C with increasing amounts of hirudin and S-2238 (0.2 mM). The rate of pNA liberation was measured with a microwell plate reader (Dynatech). The effect of bothrojaracin was measured by preincubating thrombin with bothrojaracin (100 nM) for 2 min prior to the addition of hirudin and S-2238.

RESULTS

Purification of Bothrojaracin. Seven fractions were characterized after gel filtration of *B. jararaca* venom in a

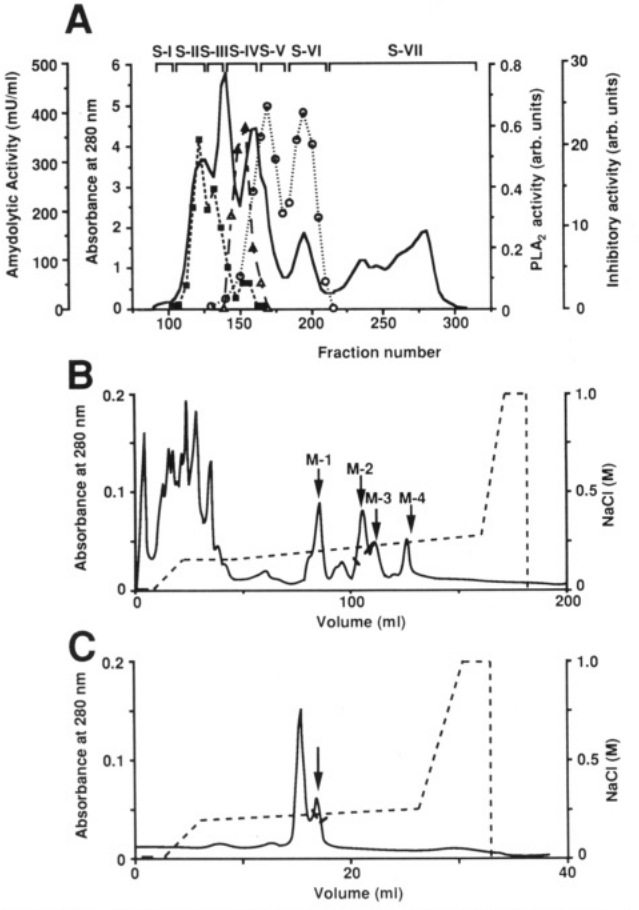

FIGURE 1: Purification of bothrojaracin. (A) Gel filtration in a Sephacyl S-200 HR column. Two grams of *B. jararaca* venom obtained from Latoxan was dissolved in 40 mL of 20 mM Tris-HCl, pH 8.8, containing 150 mM NaCl and centrifuged for 10 min at 7700*g*. The supernatant was applied to a Sephacryl column (5 × 110 cm) preequilibrated with the same buffer, and elution was carried out at a flow rate of 100 mL/h; 8-mL fractions were collected and analyzed for their protein content (—), their phospholipase A_2 activity (○), their amidolytic activity on S-2238 (■), and their inhibitory action on platelet aggregation induced by 0.04 NIH/mL α-thrombin (△), as described in Materials and Methods. Fractions were pooled as indicated. (B) Pool S-IV in (A) was concentrated by lyophilization and dialyzed against 20 mM Tris-HCl, pH 7.5. Samples (50 mg) were applied to a preparative Mono-Q column (HR 16/10) preequilibrated with this buffer, and elution was performed at a flow rate of 3 mL/min by increasing the NaCl concentration in the Tris-HCl buffer, as indicated on the figure (- - -). Protein content (—) was determined by absorbance at 280 nm. Fractions M-1 to M-4, indicated by arrows, contained the inhibitory activity of bothrojaracin. (C) Fraction M-2 (B) was concentrated by lyophilization, dialyzed against 20 mM Bis-Tris-HCl, pH 6.0, and applied to an analytical Mono-Q column (HR 5/5). The elution was achieved at a flow rate of 1 mL/min by increasing the NaCl concentration in Bis-Tris-HCl buffer (- - -). Protein content (—) was determined by absorbance at 280 nm. The arrow indicates the protein peak corresponding to bothrojaracin.

Sephacryl S-200 column (Figure 1A). The inhibitory effect of the venom on thrombin-induced platelet aggregation was predominantly found in fraction S-IV. This fraction also contained most of the venom platelet activating activity (results not shown). Amidolytic activity was mainly eluted in fractions S-II and S-III, but a lesser proportion was also present in fraction S-IV. Phospholipase A_2 activity was found in fractions S-V and S-VI (Figure 1A).

Fraction S-IV was further fractionated by ion-exchange chromatography in a Mono-Q column. As shown in Figure 1B, four fractions, M-1 to M-4, were able to inhibit platelet aggregation induced by α-thrombin. They were devoid of amidolytic and phospholipase A_2 activities and did not induce platelet aggregation. When submitted by SDS–PAGE under nonreducing conditions, fractions M-1 to M-4 showed a single protein band with an apparent molecular mass of 27 kDa, while two polypeptides of 13 and 15 kDa were observed after reduction with 2% β-mercaptoethanol. On the other hand, isoelectric focusing on polyacrylamide gels showed that all these fractions were mixtures of two, three, or more proteins, with isoelectric points between 4.0 and 4.5 (results not shown).

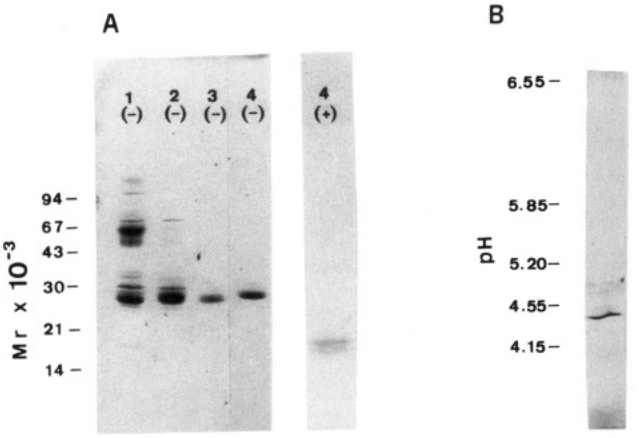

FIGURE 2: SDS–PAGE and isoelectric focusing of bothrojaracin and *B. jararaca* venom fractions. (A) SDS–PAGE was carried out with 20% acrylamide gels in the absence (–) or in the presence (+) of 5% β-mercaptoethanol. Lanes: 1, *B. jararaca* venom; 2, fraction S-IV; 3, fraction M-2; 4, bothrojaracin. (B) Isoelectric focusing of bothrojaracin. The pH gradient, from 4.0 to 6.5, was determined with a contact electrode immediately after electrophoresis and with standard proteins.

Fraction M-2 presented the highest capacity to inhibit thrombin-induced platelet aggregation (Table I) and appeared more homogeneous than other fractions in isoelectric focusing. This fraction was further fractionated by a second ion-exchange chromatography in a Mono-Q column (Figure 1C) and was found to contain two proteins: the protein which eluted at the highest ionic strength was able to prevent thrombin-induced platelet aggregation and was named bothrojaracin; the other protein was inactive.

Table I shows that the specific activity of purified bothrojaracin, which was obtained with a purification yield of 20%, was 100-fold that of the crude *B. jararaca* venom. Several other fractions (M-1, M-3, and M-4) were, however, observed to present the same activity: altogether, the four fractions, M-1 to M-4, represented, in fact, more than 40% of the capacity of the crude venom to inhibit thrombin-induced platelet aggregation (Table I). Proteins contained in these fractions, having similar physicochemical properties and biological activity, might therefore represent bothrojaracin isoforms (or variants), as expected for a venom collected from several snakes.

Molecular Properties of Bothrojaracin. When analyzed by SDS–PAGE under reducing and nonreducing conditions, purified bothrojaracin proved to be a homogeneous protein of 27 ± 2 kDa made of two polypeptide chains of 13 ± 1 and 15 ± 1 kDa, linked by disulfide bridges (Figure 2A). Bothrojaracin was also homogeneous when tested by isoelectric focusing, with an isoelectric point of 4.2 ± 0.1 (Figure 2B). Treatment of bothrojaracin with *N*- and *O*-glycanases did not modify the electrophoretic mobility of the protein, suggesting the absence of glycosylation (results not shown).

Bothrojaracin did not show any phospholipase A_2 or amidolytic activity. It did not possess any procoagulant action,

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                                  1 . . . 5 . . . .10 . . . .15 . . . .20
                                  |       |         |         |         |
Bothrojaracin          13kDa      D C P ? D W S ? Y ...
                       15kDa      D C P S D W S P Y G Q G C Y ? F ...

Botrocetin             α chain    D C P S G W S S Y E G N C Y K F F Q Q K ...
                       β chain    D C P P D W S S Y E G H C Y D F F K E W ...
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FIGURE 3: Amino acid NH_2-terminal sequence of bothrojaracin. The NH_2 sequence of the two polypeptide chains of bothrojaracin was determined by Edman degradation, as indicated in Materials and Methods. The sequences are compared with the NH_2 sequences determined for botrocetin by Fujimura *et al.* (1991).

Table II: Platelet Agglutination by Botrocetin-like Activity in *B. jararaca* Venom Fractions

venom fraction	concn (μg/mL)	agglutination time (s)	venom fraction	concn (μg/mL)	agglutination time (s)
S-IV	4.5	20	M-3	1.0	>180
M-1	1.0	35	bothrojaracin	1.0	>180
M-2	1.0	>180			

Table III: The Anti-Aggregating Activity of Bothrojaracin Is Specific for Thrombin

platelet agonist	agonist concn[a]	bothrojaracin concn[b] (nM)	inhibition[c] (%)
thrombin	2 nM	10	100 ± 5
collagen	13 μg/mL	1500	0 ± 5
PAF	0.1 nM	750	0 ± 5
AA	60 μM	750	0 ± 5
ADP	7.5 μM	750	0 ± 5
cerastocytin	20 μM	75	0 ± 5

[a] The concentrations of agonists were chosen in order to induce 80% of maximal aggregation. [b] Bothrojaracin was added to rabbit platelets 2 min before introduction of agonist. [c] The inhibitory effect of bothrojaracin was measured as described in Materials and Methods.

was unable to clot fibrinogen, and did not show any fibrino-(geno)lytic activity. Treatment of bothrojaracin for 20 h at 4 °C with 3 mM PMSF or 50 mM DFP did not modify its ability to inhibit platelet aggregation induced by α-thrombin (results not shown).

The NH_2-terminal amino acid sequences of the two polypeptide chains which constitute bothrojaracin were determined by Edman degradation (Figure 3). These sequences clearly show considerable similarity with botrocetin (Andrews *et al.*, 1989; Fujimura *et al.*, 1991). Bothrojaracin was, however, unable to cause the agglutination of fixed platelets in the presence of plasma, as does botrocetin (Table II). In addition the two activities were separated in the Mono-Q column: botrocetin-like activity eluted in fraction M-1 while bothrojaracin was recovered in fraction M-2 (Table II). These results clearly indicate that bothrojaracin and botrocetin are distinct proteins.

Effect of Bothrojaracin on Blood Platelets. Bothrojaracin completely inhibited the aggregation of rabbit platelets induced by 2 nM human α-thrombin (Table III). In contrast, bothrojaracin was unable to inhibit platelet aggregation induced by other agonists used at submaximal concentrations: collagen, PAF, arachidonic acid, ADP, and ceratocytin, a thrombin-like serine protease from the venom of *Cerastes vipera*, even when the bothrojaracin concentration was 100-fold higher than that required for maximal inhibition of platelet aggregation induced by thrombin (Table III). These observations indicate that the inhibitory effect of bothrojaracin on platelet aggregation is very specific for α-thrombin.

Preincubation of bothrojaracin with α-thrombin inhibited aggregation of human platelets, in a dose-dependent manner,

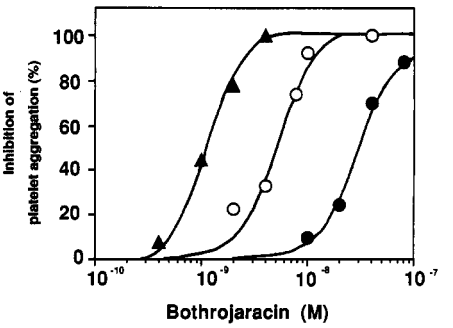

FIGURE 4: Inhibition by bothrojaracin of α-thrombin-induced platelet aggregation. The indicated concentrations of bothrojaracin were preincubated for 3 min at 37 °C with α-thrombin: (▲) 0.5 nM; (○) 1 nM; (●) 5.0 nM. The mixtures were added to human platelets, and the extent of aggregation was measured after 3 min. Dose–response curves of inhibition in function of bothrojaracin concentration are presented.

as shown on Figure 4. The concentrations of bothrojaracin required to achieve 50% inhibition (IC_{50}) of aggregation induced by 0.5, 1.0, and 5.0 nM α-thrombin were 1.0, 4.0, and 20 nM, respectively. Bothrojaracin (4 nM) also inhibited by 70% the time-dependent platelet secretion of [^{14}C]-5-HT induced by (1 nM) α-thrombin throughout the course of the reaction (data not shown). In contrast to the results obtained with α-thrombin, bothrojaracin (400 nM) did not inhibit aggregation nor 5-HT secretion induced by 50 nM γ-thrombin (not shown).

Equilibrium binding of ^{125}I-labeled α-thrombin to rabbit platelets was performed in the presence of increasing concentrations of bothrojaracin. The specific binding of ^{125}I-labeled thrombin to platelets was dose dependently inhibited (Figure 5). Bifunctional chemical agents allow stabilization of ^{125}I-labeled α-thrombin to platelet proteins, some of which are identified such as GP-Ib and protease nexine I (Jandrot-Perrus et al., 1988). This covalent binding was dose dependently inhibited by bothrojaracin (results not shown). The inhibition of thrombin binding to platelets and of thrombin-induced platelet aggregation could thus be the result of a direct interaction of bothrojaracin with either thrombin-receptor sites on platelets or thrombin itself. A direct interaction of bothrojaracin with platelets was investigated.

Platelet aggregation inhibition was found to be independent of the incubation time of platelets with bothrojaracin before α-thrombin addition. Platelets incubated for 2 min with 10 nM bothrojaracin, a concentration which totally inhibits thrombin-induced aggregation, then centrifuged, and resuspended in a bothrojaracin-free medium were still able to aggregate when stimulated with α-thrombin (results not shown), indicating that if bothrojaracin interacts with platelets, its effect is reversible. At least, binding experiments showed that ^{125}I-labeled bothrojaracin (30–300 nM), which retained its ability to prevent thrombin-induced aggregation, did not

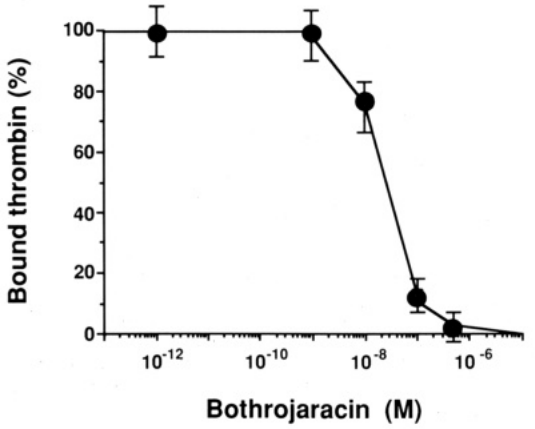

FIGURE 5: Inhibition by bothrojaracin of ^{125}I-labeled α-thrombin binding to platelets. Inhibition of the binding of ^{125}I-labeled α-thrombin (2.5 nM) to rabbit platelets by bothrojaracin was analyzed as described in Materials and Methods. Specific binding was determined by subtraction of the nonspecific binding, measured in the presence of an excess of unlabeled α-thrombin (1 mM), from total binding and was expressed as a percent of the control value, determined in the absence of bothrojaracin. Values are means of three independent determinations ± standard errors.

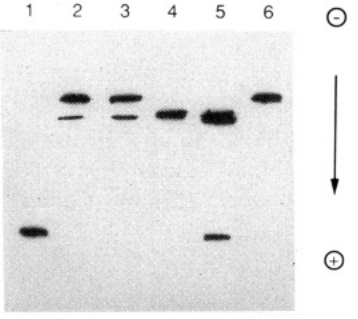

FIGURE 6: Nondenaturing gel electrophoresis of α-thrombin and bothrojaracin. After incubation of α-thrombin with bothrojaracin at 37 °C for 20 min in 20 mM Tris-HCl, pH 7.5, nondenaturing PAGE was performed in precast gels (20% acrylamide). Aliquots (4 μL) were applied. Lanes: 1, 6.2 μM bothrojaracin; 2–5, bothrojaracin (0.75, 1.5, 3.0, and 6.2 μM) incubated with 4 μM α-thrombin; 6, 4 μM α-thrombin.

specifically bind to platelets: specific and nonspecific bindings were identical and corresponded to about 1% of free ^{125}I-labeled bothrojaracin (results not shown). Altogether, these results indicate that the inhibitory effect of bothrojaracin could result from a direct interaction with thrombin and not with platelets.

Identification of α-Thrombin–Bothrojaracin Complexes. Incubation of α-thrombin (4.0 μM) with different concentrations of bothrojaracin (0.75–6.2 μM) leads to the formation of a stable complex that can be detected by PAGE under nondenaturing conditions. When bothrojaracin was present in a 1.5 molar excess over α-thrombin, two protein bands were observed (Figure 6, lane 5): a diffuse, slow-migrating band that corresponds to α-thrombin complexed with bothrojaracin and a sharp band near the migration front that corresponds to bothrojaracin alone. At lower bothrojaracin concentrations, a band corresponding to α-thrombin alone was observed while the band corresponding to the thrombin–bothrojaracin complex decreased in intensity (Figure 6, lanes 2–4).

The thrombin–bothrojaracin complex is not covalent since it was not observed on SDS–PAGE. In addition the migration of the thrombin band was not modified under nonreducing and reducing conditions, even after a long incubation time (150 min at 37 °C) with bothrojaracin, excluding a proteolytic degradation of α-thrombin in the complex. In order to

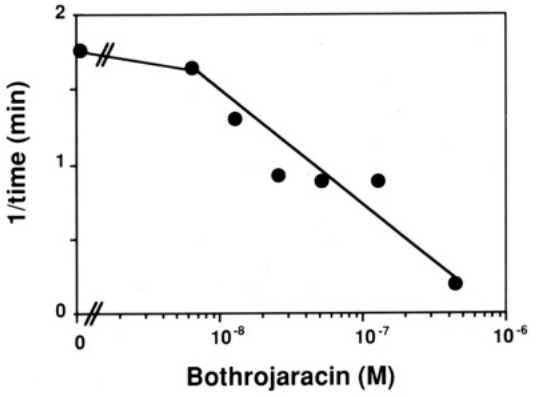

FIGURE 7: Effect of bothrojaracin on fibrinogen clotting time. After preincubation of 0.2 mL of 0.4% fibrinogen at 37 °C for 30 s, 0.2 mL of α-thrombin (0.6 NIH/mL) was added in the presence or absence of bothrojaracin, and the clotting time was measured. Values are the means of three determinations.

elucidate the mechanism of action of bothrojaracin on thrombin, its effects on the biological activities of thrombin were tested.

Effect of Bothrojaracin on the Amidolytic Activity of α-Thrombin. Preincubation of α-thrombin with a 25-fold molar excess of bothrojaracin did not produce any change in the initial velocity of the hydrolysis of S-2238 used at various concentrations below the K_m, indicating that the amidolytic activity of α-thrombin was not impaired by its binding with bothrojaracin (not shown).

Effect of Bothrojaracin on α-Thrombin Interaction with Fibrin(ogen), Thrombomodulin, and Protein C. Bothrojaracin exerts an anticoagulant activity. The clotting time of human fibrinogen was measured after addition of bovine thrombin (0.3 NIH/mL), in the presence of different concentrations of bothrojaracin: it was increased 2-fold in the presence of 50 nM bothrojaracin (Figure 7) and 10-fold in the presence of 500 nM bothrojaracin. Fibrinogen proteolysis by thrombin results from the combination of a binding step and a catalytic step (Fenton *et al.*, 1988). Since bothrojaracin does not inhibit thrombin amidolytic activity, but prevents the conversion of fibrinogen to fibrin, we examined whether it blocked the binding of α-thrombin to fibrin(ogen). For this purpose, fibrin monomers were allowed to polymerize in the presence of ^{125}I-labeled human α-thrombin. Bothrojaracin was found to behave as a competitive inhibitor of the binding of α-thrombin to fibrin, with a K_i of 15 nM (Figure 8).

Bothrojaracin also prevented the binding of α-thrombin to thrombomodulin as shown on Figure 9. Inhibition was dose dependent, with an IC_{50} of 12 nM for 0.25 nM α-thrombin.

The rate of protein C activation in the absence of thrombomodulin was measured in the absence or presence of bothrojaracin: activation by α-thrombin was 2-fold slower in the presence of 50 nM bothrojaracin.

Effect of Bothrojaracin on α-Thrombin Inhibition by Hirudin. The inhibition of thrombin catalytic activity on S-2238 by increasing concentrations of hirudin is shown in Figure 10. The presence of bothrojaracin significantly reduced the inhibitory effect of hirudin, three times more hirudin being required to produce the same inhibition in the presence of bothrojaracin than in its absence. This result indicates that bothrojaracin impairs hirudin binding to thrombin.

DISCUSSION

Crude venom from *B. jararaca* interferes with platelet functions by several mechanisms since it has been reported to

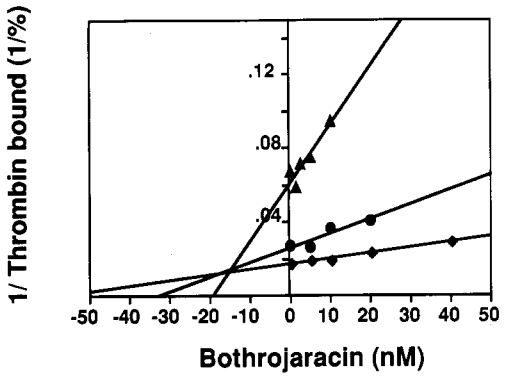

FIGURE 8: Effect of bothrojaracin on the binding of α-thrombin to fibrin monomers. Fibrin monomers were allowed to polymerize at 37 °C in 50 mM Tris-HCl pH 7.5, containing 50 mM NaCl and 1% BSA in the presence of 2 nM ^{125}I-labeled α-thrombin and various amounts of bothrojaracin. After centrifugation (12000*g* and 20 min) fibrin-bound and free thrombin were quantified by measuring the radioactivity of the pellet and of the supernatant. Dixon plots represent the binding of ^{125}I-labeled α-thrombin to fibrin as a function of bothrojaracin concentration, for various concentrations of fibrin monomers: (◆) 1 μM; (●) 0.5 μM; (▲) 0.125 μM. Three different experiments yielded essentially the same results.

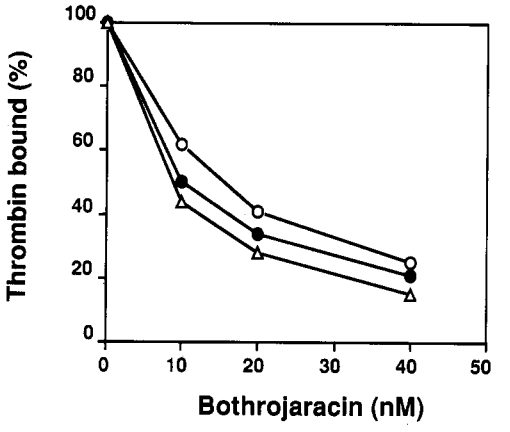

FIGURE 9: Effect of bothrojaracin on the binding of α-thrombin to thrombomodulin. Thrombomodulin (25 ng) was coated in microwell titer plates and incubated for 90 min at room temperature with α-thrombin and various concentrations of bothrojaracin. The activity of bound thrombin was measured using the hydrolysis of S-2238. α-Thrombin concentrations were (○) 0.5 nM, (●) 0.25 nM, and (△) 0.125 nM.

contain three different platelet activators and a PLA_2 which inhibits platelet aggregation induced by collagen (Zingali *et al.*, 1988, 1990). In the current study, we identified a protein from *B. jararaca* venom that is capable of inhibiting platelet aggregation. The purified protein, bothrojaracin, appears homologous to botrocetin, a platelet agglutinating protein isolated from the same venom (Read *et al.*, 1978). These two proteins have the same molecular weight and a strong similarity in their N-terminal amino acid polypeptide sequence. However, they are clearly separated during the purification procedure, and they differ by their p*I*: 4.2 for bothrojaracin (this study) compared to values ranging from 4.6 to 7.8 for botrocetin (Fujimura *et al.*, 1991). In addition, their biological activities are quite different since, in contrast to botrocetin, bothrojaracin does not induce platelet agglutination either directly or indirectly, in the presence of plasma.

We observed that *B. jararaca* venom contains at least four proteins that possess the same activity and the same molecular weight as bothrojaracin but differ by their p*I*. Andrews *et al.* (1989) purified botrocetin and described the presence of an inactive botrocetin analog, with the same structural characteristics and a homologous N-terminal sequence. We propose that this analog of botrocetin might be bothrojaracin. On the other hand, Fujimura *et al.* (1991) described the presence of at least two different forms of botrocetin, both promoting the binding of von Willebrand factor to platelets. Altogether, these data indicate the existence of a protein family presenting structural homology but different biological activities.

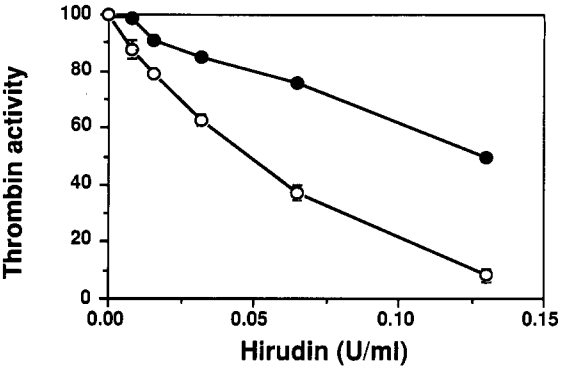

FIGURE 10: Effect of bothrojaracin on thrombin inhibition by hirudin. Thrombin (0.5 nM) was incubated in microwell titer plates at 37 °C with 0.2 mM S-2238 in the presence of increasing concentrations of hirudin, and the initial rate of pNA liberation was measured at 405 nm. Data are presented as the percent of thrombin amidolytic activity inhibited by hirudin. They are the mean ± SD of three experiments performed in duplicate in the absence of bothrojaracin (○) and of two experiments in the presence of 100 nM bothrojaracin (●).

Bothrojaracin specifically inhibits platelet aggregation induced by α-thrombin. The restriction of its inhibitory effect to only one platelet agonist, the reversibility of the effect on platelets, and the absence of binding of bothrojaracin to platelets indicate that platelets are not the target of bothrojaracin action. In fact, bothrojaracin forms a complex with α-thrombin, as shown by nondenaturing PAGE, which is, however, dissociated by SDS, indicating that it is noncovalent. Thus bothrojaracin is not a serpin, such as the natural anticoagulant antithrombin III. The different mode of action of bothrojaracin compared to antithrombin III is confirmed by the lack of effect of bothrojaracin on the α-thrombin catalytic site, which retains full amidolytic activity.

The interaction of α-thrombin with its protein substrates, inhibitors, or receptors involves multiple contacts with the classical catalytic center of the enzyme and/or with the noncontiguous exosite(s) (Fenton *et al.*, 1991). This unique mode of interaction has been demonstrated by crystallography of an α-thrombin–hirudin complex (Rydel *et al.*, 1990; Grutter *et al.*, 1990). Hirudin, a potent and specific thrombin inhibitor from the leech *Hirudo medicinalis*, consists of two domains. The N-terminal domain interacts with the catalytic center of α-thrombin, whereas the highly acidic C-terminal tail makes numerous contacts with a distinct positively charged surface region of α-thrombin, designated as the "anion binding exo site". Peptides mimicking the C-terminal tail of hirudin have been shown to inhibit the binding of α-thrombin to fibrin(ogen) (Naski *et al.*, 1990), thrombomodulin (Tsiang *et al.*, 1990), platelet GPIb (Jandrot-Perrus *et al.*, 1991), and thrombin receptor (Vu *et al.*, 1991), as well as activation of protein C (Hortin & Trimpe, 1991). These peptides, however, do not inhibit α-thrombin-catalyzed hydrolysis of small synthetic substrates (Krstenansky *et al.*, 1987; Maraganore *et al.*, 1989). In the present paper, we show that bothrojaracin does not interfere with the catalytic site of thrombin but inhibits its interaction with fibrin(ogen), protein C, thrombomodulin, and GPIb (data not shown). Bothrojaracin acts, therefore, via a mechanism very similar to that of the hirudin C-terminal

tail, *i.e.*, binding to an exosite or exosites distinct from the catalytic site. In fact, this is confirmed by the blocking effect of bothrojaracin on thrombin inhibition by hirudin, which indicates a competition between the two inhibitors for binding to thrombin exosite(s).

Controlled proteolysis of human α-thrombin by trypsin operates limited cleavages of the thrombin B chain at Arg_{73} and in its vicinity and removes a region extending from Ile_{124} to Lys_{154} (Fenton *et al.*, 1977; Braun *et al.*, 1988), yielding γ-thrombin. γ-Thrombin retains most of the activity of α-thrombin toward small synthetic substrates but is inactive on protein substrates, such as fibrinogen and protein C, or on ligands, such as thrombomodulin and platelet GPIb (Bezeaud *et al.*, 1985; Bezeaud & Guillin, 1988; Fenton *et al.*, 1988; Hofsteenge *et al.*, 1988; Jandrot-Perrus *et al.*, 1988). In addition, conversion of α- to γ-thrombin dramatically decreases the binding of the C-terminal part of hirudin (Stone & Hofsteenge, 1991). Similarly, we were not able to inhibit γ-thrombin-induced platelet activation with bothrojaracin, indicating that the affinity of bothrojaracin for γ-thrombin is extremely reduced compared to that for α-thrombin and that the proteolytic cleavages accompanying the conversion of α- to γ-thrombin disrupt or alter the conformation of the binding site(s) for bothrojaracin.

Bothrojaracin is a more potent thrombin inhibitor than peptides derived from the C-terminal tail of hirudin: (i) the IC_{50} values for inhibition of platelet activation (Jakubowski *et al.*, 1990; Jandrot-Perrus *et al.*, 1991) or thrombomodulin binding (Tsiang *et al.*, 1990) are 3 orders of magnitude higher for hirudin C-terminal peptides than for bothrojaracin; (ii) the inhibition constants (K_i) for α-thrombin interaction with fibrin(ogen) are 20 and 540 nM for bothrojaracin (this study) and hirudin C-terminal peptide (Naski *et al.*, 1990), respectively.

Platelet responses to α-thrombin are now known to be mainly elicited via a specific receptor which belongs to the family of the seven transmembrane domain receptors (Vu *et al.*, 1991). Proteolytic attack of the receptor has been proposed to be favored by the binding of α-thrombin to a hirudin C terminus-like sequence on the receptor, via the anion-binding exo site (Liu *et al.*, 1991). The complete inhibition of platelet activation produced by bothrojaracin strongly suggests that thrombin interaction with the receptor might be inhibited by bothrojaracin, probably by a competitive binding to the α-thrombin anion-binding exosite.

The observations presented here reveal the mechanism of action of a new natural thrombin inhibitor, bothrojaracin. The venom of *B. jararaca* has previously been shown to contain many other proteins which induce blood coagulation, platelet aggregation, and platelet agglutination (botrocetin), but bothrojaracin is the first anti-thrombin protein reported in a snake venom. Bothrojaracin will be a useful tool for studying thrombin functions, and because of its very potent and specific action on thrombin, it may possibly be used as an antithrombotic compound. For this purpose, additional information on the structure of the protein and on its functional domain(s) will be required.

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