Purification and Characterization of Bothrombin, a Fibrinogen-Clotting Serine Protease from the Venom of *Bothrops jararaca*†

Sachiyo Nishida,‡ Yoshihiro Fujimura,‡ Shuji Miura,‡ Yukio Ozaki,‖ Yoshiko Usami,⊥ Masami Suzuki,§ Koiti Titani,*,§ Eri Yoshida,‡ Mitsuhiko Sugimoto,‡ Akira Yoshioka,‡ and Hiromu Fukui‡

Departments of Blood Transfusion and Pediatrics, Nara Medical College, Kashihara, Nara 634, Japan, Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University School of Medicine, Toyoake, Aichi 470-11, Japan, Department of Pharmaceutics, Gifu Pharmaceutical University, Gifu, Gifu 502, Japan, and Department of Clinical & Laboratory Medicine, Yamanashi Medical College, Nakagoma-gun, Yamanashi 409-38, Japan

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ABSTRACT: A fibrinogen-clotting enzyme (bothrombin) was purified from the venom of *Bothrops jararaca*. Bothrombin showed M_r values of 33 000 under nonreducing and 35 000 under reducing conditions on SDS polyacrylamide gel electrophoresis and specific fibrinogen-clotting activity equivalent to 814-904 NIH α-thrombin units/mg. Diisopropyl fluorophosphate totally abolished its activity, but hirudin, a specific α-thrombin inhibitor, had negligible effect on bothrombin activity. Unlike α-thrombin, bothrombin split off fibrinopeptide A without releasing fibrinopeptide B. Bothrombin activated blood coagulation factor VIII, but its activity was about 950 times less than that of α-thrombin. Bothrombin did not induce aggregation or serotonin release of washed normal platelets by itself, but did aggregate platelets in the presence of exogenous fibrinogen. This latter activity was completely inhibited by either anti-glycoprotein (GP) IIb/IIIa monoclonal antibody (which blocks fibrinogen binding to GP IIb/IIIa) or anti-GP Ib monoclonal antibody (which specifically inhibits α-thrombin binding to GP Ib). Prostaglandin E1 (1 μM) and EDTA (10 mM) also abolished platelet aggregation without affecting clotting activity. Washed platelets from a patient with Bernard-Soulier syndrome did not respond to bothrombin even in the presence of exogenous fibrinogen, suggesting that the initial binding of bothrombin on platelets is GP Ib, but not a recently cloned thrombin receptor. The complete amino acid sequence of bothrombin was determined by analysis of (*S*)-pyridylethylated protein and peptides generated by digestion with cyanogen bromide and *Achromobacter* protease I, respectively. Bothrombin is composed of 232 amino acid residues and contains three Asn-linked oligosaccharide chains. The sequence is homologous to those of other serine proteases; in particular, batroxobin from the *Bothrops atrox moojeni* venom.

Although a number of snake venoms are known to exhibit Fg[1]-clotting activity, only four enzymes with such activity have been purified and characterized: batroxobin from *Bothrops atrox moojeni* (Itoh et al., 1988), ancrod from *Agkistrodon rhodostoma* (Hatton et al., 1973), crotalase from *Crotalus adamanteus* (Markland & Damus, 1971), and flavoxobin from *Trimeresurus flavoviridis* (Shieh et al., 1988). These four enzymes have no or minimal effect on platelet activation, in contrast to α-thrombin.

Teng & Ko (1988) reported that batroxobin aggregates washed rabbit platelets in a dose-dependent manner, but only at an extremely high concentration, equivalent to 10-100 NIH α-thrombin units/mL (NIH equiv units/mL). In a recent review, Pirkle & Stocker (1991) stated that *Bothrops atrox* (Linneaus) can be divided into five subspecies: *B. atrox* (Hoge), *B. atrox asper*, *B. atrox marajoensis*, *B. atrox moojeni*, and *B. atrox paradoi*. The name batroxobin could be used for a Fg-clotting[1] enzyme from any of these subspecies. Ideally, the enzyme source should be clarified by indicating the subspecies in parentheses. However, neither the amino acid sequences nor biological characteristics of batroxobins from the other four subspecies have been described.

von Klobusitzky & Köning (1936) reported the partial extraction of a component with strong Fg-clotting activity from the venom of the congeneric species *Bothrops jararaca*. Subsequent studies (Blombäck et al., 1957; Furukawa & Hayashi, 1977) have reported purification of this component, but without solid evidence of purity.

Andrews et al. (1989) and our group (Fujimura et al., 1991) recently purified botrocetin, a vWF modulator from the venom of *B. jararaca*. During the initial stages of purification of botrocetin, we observed most Fg-clotting activity in fractions eluted with 0.1 M NaCl from a DEAE-Sepharose CL-6B column (Fujimura et al., 1991). From these fractions, we extracted a Fg-clotting enzyme composed of a single polypeptide with M_r 35 000. Blombäck et al. (1957) first gave the term reptilase to this enzyme. This term, however, has been used incorrectly in many literatures as the trivial name synonymous with batroxobin from the venom of *B. atrox*

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* To whom correspondence should be addressed at Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-11, Japan. Telephone: 81 (Japan)-562-93-9379, Facsimile: 81 (Japan)-562-93-8832.

‡ Nara Medical College.

§ Fujita Health university.

⊥ Gifu Pharmaceutical University.

‖ Yamanashi Medical College.

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[1] Abbreviations: API, *Achromobacter* protease I; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; Fg, fibrinogen; GP, glycoprotein; MoAb, monoclonal antibody; PGE1, prostaglandin E1; PE, pyridylethyl; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TFA, trifluoroacetic acid; vWF, von Willebrand factor.

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Table 1: Purification of Bothrombin

step	total protein (mg)	specific activity (NIH equiv units/mg)	yield (%)
crude venom	3,000	6	100
DEAE-sepharose CL-6B	113	137	86
sephacryl S-300	58	219	71
heparin-sepharose CL-6B	13	886	64
SynChropak RP-8	7	904	35

moojeni (Pirkle & Stocker, 1991). To avoid this terminological confusion, we hereby use the term bothrombin for the purified Fg-clotting enzyme from *B. jararaca*.

In this paper, we describe purification and biochemical characterization of bothrombin (in particular, its unique action on platelets) and a possible platelet activation mechanism through the functional linkage between GP Ib and GP IIb/IIIa. We also present the complete amino acid sequence determined by direct analysis of bothrombin and its homology to other serine proteases.

RESULTS

Purification of Bothrombin. Most Fg-clotting activity was present in the fractions eluted at 0.1 M NaCl by DEAE-Sepharose CL-6B chromatography (Fujimura et al., 1991). At the second chromatographic step on Sephacryl S-300 superfine gel (data not shown), clotting activity was seen when the ratio of elution volume to total bed volume was between 0.7 to 0.82. During subsequent separation on a heparin-Sepharose CL-6B column (supplementary material, Figure 1A), six major protein peaks were separated by gradient elution from 0 to 0.7 M NaCl; strongest clotting activity was recovered mainly in the third peak. These fractions were pooled, and a portion of the material (about 2 mg of protein) was applied directly to RP-HPLC on a SynChropak RP-8 column. One major protein peak appeared at an acetonitrile concentration of 51%, with several small trailing peaks (supplementary material, Figure 1B). Clotting activity was present in the major peak, which showed a single band on SDS-15% PAGE under both nonreducing and reducing conditions. Estimated M_r was 33 000 before and 35 000 after reduction (supplementary material, Figure 2). On analytical isoelectric focusing, the purified protein showed a single band with p*I* 5.54 (data not shown). Specific activity of purified bothrombin was 151 times greater than that of the crude venom, and recovery of activity was 35% of the starting material (Table 1).

Biological Characteristics of Bothrombin. (*a*) *Specific Fg-Clotting Activity.* In five separate experiments, purified bothrombin showed specific activity ranging from 814 to 904 NIH equiv units/mg of protein.

(*b*) *Substrate Specificity for Fg from Various Animal Sources.* Bothrombin coagulated rabbit Fg very weakly compared to human, rat, or bovine Fg, whereas human α-thrombin showed no such substrate specificity (supplementary material, Table 1).

(*c*) *Optimal pH.* Optimal pH for the clotting activity of bothrombin was in the range 7.4–8.0 (data not shown).

(*d*) *Heat Stability.* Clotting activity of bothrombin was fairly stable at 56 °C during a 120-min incubation, but deteriorated progressively at temperatures above 75 °C (data not shown).

(*e*) *Peptidase Activity on S-2238.* Release of *p*-nitroaniline from chromogenic substrate S-2238 by bothrombin was essentially identical to that by human α-thrombin (data not shown).

(*f*) *Inhibition of Clotting Activity by DFP or Hirudin.* DFP totally blocked clotting activity of bothrombin as well as human α-thrombin at concentrations above 0.5 mM (supplementary material, Figure 3A). In contrast, hirudin had minimal effect on bothrombin activity (supplementary material, Figure 3B).

(*g*) *Release of Fibrinopeptide(s) by Bothrombin.* RP-HPLC analysis of bothrombin-treated Fg supernatant on a Cosmosil 5C18-p-300 column revealed three peaks (supplementary material, Figure 4A). The first peak was injection noise. The N-terminal amino acid sequence of the second peak was ADSGEGDFLAEGGGVR, identical to fibrinopeptide A (Kehl et al., 1981). No sequence information was obtained for the third peak by either amino acid sequencing or mass spectral analysis. RP-HPLC analysis of human α-thrombin-treated Fg supernatant showed four major peaks (supplementary material, Figure 4B). The retention times of peaks 1, 2, and 4 were exactly the same as for the peaks in supplementary material Figure 4A. For peak 3, no sequence information was obtained by amino acid sequencing, but mass spectral analysis identified the amino acid sequence as Q*GVNDNEEGFFSAR, where Q* denotes pyrroglutamyl residue; this is the sequence of fibrinopeptide B (Kehl et al., 1981). These results indicate that bothrombin split off fibrinopeptide A alone, without releasing fibrinopeptide B.

(*h*) *Factor VIII Activation.* Bothrombin activated purified factor VIII, but the activity was far less (about 1/950) than that of human α-thrombin (supplementary material, Figure 5).

(*i*) *Serotonin Release from Platelets by Bothrombin.* There was no release of [^{14}C]serotonin during a 10-min incubation of normal washed platelets, regardless of bothrombin concentration (data not shown).

Effect of Bothrombin on Platelet Aggregation. Bothrombin (up to 200 NIH equiv units/mL) alone did not induce appreciable aggregation of freshly-prepared human washed platelets (data not shown). However, when exogenous human (or bovine) Fg (final concentration 0.4 mg/mL) was added 2 min after bothrombin treatment, there was clear platelet aggregation correlated with bothrombin concentration (supplementary material, Figure 6). This Fg-dependent aggregation was completely inhibited by pretreatment of platelets with 1 μM PGE1 or 10 mM EDTA. These concentrations of PGE1 or EDTA did not affect clotting activity of bothrombin. DFP-treated bothrombin also inhibited Fg-dependent platelet aggregation (Figure 1A).

Preincubation of platelets with anti-GP IIb/IIIa MoAb LJ-CP8, which blocks Fg binding to GP IIb/IIIa receptor (Trapani-Lombardo et al., 1985), or with MoAb LJ-Ib 10, which specifically blocks α-thrombin binding to GP Ib (De Marco et al., 1991), almost completely abolished exogenous Fg-dependent platelet aggregation by bothrombin. However, anti-GP Ib MoAb AP-1 (Montgomery et al., 1983), a specific inhibitor of vWF binding to GP Ib, or anti-GP IIb/IIIa MoAb 3F11, which has no effect on Fg or vWF binding to GP IIb/IIIa, did not affect Fg-dependent aggregation (Figure 1B).

When platelets from patients with Bernard–Soulier syndrome or Glanzmann thrombasthenia were used, exogenous Fg-dependent platelet aggregation by bothrombin did not occur (Figure 2). However, subsequent addition of bovine vWF (final concentration 30 μg/mL) resulted in clear aggregation of Glanzmann thrombasthenia platelets (Figure 2B), but not Bernard–Soulier platelets (Figure 2A).

Sequence Analysis of Bothrombin. Reduced and (*S*)-pyridylethylated bothrombin was separated by RP-HPLC as shown in supplementary material Figure 7. The major peak

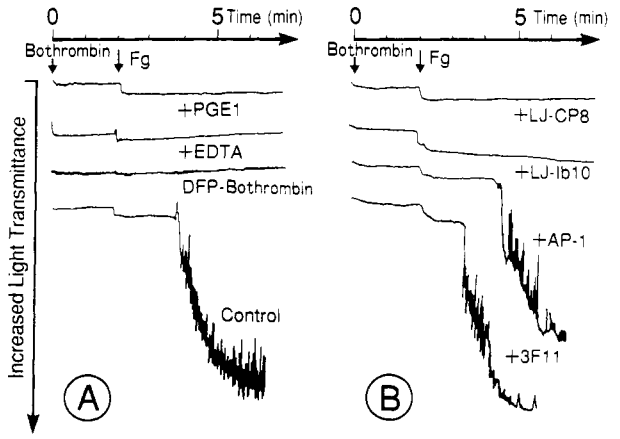

FIGURE 1: Effects of PGE1, EDTA, DFP, and various antiplatelet MoAbs on bothrombin-induced, exogenous Fg-dependent platelet aggregation. Procedure was similar to that of Supplementary Material Figure 6, except that all samples contained fixed concentrations of bothrombin (0.38 μg/mL) and human Fg (0.4 mg/mL). Panel A: Preincubation of platelets with PGE1 (1 μM), EDTA (10 mM), or bothrombin pretreated with DFP for 2 min. Panel B: Preincubation of platelets with various anti-platelet MoAbs: LJ-CP8 (100 μg/mL), LJ-Ib10 (200 μg/mL), AP-1 (100 μg/mL), or 3F11 (100 μg/mL).

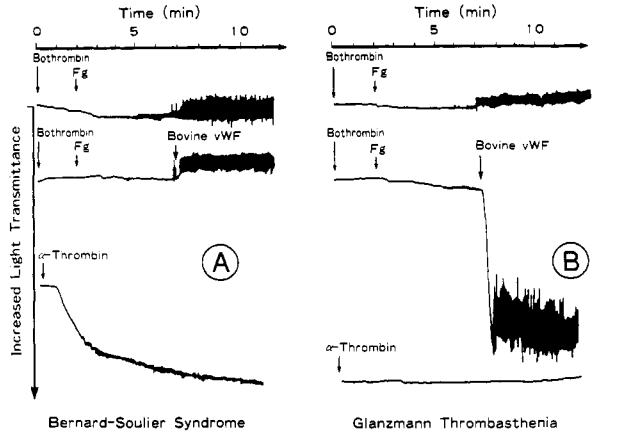

FIGURE 2: Bothrombin-induced aggregation of platelets from Bernard-Soulier syndrome (panel A) or Glanzmann thrombasthenia (panel B) patients. Procedure was similar to that of Figure 1. Bovine vWF (final concentration 30 μg/mL) was added at 7 min. Human α-thrombin-induced platelet aggregation was used as a control.

was divided into three fractions. By sequence analysis, fraction 1 showed two N-terminal sequences of Asn-Val-Ile-Tyr-Asp----- (residues 80-232) and Val-Ile-Gly-Gly-Asp----- (residues 1-79) at a molar ratio of about 2:1, indicating that this fraction contained some autolysis products. Both fractions 2 and 3 showed a single N-terminal sequence of Val-Ile-Gly-Gly-Asp----- (residues 1-232). The three fractions were combined for further studies.

Repeated sequence analysis of the intact PE-protein fraction 2 (ca. 30 pmol) yielded an N-terminal sequence of 49 residues except for weak or ambiguous identification at residues 7, 26, 34, 35, 41, 42, 43, and 48.

The cyanogen bromide digest of the PE-protein (ca. 5 nmol) was separated into nine major fractions by RP-HPLC (supplementary material, Figure 8). These fractions, designated as M1–M6 on the basis of the location in the final sequence (Figure 3), were subjected to sequence determination. Since fraction 1 in supplementary material Figure 7 was combined with fractions 2 and 3 and used for digestion, M4N and M4C, the N- and C-terminal portions of M4, respectively, were also obtained. Peptide M6 was partially cleaved at the Asp–Pro bond (residues 197 and 198). Peptide M2/3 was produced by incomplete cleavage at the Met–Thr bond (residues 28 and 29). Peptide M3 was identified as a minor component in peptide M2/3.

The PE-protein (ca. 6 nmol) was digested wtih API. The digest was separated by RP-HPLC and analyzed (Figure 3). Peptides K2 and K7 were found to provide overlaps of cyanogen bromide peptides M3–M4 and M5–M6, respectively. C-Terminal peptide K11 appeared to be eluted with urea in the breakthrough peak and not isolated. Peptide M6C was subdigested with API in the absence of urea. A peptide identical to peptide K11 was isolated by RP-HPLC on a Cosmosil 5C18-P-300 column and determined by sequence and mass spectral analysis.

Mass Spectral Analysis. MH^+ values were measured by ion-spray mass spectrometry for peptides obtained by cyanogen bromide and API digestion (supplementary material, Table 2). No signal was obtained for some large glycopeptides such as peptides M6, M6C, and K7. These data facilitated the sequence proof by either providing overlaps of peptides or confirming the sequences obtained with a protein sequencer. For example, the MH^+ value of peptide M4 indicated that peptide M4N was directly followed by M4C. The presence of an extra Pro residue at the C-terminus was indicated by mass spectral analysis in addition to the amino acid composition of peptide K11 (supplementary material, Table 3). The locations of Trp predicted at residues 35 and 194 by sequence homology with other serine proteases (Figure 4) were also confirmed by mass spectral analysis of peptides K1 and K8, respectively.

Asn 98, 146, and 225 were not identified by sequence analysis of either cyanogen bromide or API peptides. Both amino acid composition and mass spectral data (supplementary material, Tables 2 and 3) indicated that these residues were most likely to be glycosylated Asn.

Sequence Homology. The amino acid sequence of bothrombin was compared with those of several serine proteases (Figure 4). Bothrombin showed high homology to batroxobin (*B. atrox moojeni*) without deletion or insertion and moderate homology to flavoxobin, human α-thrombin, and human trypsin I (see Discussion).

DISCUSSION

We have isolated and characterized a new Fg-clotting serine protease, named bothrombin, from the venom of *Bothrops jararaca*. Batroxobin (*B. atrox moojeni*), a similar Fg-clotting enzyme, has been isolated from a different species of *Bothrops*, and its amino acid sequence has been deduced by cDNA cloning (Itoh et al., 1988). Batroxobin is used as an antithrombotic drug because it induces formation of non-cross-linked fibrin and thereby reduces the plasma Fg level (Bell, 1982). Like batroxobin, but in contrast to α-thrombin, bothrombin preferentially splits off fibrinopeptide A without releasing fibrinopeptide B. Effects of batroxobin on human platelets have been reported to be negligible. However, Huang et al. (1991) have recently reported that it can induce platelet aggregation only when a large amount of the enzyme is used.

Hill-Eubanks et al. (1989) reported that another Fg-clotting enzyme (designated as BVJ-VIIIcp) purified from the venom of *B. jararacussu* activates blood coagulation factor VIII in a different fashion from that of α-thrombin. The structure of BVJ-VIIIcp was not described in detail except for its molecular mass (28 kDa before and 33 kDa after reduction). Bothrombin seems to be distinct from BVJ-VIIIcp in that BVJ-VIIIcp directly aggregates washed human platelets in a

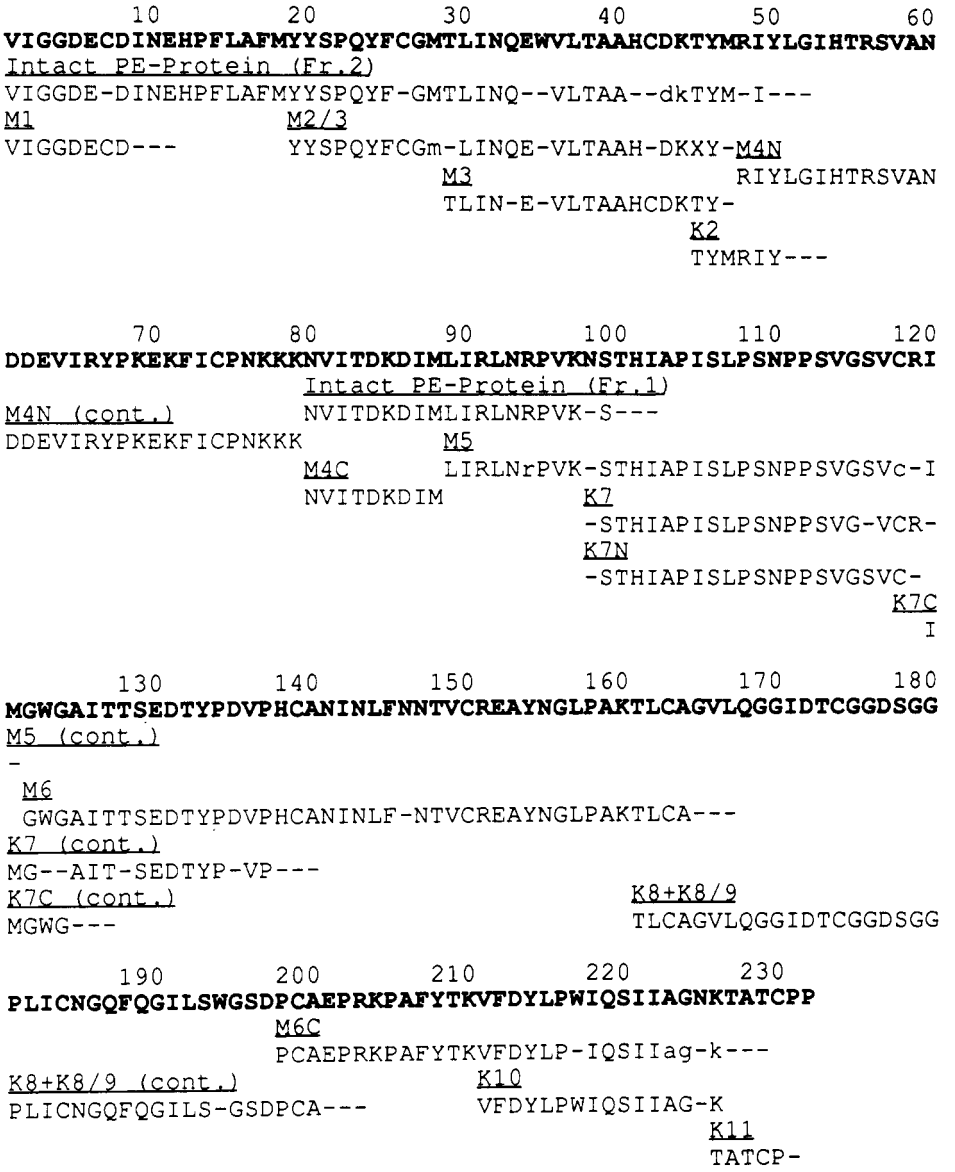

FIGURE 3: Sequence proof of bothrombin. The proven sequences of specific peptides (underlined) are given in one-letter code below the summary sequence. Prefixes K and M denote peptides generated by cleavage of bothrombin at lysyl and methionyl bonds, respectively. Products of lysyl and methionyl cleavage are numbered from the N-terminus toward the C-terminus of the protein. Sequences written in upper case letters were proven by Edman degradation; those in lower case letters are tentative identifications. Unidentified residues are shown by dashes.

manner similar to that previously described for thrombocytin from *B. atrox* (Kirby et al., 1979; Niewiarowski et al., 1979). As shown here, bothrombin activates factor VIII, but with specific activity roughly three orders of magnitude less than that of α-thrombin.

Platelet aggregation studies revealed several important functional differences between bothrombin and α-thrombin. (1) Bothrombin alone, even at a final concentration of 200 NIH equiv units/mL, induced neither serotonin release from nor appreciable aggregation of platelets. (2) Bothrombin induced platelet aggregation only in the presence of exogenous human (or bovine) Fg. (3) The exogenous Fg-dependent platelet aggregation induced by bothrombin was almost completely inhibited by anti-GP IIb/IIIa MoAb LJ-CP8, which blocks Fg binding to GP IIb/IIIa (Trapani-Lombardo et al., 1985) or by anti-GP Ib MoAb LJ-Ib10, which specifically abolishes α-thrombin binding to GP Ib (De Marco et al., 1991).

Preincubation of platelets with PGE1 or EDTA abolished bothrombin-induced aggregation. Neither formalin-fixed normal platelets, washed Bernard–Soulier platelets, nor washed Glanzmann thrombasthenia platelets were aggregated by bothrombin, even in the presence of exogenous Fg. Further addition of bovine vWF induced aggregation of Glanzmann thrombasthenia platelets, but not Bernard–Soulier platelets. These experiments ruled out the possibility that formation of non-cross-linked fibrin by bothrombin might reduce platelet aggregation.

At present, the mechanism of platelet activation by α-thrombin is only partially understood. Platelets contain at least three distinct binding sites for α-thrombin, i.e., high-, intermediate-, and low-affinity binding receptors (Greco & Jamieson, 1991). The high-affinity receptor appears to be located on GP Ib, but the location of the other binding sites is unknown. cDNA cloning of a functional thrombin receptor by Vu et al. (1991b) provided a breakthrough in our understanding of the molecular mechanism of thrombin-induced platelet activation. This receptor is now considered to play a key role in platelet activation by α-thrombin. Since Bernard–Soulier platelets, which lack GPIb-IX complex, respond to α-thrombin with less intensity than normal platelets, the low-affinity binding receptor may provide a nonspecific

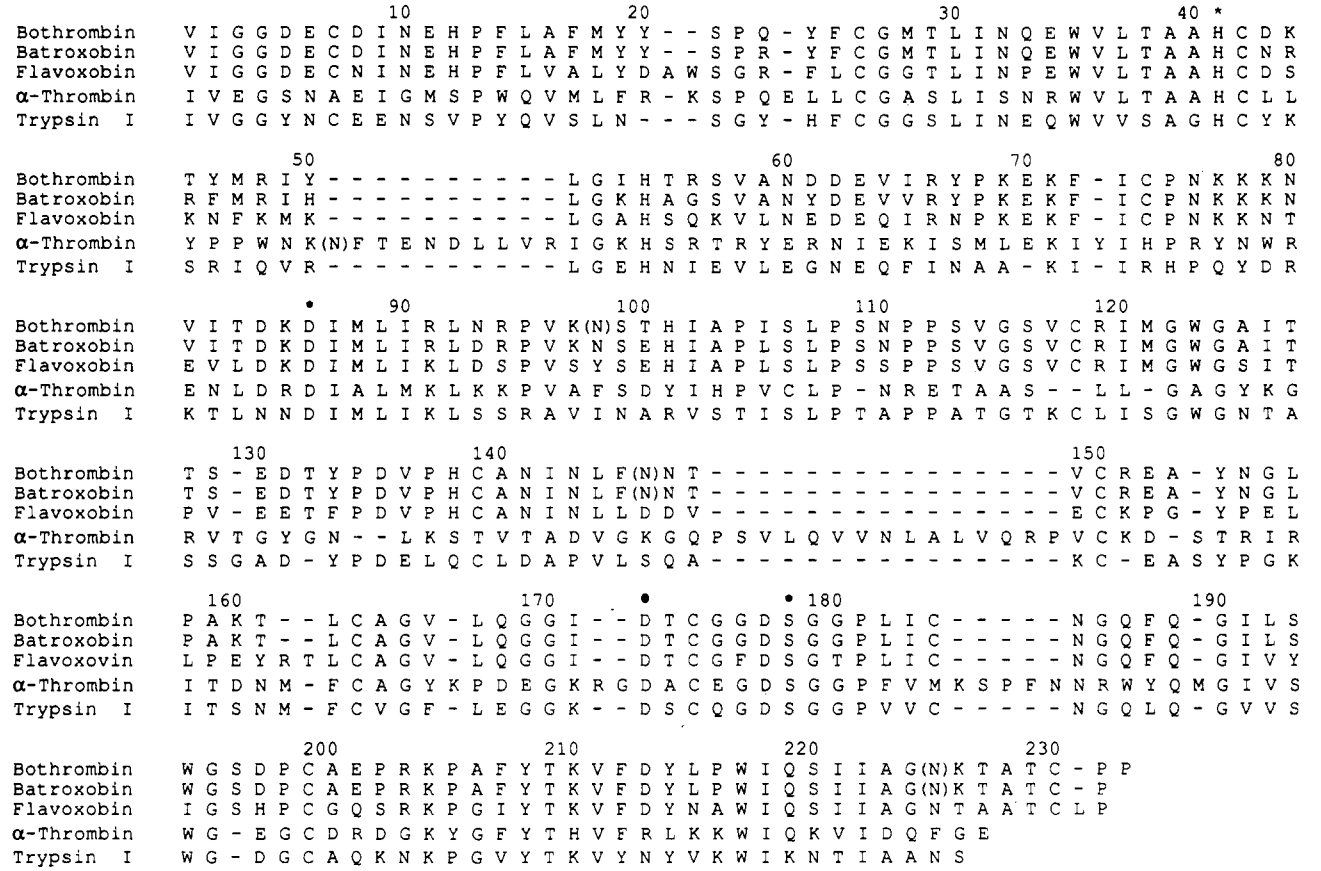

FIGURE 4: Amino acid sequence homology between bothrombin and other known serine proteases. Residue numbers shown are those of bothrombin (see Figure 3). Gaps have been inserted to maximize homology. Asterisks and filled circle indicate residues known to be the catalytic triad and the primary substrate binding site of mammalian trypsins, respectively (see Discussion). Potential N-glycosylation sites are indicated by parentheses.

binding site. Thus, the cloned thrombin receptor appears to be the intermediate-affinity binding site (Vu et al., 1991a). This scenario makes unclear the physiological relevance of α-thrombin binding to GP Ib.

Several of the present findings suggest that bothrombin initiates exogenous Fg-dependent platelet aggregation through interaction with GP Ib, not through interaction with the cloned thrombin receptor. This interpretation is supported by the observation that hirudin, a specific α-thrombin inhibitor, affects neither bothrombin-induced clotting activity nor exogenous Fg-dependent platelet aggregation. Since the cloned thrombin receptor has an amino acid sequence homologous to that of a possible thrombin-binding site of hirudin (Rydel et al., 1990; Vu et al., 1991b), we can speculate that bothrombin lacks a structure favorable for the cloned thrombin receptor or for hirudin. It is unclear why DFP-treated bothrombin does not induce exogenous Fg-dependent platelet aggregation. However, DFP-treated α-thrombin was similarly unable to activate platelets, in spite of retaining GP Ib-binding ability (Harmon & Jamieson, 1986). We do not know whether intact or DFP-treated bothrombin bind directly to GP Ib or not, because the number of high-affinity thrombin binding receptors on a single platelet is small (ca. 50) (Harmon & Jamieson, 1985). Classic calculation by Scatchard plots is therefore not reliable. Attempted use of specific techniques such as radiation inactivation of platelets (Harmon & Jamieson, 1985) has so far been unsuccessful.

We presume that interaction of bothrombin with GP Ib is directly or indirectly linked to activation of GP IIb/IIIa, followed by binding of Fg to this receptor. Bothrombin seems incapable of inducing platelet activation, as demonstrated by the lack of serotonin release from bothrombin-treated platelets. Thus, endogenous Fg in platelets probably does not play a role in exogenous Fg-dependent platelet aggregation.

Results of the present study suggest the existence of a signal transduction pathway from GP Ib to GP IIb/IIIa without release reactions from α-granule in platelets, which would clarify the physiological relevance of interactions between α-thrombin or thromnin-like protease and GP Ib. Bothrombin may provide a powerful tool for further studies of the complex mechanism of platelet activation.

Bothrombin is a member of the serine protease family, as evidenced by the inactivation of bothrombin by DFP. Although separated from pancreatic (e.g. trypsin) and plasma (e.g. thrombin) serine proteases by long time spans of evolutionary time, bothrombin nevertheless retains characteristics of their common origin. An example of these common characteristics is furnished by the complete amino acid sequence of bothrombin as presented here. Comparative analysis of the bothrombin sequence reveals homology with batroxobin (*B. atrox moojeni*) (Itoh et al., 1988) and flavoxobin (*T. flavoviridis*) (Shieh et al., 1988), Fg-clotting enzymes from other snake venoms, as well as with mammalian serine proteases such as human α-thrombin (Degen et al., 1983) and trypsin I (Emi et al., 1986).

Most of the bothrombin sequence reported here is unambiguous, being based on replicate or overlapping sequence analysis. Some of the data contain unidentified Trp or glycosylated Asn residues or are derived from single analysis only, but in each such case the sequences given are consistent with the composition and/or molecular mass of small peptides derived from corresponding regions.

Bothrombin and batroxobin are highly homologous, having almost the same chain length (232 and 231 residues) and differing only in 15 residues (94% identity). Out of these 15 differences, 14 occur within the N-terminal 105 residues; the C-terminal 126 residues of the two enzymes are almost identical except for the presence of an extra Pro residue at the C-terminus of bothrombin.

The N-terminal Val residue of the mature form of batroxobin is preceded by 24 amino acids in the precursor (Itoh et al., 1987). Since the precursors of mammalian serine proteases have been shown to have additional amino acids at their N-termini that represent prepeptides and propeptides (MacDonald et al., 1982; Swift et al., 1982), it is likely that the N-terminal Val residue of bothrombin is also preceded by several additional amino acids in the precursor form.

Bothrombin and the other three serine proteases compared (flavoxobin, human trypsin I, and human α-thrombin) differ in chain length (232, 236, 224, and 259 residues, respectively). When the sequences of these enzymes are aligned as shown in Figure 4, and allowance is made for insertions and deletions, the following percentages of homology are calculated: bothrombin/flavoxobin, 68%; bothrombin/human α-thrombin, 33%; bothrombin/human trypsin I, 40%.

The number of half-cystine residues and their positions in the sequence of bothrombin coincide with those of batroxobin. It has been predicted on the basis of sequence homology to trypsin that they are paired in the same fashion as in trypsin, i.e., Cys 7–Cys 139, Cys 26–Cys 42, Cys 74–Cys 230, Cys 118–Cys 184, Cys 150–Cys 163, and Cys 174–Cys 199 (Itoh et al., 1987).

As expected from functional considerations, the constituents of active sites found in mammalian trypsins are present in corresponding positions in the three snake venom enzymes and human α-thrombin, as shown in Figure 4. These include the components of the catalytic triad, His 41, Asp 86, and Ser 178 (bothrombin/batroxobin numbering system), and residues Val 1 and Asp 177, which are known to form a salt bridge stabilizing the catalytic site. Bothrombin also shares with mammalian trypsins the residues which determine the primary and secondary substrate binding sites, i.e., Asp 172 and Gly 195.

The Fg-clotting activity of bothrombin, as well as that of batroxobin, were almost unaffected by hirudin, a highly specific α-thrombin inhibitor from *Hirudo medicinalis*. Recent studies (Fenton II et al., 1988, 1991a,b; Mao et al., 1988) have indicated that hirudin (consisting of an apolar tridisulfide-linked core structure with an anionic tail) interacts with the catalytic site and associated regions of α-thrombin through the apolar core structure, and with the anion-binding exosite through the anionic tail, with a stoichiometry of 1:1. On the other hand, γ-thrombin, which lacks Ile 63-Arg 73 and Glu 124-Lys 154 of α-thrombin B-chain (residues 51–61 and 112–146, respectively, in bothrombin numbering [Figure 4]), has much lower affinity to hirudin, suggesting that these two regions of α-thrombin may interact with hirudin. In bothrombin, which is not inhibited by hirudin, Leu 51–Asp 61 shows 36% identity with Ile 63–Arg 73 of α-thrombin, but it is much less anionic. Pro 112–Asn 146 of bothrombin shows only 8% identity to Glu 124–Lys 154 of α-thrombin. These differences suggest that these two regions of α-thrombin are crucial for interaction with hirudin, in accord with previous observations.

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SUPPLEMENTARY MATERIAL AVAILABLE

Materials and methods, additional results, clotting times of fibrinogen (Table 1), MS data (Table 2), amino acid composition of PE-bothrombin (Table 3), purification of bothrombin (Figure 1), SDS-PAGE analysis of bothrombin (Figure 2), inhibitory effect of DFP or hirudin on Fg-clotting activity of bothrombin and human α-thrombin (Figure 3), RP-HPLC profiles (Figure 4), activation of factor VIII (Figure 5), platelet aggregation induced by bothrombin (Figure 6), separation of PE-bothrombin by RP-HPLC (Figure 7), and separation of peptides generated by cyanogen bromide cleavage of PE-bothrombin (Figure 8) (23 pages). Ordering information is given on any current masthead page.

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