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Isolation and Chemical Characterization of Two Structurally and Functionally Distinct Forms of Botrocetin, the Platelet Coagglutinin Isolated from the Venom of *Bothrops jararaca*†

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ABSTRACT: Two distinct forms of botrocetin, the von Willebrand factor (vWF)-dependent platelet coagglutinin isolated from the venom of the snake *Bothrops jararaca*, were purified and characterized structurally and functionally. The apparent molecular mass of the one-chain botrocetin was 28 kDa before and 32 kDa after reduction of disulfide bonds, while that of the two-chain botrocetin was 27 kDa before and 15/14.5 kDa after reduction. Amino acid composition of the two species revealed a similar high content of potentially acidic residues (greater than 60 Asx and Glx residues/molecule) but significant differences in the content of Cys and Phe residues. The NH_2-terminal sequence of the one-chain botrocetin was Ile-Ile/Val-Ser-Pro-Pro-Val-Cys-Gly-Asn-Glu-. Two constituent polypeptides of the two-chain botrocetin showed similar but different NH_2-terminal sequences, distinct from that of the one-chain species: (α) Asp-Cys-Pro-Ser-Gly-Trp-Ser-Ser-Tyr-Glu- and (β) Asp-Cys-Pro-Pro-Asp-Trp-Ser-Ser-Tyr-Glu-. The carbohydrate content of both species was less than 2% of the total mass, and the p*I* was 4.0–4.1 for the one-chain species, and 4.6, 5.3–5.4, and 7.7–7.8 for the two-chain species. No free sulfhydryl group was detected in each species. Both types of botrocetin were resistant to proteolysis at neutral pH. Incubation of ^{125}I-labeled one-chain botrocetin with the crude venom solution resulted in no detectable structural change. On a weight basis, the two-chain botrocetin was 34 times more active than the one-chain form in promoting vWF binding to platelets. A new experimental approach revealed that vWF and botrocetin form a soluble complex, and the binding of ^{125}I-labeled two-chain botrocetin to vWF was clearly inhibited in a dose–response manner by one-chain botrocetin as well as the unlabeled two-chain counterpart. Furthermore, when the concentration of botrocetin was not limiting, the parameters of vWF binding to platelets were identical with either species. These results clearly indicate the existence of two different forms of botrocetin which differ in their molecular structure and affinity for vWF, and provide their initial chemical characterization.

The von Willebrand factor (vWF)[1] mediates platelet adhesion to exposed subendothelium, and its interaction with the platelet GP Ib–IX complex is of critical importance in this process. To reproduce this event in vitro, the antibiotic ristocetin has

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; CAM, carbamoylmethyl; PE, pyridylethyl; CM, carboxymethyl; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; GP, glycoprotein; vWF, von Willebrand factor; HPLC, high-performance liquid chromatography; TBS, 0.05 M Tris-buffered saline, pH 7.4; TPCK, N^{α}-tosylphenylalanine chlorometyl ketone; PRP, platelet-rich plasma; BSA, bovine serum albumin; endo F, endo-β-*N*-acetylglucosaminidase F; DEAE, diethylaminoethyl; BM_{50}, concentration of botrocetin resulting in half-maximal binding to vWF; IC_{50}, concentration of unlabeled botrocetin inhibiting binding of radiolabeled botrocetin to vWF by 50%; K_d, dissociation constant; B_{max}, maximal binding at saturation; PVDF, poly(vinylidene difluoride).

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been widely used (Howard & Firkin, 1971; Kao et al., 1979; Ruggeri et al., 1983). As an alternative, Read et al. (1978) partially purified a substance from the venom of the snake *Bothrops jararaca* which had the property of promoting platelet agglutination in the presence of vWF. They suggested its use as a substitute for ristocetin, and named it "botrocetin". Even though Brinkhous et al. (1983) and Howard et al. (1984) noticed several functional differences between these two agents, both ristocetin and botrocetin induce vWF binding to GP Ib (Read et al., 1983; Howard et al., 1984; Fujimura et al., 1987). Moreover, the binding of vWF to Gp Ib in the presence of either ristocetin or botrocetin is mediated by the same domain of the molecule, the 52/48-kDa fragment comprising residues Val449–Lys728 of the constituent subunit (Fujimura et al., 1986, 1987; Mohri et al., 1988).

Some controversial results have been obtained concerning the chemical structure of botrocetin. Read et al. (1989) reported that purified botrocetin prepared by anion-exchange chromatography shows a single band at 26.5 kDa by SDS–PAGE, and demonstrated that it binds to purified vWF and forms an "activated" complex that, in turn, binds to GP Ib. In contrast, Andrews et al. (1989) characterized a preparation of purified botrocetin which was composed of a 25-kDa disulfide-linked dimer with apparent subunits of 14 and 14.5 kDa, and demonstrated that the 52/48-kDa tryptic fragment of vWF binds to botrocetin-coated beads, whereas GP Ib fragments containing the vWF binding site do not. In view of these discrepancies, the biochemical and functional properties of "purified" botrocetin remain to be clarified.

In the present report, we demonstrate the existence of two forms of botrocetin and provide their comparative chemical and functional characterization. The two-chain botrocetin binds to vWF with much greater affinity than the one-chain species, but both mediate vWF binding to GP Ib in a similar manner. The two forms of botrocetin appear to be related but distinct molecules.

Materials and Methods

Crude *Bothrops jararaca* venom (lots 75F03409, 68F0557, 98F0261, and 119-F0599), bovine fibrinogen (type I), and DFP were obtained from Sigma. TPCK-trypsin and α-chymotrypsin were purchased from Worthington. Thermolysin and pepsin were obtained from Seikagaku Kogyo, and arginylendopeptidase from Takara Shuzo. *Achromobacter* protease I was a gift from Dr. T. Masaki at Ibaraki University Ibaraki, Japan. *Staphylococcus aureus* V8 protease was from Miles. BSA, neuraminidase, and endo F were from Calbiochem. Cyanogen bromide and Iodogen were obtained from Pierce. Carrier-free $Na^{125}I$ was from Amersham. DEAE-Sepharose CL-6B gel and the phenyl-Superose HR5/5 column were products of Pharmacia–LKB. TSK G3000SW and G2000SW columns were obtained from Toyo Soda.

Protein concentration was determined by the dye binding assay kit from Bio-Rad using BSA as a standard. Highly purified vWF was isolated from cryoprecipitate (a generous gift from Nara Red Cross Blood Center, Nara, Japan) as described (Fujimura et al., 1986). Other chemicals used were of analytical grade commercially available.

Iodination of Proteins. Protein labeling with ^{125}I was performed by the Iodogen method (Fraker & Speak, 1978). The labeled proteins had a specific activity of (0.62–0.94) × 10^9 cpm/mg.

Amino Acid Analysis and Sequence Determination. Amino acid compositions were determined with a Hitachi amino acid analyzer Model L-8500 or with a Waters Pico-tag system (Bidlingmeyer et al., 1984) after 24-h acid hydrolysis. Amino acid sequences were determined with an Applied Biosystems protein sequencer Model 470 A after blotting onto a PVDF membrane (Matsudaira, 1987) or after separation by reversed-phase HPLC.

Homology Search. Sequence homology (in 25- or 40-residue segments) was evaluated by a computer search in the Protein Sequence Database.

Cysteine and Cystine Content. Protein was first dissolved in 0.3 M Tris-HCl, pH 8.3, containing 6 M guanidine hydrochloride. Aliquots of the solution, either before or after reduction of disulfide bonds with tri-*n*-butylphosphin (Ruegg & Rudinger, 1977), were treated with 4-vinylpyridine (Hermodson et al., 1973), and the content of S-PE cysteine was quantitated by amino acid analysis.

Carbohydrate Content. Neutral sugar content was estimated by the method of Dubois et al. (1946).

Assay of Fibrinogen Clotting Activity. Thrombin-like activity was measured with a modification of the method of Esnouf and Tunnah (1967). Each sample was first diluted 10-fold with TBS, composed of 0.05 M Tris and 0.15 M NaCl, pH 7.4. A 100-μL aliquot of the diluted sample was then added to test tubes which contained 100 μL of bovine fibrinogen (600 μg/mL), and the clotting time was recorded.

Assay for Botrocetin-Induced vWF Binding to Platelet GP Ib. Formalin-fixed platelets (1×10^8/mL) and ^{125}I-labeled vWF (final concentration 5 μg/mL) were mixed in Eppendorf tubes, and the final volume was adjusted to 112.5 μL with TBS. A 12.5-μL aliquot of each botrocetin sample, diluted 10-fold with TBS, was added to this mixture to initiate vWF binding to GP Ib. After 30-min incubation at room temperature, the mixture was divided into duplicate 50-μL aliquots, and the radioactivity bound to the platelets was separated from free radioactivity by centrifugation for 5 min at 13000*g* through a 20% sucrose cushion using a Sarstedt microcentrifuge tube, as previously described (Fujimura et al., 1987). Nonspecific binding was defined as the amount measured in the presence of a 50-fold excess of unlabeled vWF. Specific binding was calculated by subtracting the nonspecific binding from the total.

Proteolytic Cleavage. Purified botrocetin was dissolved in 0.1 M NH_4HCO_3 containing 0.1 mM $CaCl_2$, pH 7.8 (1 mg of protein/mL). An enzyme to substrate (E:S) ratio of 1:100 by weight was used for cleavage with TPCK-trypsin, lysylendopeptidase, V8 protease, arginylendopeptidase, or α-chymotrypsin. The digestion was allowed to proceed for 1 h at 37 °C and stopped by addition of 5 mM DFP. Thermolysin digestion was carried out at an E:S ratio of 1:100 for 1 h at 37 °C and stopped by addition of 5 mM Na_2EDTA. Pepsin digestion was carried out at an E:S ratio of 1:100 for 1 h at 37 °C in 5% formic acid, pH 2, and the digest was then lyophilized.

Chemical Modification and Cleavage. Reduction of disulfide bonds with DTT followed by S-carbamoylmethylation with iodoacetamide was carried out as described by Titani et al. (1984). Cleavage at methionyl bonds with cyanogen bromide followed the method of Gross (1967).

Treatment with Glycosidases. Purified botrocetin was dissolved in 0.1 M sodium acetate buffer, pH 5.0 (1 mg of protein/mL). Neuraminidase, 0.05 unit/mg of botrocetin, was then added and incubated overnight at 37 °C. Treatment with endo F was accomplished by incubating 1 mg of protein with 50 units of enzyme in 0.1 M sodium phosphate buffer, 5 mM Na_2EDTA, and 0.02% NaN_3, pH 6.1, for 18 h at 37 °C.

Gel Electrophoresis. SDS–PAGE (4–20% acrylamide gradient) was performed by the method of Laemmli (1970).

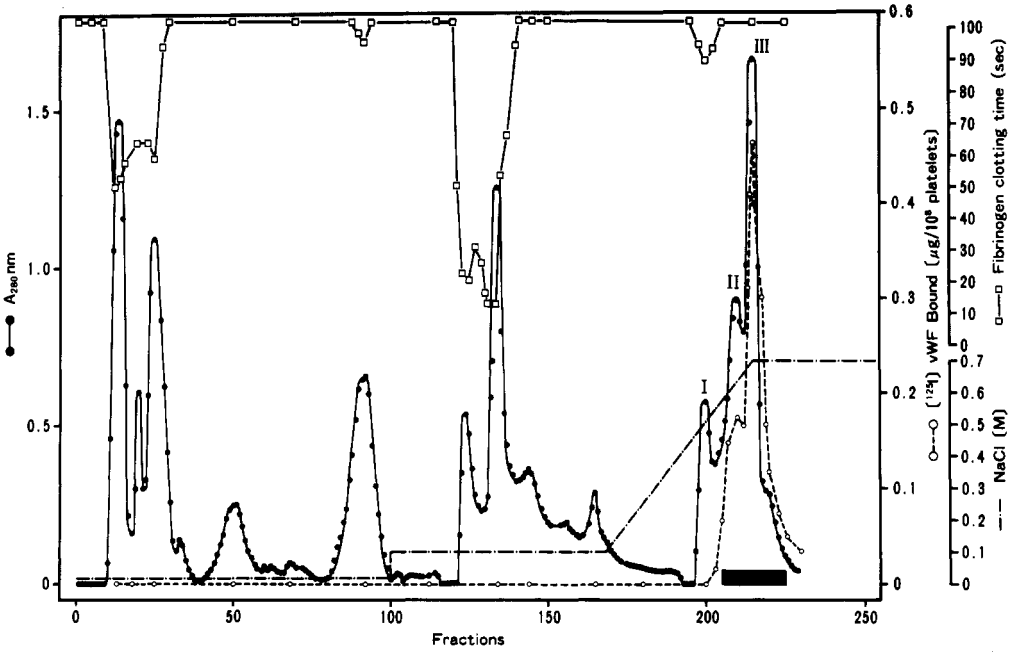

FIGURE 1: Fractionation of the crude venom of *Bothrops jararaca* by DEAE chromatography. All the procedures in this step were performed at 4 °C. Five hundred milligrams of the crude venom was dissolved in 50 mL of 84 mM imidazole hydrochloride buffer containing 0.02% NaN_3 and 2 mM benzamidine hydrocyloride, pH 7.4 (buffer A). The insoluble material was removed by centrifugation at 2500*g* for 15 min. The supernatant was applied to a column of DEAE-Sepharose CL-6B (2.6 × 35 cm) equilibrated with the same buffer. The column was washed with buffer A for 18 h at a flow rate of 50 mL/h. Then, a stepwise elution with buffer A containing 0.1 M NaCl was carried out for 12.5 h. The column was then developed with a linear gradient from 0.1 to 0.7 M NaCl in buffer A, using 150 mL each of the initial and final buffer. Fractions (9 mL) were collected and monitored for protein (●), fibrinogen clotting activity (□), and the activity promoting ^{125}I-vWF binding to platelets (○) as described under Materials and Methods.

Molecular weight standards contained phosphorylase *b* (97K), BSA (66K), ovalbumin (43K), carbonic anhydrase (31K), soybean trypsin inhibitor (22K), and lysozyme (14K). SDS–agarose gel electrophoresis was carried out by the method of Ruggeri and Zimmerman (1981). Isoelectric focusing was performed with a Phast system (Pharmacia–LKB).

Monoclonal Antibodies. The anti-vWF monoclonal antibodies designated as NMC-4 and 2-2-9 were produced and characterized as previously described (Shima et al., 1985; Fulcher & Zimmerman, 1982). The anti-GP Ib monoclonal antibody AP-1 was kindly provided by Dr. Robert R. Montgomery (The Blood Center of South Eastern Wisconsin, Milwaukee, WI) and was also well characterized in previous publications (Montgomery et al., 1983; Fujimura et al., 1987).

Platelet Aggregation. Platelet-rich plasma (PRP) was prepared from normal individuals or patients by mixing whole blood with 0.1 volume of 3.8% trisodium citrate followed by centrifugation at room temperature (De Marco et al., 1986). Platelet aggregation studies were carried out with a NKK aggregometer using 0.3 mL of PRP at 37 °C. Two patients with Bernard–Soulier syndrome (T.H. and Y.T.) and one patient with Glanzmann thrombasthenia (A.K.) fulfilled all the accepted criteria for the respective diagonosis.

Measurement of Botrocetin–vWF Complex Formation. Polystyrene microtiter plates with removable flat-bottom wells (Immulon I Removawell strips, Dynatech Laboratories) were coated for 2 h at room temperature with 100 μL of a solution of purified monoclonal antibody 2-2-9 IgG, 10 μg/mL, in 0.05 M bicarbonate buffer (pH 9.0). This monoclonal antibody binds to the carboxyl-terminal region of the vWF subunit and has no effect on vWF binding to platelets in the presence of either ristocetin or botrocetin. The coating solution was removed, and the plastic surface was covered with 200 μL of 1% BSA in TBS for 1 h at room temperature. The BSA solution was then removed, and the wells were washed 3 times with TBS containing 0.05% NP-40 (TBS-NP-40). Formation of the botrocetin–vWF complex in fluid phase was obtained by mixing purified vWF (5 μg/mL), ^{125}I-labeled two-chain botrocetin (3.2 μg/mL), and BSA (5 mg/mL) in an incubation volume of 120 μL in plastic Eppendorf tubes, at room temperature. For competitive inhibition studies, various amounts of unlabeled one-chain or two-chain botrocetin were added to the incubation mixture. After 30 min, two 50-μL aliquots of each mixture were added into the 2-2-9 IgG coated wells, in order to bind vWF through its subunit carboxyl-terminal portion (Nishio et al., 1990). After an additional 30 min at room temperature, the wells were washed 5 times with 500 μL of TBS/NP-40, and the radioactivity bound to the wells was measured to calculate the amount of radiolabeled botrocetin complexed to vWF. Nonspecific binding was determined by omitting vWF in the incubation mixtures. Specific binding was calculated by subtracting nonspecific from total binding.

Results

Purification of One-Chain Botrocetin. Botrocetin was purified by anion-exchange chromatography followed by size-exclusion and hydrophobics-interaction HPLC. The results of fractionation of crude *Bothrops jararaca* venom (500 mg; lots 75F03409 and/or 68F0557) by anion-exchange chromatography on a DEAE-Sepharose CL-6B column are shown in Figure 1. More than 90% of the fibrinogen clotting activity present in the starting material eluted from the column at 0.1 M NaCl concentration. By a gradient elution from 0.1 to 0.7 M NaCl, three major protein peaks were obtained and pooled as peaks I, II, and III. Peak I showed only residual fibrinogen clotting activity. Peaks II and III showed activity for inducing vWF binding to platelets and vWF-dependent platelet agglutination (botrocetin cofactor activity), but no fibrinogen clotting activity. Peaks II and III were pooled, dialyzed against 0.1 M NH_4HCO_3, pH 7.8, at 4 °C for 2 days, and lyophilized. A portion (0.8–3 mg each) of the lyophilized protein was subjected to size-exclusion HPLC on tandem columns of TSK

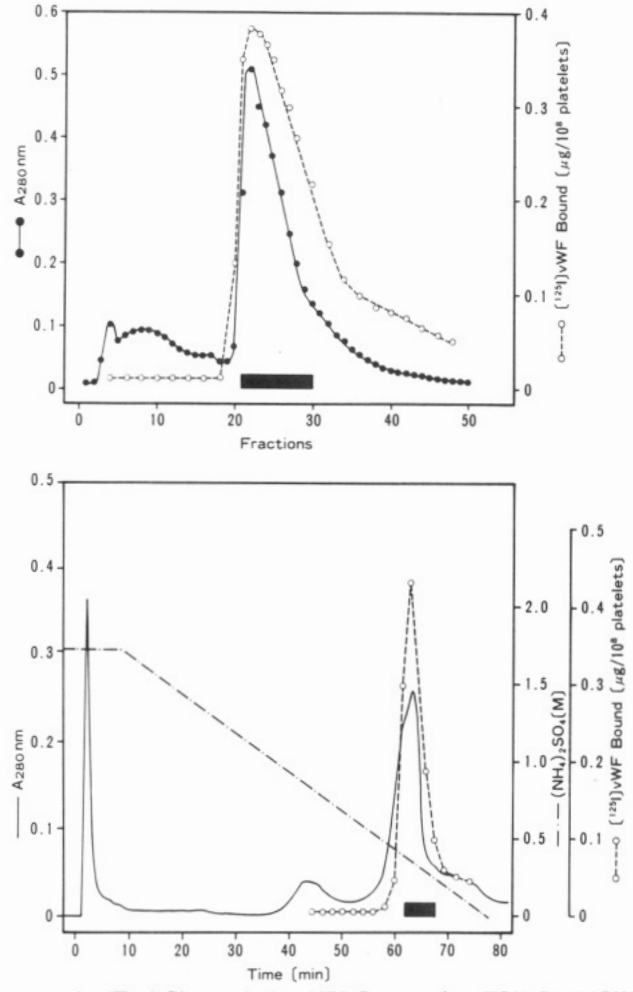

FIGURE 2: (Top) Size-exclusion HPLC on tandem TSK G3000SW and G2000SW columns. The lyophilized protein obtained in Figure 1 was dissolved in 0.02 M Tris-HCl buffer containing 0.5 M NaCl, pH 6.8 (buffer B). Three milligrams of protein was separated by size-exclusion HPLC at a flow rate of 1.0 mL/min and collected in 1-mL fractions. The fractions which retained the activity promoting vWF binding to platelets (○) were pooled, dialyzed, and lyophilized. (Bottom) HPLC on a phenyl-Superose HR 5/5 column. The lyophilized sample was dissolved in 50 mM phosphate buffer, pH 7.0 (buffer C), containing 1.7 M $(NH_4)_2SO_4$, and then each 500 μg of protein was applied onto the column equilibrated with the same buffer at a flow rate of 0.5 mL/min. After 5 min, a 1.7–0 M $(NH_4)_2SO_4$ gradient in buffer B was started, using 18 mL each of the initial and final buffer. The fractions containing the activity promoting vWF binding to platelets (○) were pooled, lyophilized after dialysis, and stored at −20 °C until use. These two HPLC steps were performed at room temperature.

G3000SW and G2000SW. A major protein peak was eluted which coincided with the activity promoting vWF binding to GP Ib (Figure 2, top). The major peak was pooled and further purified by hydrophobics-interaction HPLC on a phenyl-Superose HR 5/5 column. Several protein peaks were eluted with a 1.7–0 M $(NH_4)_2SO_4$ gradient, and the activity promoting vWF binding corresponded to the major peak (Figure 2, bottom). SDS–PAGE (4–20% gradient) analysis revealed that the purified protein had a molecular mass of 28 kDa before and 32 kDa after reduction with DTT, indicating that this botrocetin is composed of a single polypeptide chain (Figure 3A). In six different experiments, approximately 1–2.5 mg of highly purified one-chain botrocetin was obtained from 500 mg of the same lot of crude venom. The p*I* was estimated to be 4.0–4.1 by isoelectric focusing, and the extinction coefficient [E(1%, 1 cm)] was 10.75 at 280 nm in 0.1 M NH_4HCO_3, pH 8.0. No proteolytic activity of purified

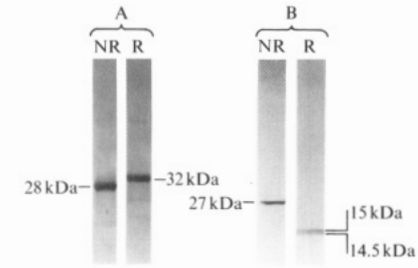

FIGURE 3: SDS–PAGE (4–20%) of purified one-chain botrocetin (A) and two-chain botrocetin (B). (NR) No reduced conditions. (R) Reduced conditions. A total of 2–4 μg of the purified protein was loaded on each lane.

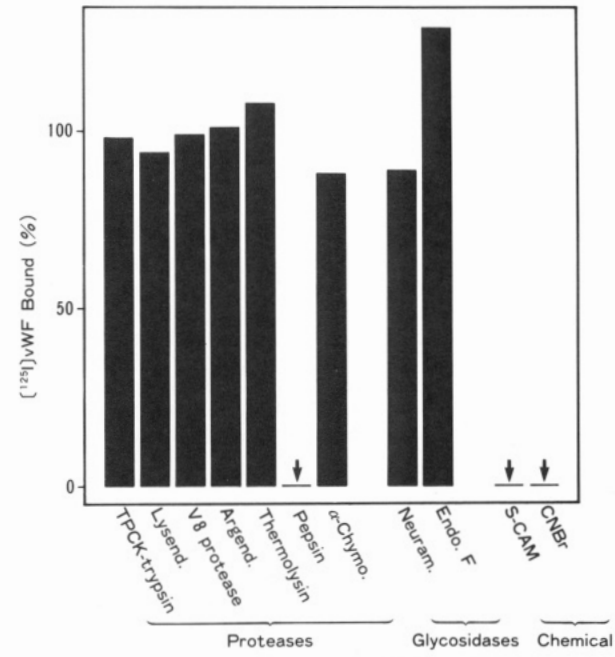

FIGURE 4: Effects of enzymatic and chemical treatment of purified one-chain botrocetin on vWF binding activity to GP Ib. Purified one-chain botrocetin was treated with several enzymes or chemicals as indicated. The conditions for treatment are described under Materials and Methods. The residual activity promoting vWF binding to platelets was then measured in all samples, as well as in a control mixture containing untreated botrocetin.

botrocetin on vWF was observed when the incubation mixture was analyzed by SDS–agarose gel electrophoresis (data not shown).

Enzymatic or Chemical Treatment of One-Chain Botrocetin. Purified one-chain botrocetin was resistant to enzymatic cleavage with proteases such as TPCK-trypsin, lysylendopeptidase, V8 protease, arginylendopeptidase, thermolysin, or chymotrypsin. Pepsin digestion, on the other hand, destroyed the protein structure and abolished the activity promoting vWF binding to GP Ib. Reduction and S-alkylation of disulfide bonds also resulted in loss of this activity, as did cyanogen bromide treatment concurrently with cleavage of peptide bonds. In contrast, treatment with glycosidases such as neuraminidase or endo F had minimal effects on botrocetin activity (Figure 4).

Amino Acid Composition and Sequence Analysis of One-Chain Botrocetin. Table I (second column) shows the amino acid composition of purified one-chain botrocetin calculated on the basis of a molecular mass of 32 kDa. The composition was characterized by a high content of potential acidic residues, Asx (39.1 residues/mol) and Glx (32.2 residues/mol), and CAM-Cys residues (33.8 residues/mol). No free sulfhydryl group was detected in the intact purified one-chain botrocetin.

Table I: Amino Acid Composition of Purified Botrocetin[a]

composition	one-chain	two-chain	composition	one-chain	two-chain
CAM-Cys[b]	12.2	5.6	Ile	2.0	3.3
Asx	14.0	12.2	Leu	4.0	4.9
Thr	3.3	3.1	Phe	2.6	7.1
Ser	6.1	9.1	Tyr	5.3	4.8
Glx	11.6	13.6	His	3.0	1.9
Pro	5.7	3.8	Lys	7.0	8.0
Gly	9.9	6.1	Arg	1.8	2.7
Ala	5.8	5.1	Trp	ND[c]	ND[c]
Val	4.4	7.1	total	100.0	100.0
Met	1.3	1.6			

[a] Values are expressed as mole percent, excluding Trp residues. [b] CAM-Cys was determined as CM-Cys by amino acid analysis. [c] Not determined.

The NH_2-terminus was not blocked, and the amino acid sequence of the first 25 residues of the S-CAM protein was determined as follows: Ile-Ile/Val-Ser-Pro-Pro-Val-Cys-Gly-Asn-Glu-Leu-Leu-Glu-Glu-Gly-Glu-Glu-Cys-Asp-X-Gly-Thr-Pro-Glu-Asn (X; unidentified). In cycle 2, two residues, Ile and Val, were identified at almost equal quantities, indicating the presence of a polymorphism. This sequence was unique, and no homologous protein was found by a computer homology search.

Platelet Aggregation Induced by One-Chain Botrocetin. Two final concentrations of purified one-chain botrocetin (10 and 50 μg/mL) were used in platelet aggregation studies (Figure 5). The platelet count was adjusted to 1×10^8/mL for all samples, because this was the maximal count attainable in the two patients with Bernard–Soulier syndrome who had a slight thrombocytopenia [$(0.7–1.4) \times 10^8$/mL]. The purified one-chain botrocetin induced agglutination in the PRP of a patient with Glanzmann thrombasthenia. Thus, the effect of botrocetin is independent of the function of the GP IIb–IIIa complex, which is essential for platelet aggregation resulting from agonist-induced activation. In contrast, botrocetin had no effect on the PRP from two patients with Bernard–Soulier syndrome. These patients lack functional expression of the GPIb–IX complex and, therefore, are defective in any activity dependent on vWF–GPIb interaction. Platelet aggregation induced by purified botrocetin in normal PRP was completely inhibited by the anti-vWF monoclonal antibody NMC-4 or the anti-GP Ib monoclonal antibody AP-1, both at an IgG concentration of 10 μg/mL (data not shown).

Isolation of Two-Chain Botrocetin and Structural Differences with the One-Chain Species. Disulfide-linked two-chain botrocetin was purified according to the scheme described for the one-chain species. This protein was isolated from different lots of crude venom (lots 98F0261 and 119F0599). SDS–PAGE (4–20% gradient) analysis revealed that the two-chain botrocetin has a molecular mass of 27 kDa before reduction and appears as a spatially closed doublet of 15/14.5 kDa after reduction (Figure 3B). Amino acid composition (Table I, third column) indicated, in analogy with the one-chain species, a high content of potential acidic residues (33.8 Asx and 37.7 Glx residues/mol). The carbohydrate content was similarly low (2% of the total mass). However, different from the one-chain species, three distinct isoelectric points (p*I*s 4.6, 5.3–5.4, and 7.7–7.8) were observed for the intact two-chain botrocetin. Furthermore, there were significant differences in the content of some amino acid residues, especially Cys, Ser, Gly, Val, and Phe, between these two different forms of botrocetin. In contrast, in accord with the findings of the one-chain species, the two-chain botrocetin had an extinction coefficient [E (1%,1 cm)] of 12.00 at 280 nm in 0.1 M

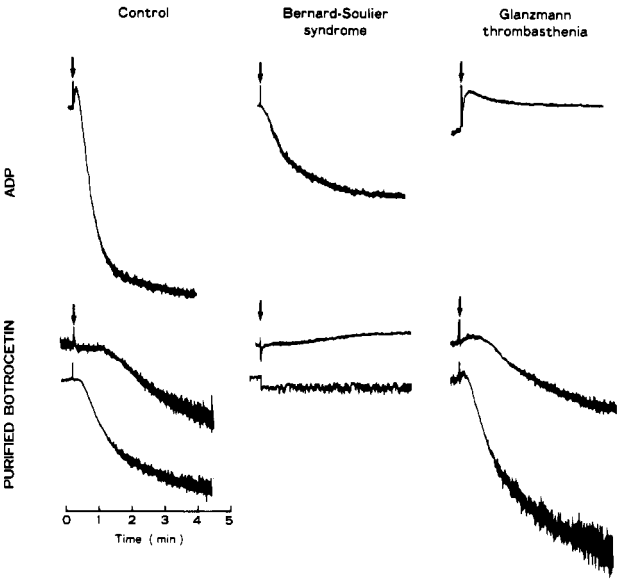

FIGURE 5: Platelet aggregation induced by purified one-chain botrocetin. Platelet aggregation studies were carried out at a final platelet count of 1×10^8/mL in an NKK aggregometer, with 0.3 mL of PRP stirred at 37 °C. From left to right, platelet aggregation curves from a normal individual, a patient (T.H.) with Bernard–Soulier syndrome, and a patient (A.K.) with Glanzmann thrombasthenia. (Top) Platelet aggregation induced by ADP (10 μg/mL) was absent in the case of Glanzmann thrombasthenia but present in Bernard–Soulier syndrome. (Bottom) Platelet aggregation induced by purified botrocetin was measured at two different final concentrations, 10 μg/mL (upper curve) and 50 μg/mL (lower curve). In contrast with ADP-induced aggregation, no response was seen in Bernard–Soulier syndrome, but normal aggregation occurred in Glanzmann thrombasthenia.

NH_4HCO_3, pH 8.0, and no free sulfhydryl group was detected in the intact protein. In addition, it was resistant to enzymatic cleavage except for pepsin, and diminished its activity after reduction and alkylation of disulfide bonds (data not shown).

The two-chain botrocetin was reduced and S-pyridylethylated, and then separated into its component subunits by reverse-phase HPLC on a SynChropak RP-8 column (data not shown). Two major peaks were noted. The NH_2-terminal 40 amino acid residue sequences of these two subunits, α and β, were similar, but different as follows: (α) Asp-Cys-Pro-Ser-Gly-Trp-Ser-Ser-Tyr-Glu-Gly-Asn-Cys-Tyr-Lys-Phe-Phe-Gln-Gln-Lys-Met-Asn-Trp-Ala-Asp-Ala-Glu-Arg-Phe-Cys-Ser-Glu-Gln-Ala-Lys-Gly-Gly-His-Leu-Val; (β) Asp-Cys-Pro-Pro-Asp-Trp-Ser-Ser-Tyr-Glu-Gly-His-Cys-Tyr-Arg-Phe-Phe-Lys-Glu-Trp-Met-His-Trp-Asp-Asp-Ala-Glu-Glu-Phe-Cys-Thr-Glu-Gln-Gln-Thr-Gly-Ala-His-Leu-Val. Thus, the two-chain botrocetin is a disulfied-linked heterodimer composed of two subunits having different NH_2-terminal sequences, distinct from that of the one-chain molecule. A computer homology search indicated that both NH_2-terminal sequences in the heterodimer form botrocetin are closely related to each other and have an apparent homology with several other proteins such as echinoidin (Giga et al., 1987), human or rat proteoglycan core protein (Krusius et al., 1987; Doege et al., 1987), and human or rat hepatic lectin (Spiess & Lodish, 1985; Halberg et al., 1987) (Figure 6).

To explore the possibility that the two-chain botrocetin is a proteolytically modified derivative of the one-chain species, possibly due to the action of a protease present in crude venom, ^{125}I-labeled one-chain botrocetin (purified from lot 68F0557) was mixed with crude venom from a different lot from which the two-chain botrocetin had been obtained (119F0599). The mixture of crude venom solution (10 mg/mL) and the one-chain botrocetin was incubated at 37 °C overnight and then

		Residue No.		
BOTROCETIN (TWO-CHAIN)	α	1	DCPSGWSSYEGNCYKFFQQKMNWADAERFC---SEQAKG-----GHLV-	40
	β	1	DCPPDWSSYEGHCYRFFKEWMHWDDAEEFC---TEQQTG-----AHLV-	40
ECHINOIDIN		2	CCPTFWTSFGSNCYRFFAVSLTWAEGEQFCQSFSVPSRGDIDSIGHLV-	49
RCPCP		1913	QCEEGWTKFQGHCYRHFPDRETWVDAERRC---REQ-----QSHLS-	1950
HPCP		457	TCDYGWHKFQGQCYKYFAHRRTWDAAERRC---RLQ---G-----AHLT-	494
HHLH2a		176	CCPVNWVEHQGSCYWFSHSGKAWAEAEKYC-----QLEN-----AHLV-	213
RHL2		169	CCPVNWVEFGGSCYWFSRDGLTWAEADQYC-----QMEN-----AHLL-	206

FIGURE 6: Comparison of the NH_2-terminal amino acid sequences of two-chain botrocetin, a lectin from the sea urchin *Anthocidaris crassispina* (echinoidin), rat cartilage proteoglycan core protein (RCPCP), human proteoglycan core protein (HPCP), human hepatic lectin H2a (HHLH2a), and rat hepatic lectin 2 (RHL2). The numbers indicate the positions of amino acid residues from the NH_2-terminus in each protein. Gaps have been inserted to maximize homology. Those residues homologous between botrocetin and other proteins have been boxed.

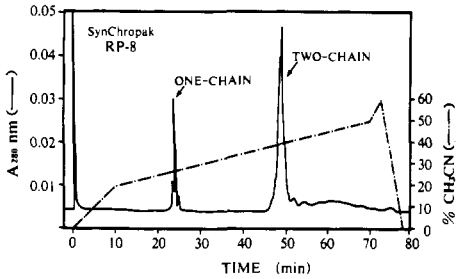

FIGURE 7: Separation of one-chain and two-chain botrocetin by reversed-phase HPLC on SynChropak RP-8 (C8). A mixture of one-chain (30 μg) and two-chain (70 μg) botrocetin was subjected to reversed-phase HPLC on SynChropak RP-8 (C8). By a gradient elution of acetonitrile from 0 to 60%, two distinct peaks were recorded at the absorbance 280 nm. The peak eluted earlier was the one-chain species, and the second peak was the two-chain species.

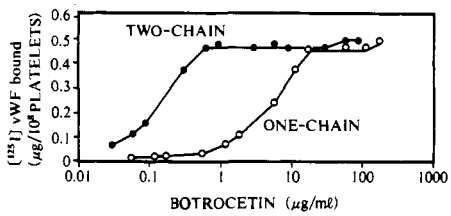

FIGURE 8: Comparison of one-chain and two-chain botrocetin activity in promoting ^{125}I-vWF binding to platelet GP Ib. Formalin-fixed platelets (1×10^8/mL) and ^{125}I-labeled vWF (final concentration 5 μg/mL) were mixed in Eppendorf tubes; then various amounts of purified one- or two-chain botrocetin were added. After 30-min incubation at room temperature (22–25 °C), the mixture was divided into duplicate 50-μL aliquots and the radioactivity bound to platelets was separated from free radioactivity by centrifugation at 13000g through a 20% sucrose cushion.

analyzed by reduced SDS-PAGE (4–20% gradient) followed by autoradiography. No change in the molecular mass of the 32-kDa band was observed, nor was the appearance of the doublet typical of the two-chain species noticed (data not shown). To exclude possible cross-contamination of either the one- or the two-chain botrocetin in the purified preparation, reversed-phase HPLC on SynChropak RP-8 was used for purity analysis. As shown in Figure 7, the mixture of purified one- and two-chain botrocetin was well separated by this column, but neither by the size nor by the hydrophobics-interaction HPLC column (data not shown).

Comparison of the Functional Activity of the Two Forms of Botrocetin. The minimal concentration of the one-chain botrocetin necessary to induce vWF binding to GP Ib was 1 μg/mL, and maximal binding was obtained with 17 μg/mL (Figure 8). Half-maximal binding capacity (BM_{50}) was estimated to correspond to 5.5 μg/mL. In contrast, the BM_{50} for the two-chain species was 0.16 μg/mL (Figure 8), indicating a 34-fold greater activity than for the one-chain botrocetin.

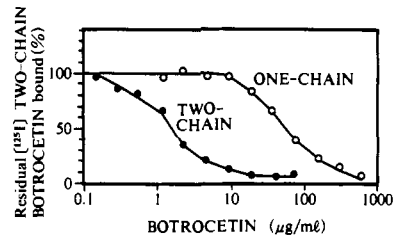

FIGURE 9: Competitive binding of one-chain and two-chain botrocetin to vWF. ^{125}I-Labeled two-chain botrocetin (3.2 μg/mL) was mixed with vWF (5 μg/mL) in the presence of increasing concentrations of unlabeled one-chain or two-chain botrocetin. After 30 min at 22–25 °C, the mixtures were added to polystyrene microtiter wells coated with an anti-vWF antibody, and the percentage of radioactivity bound to the wells, relative to a control mixture containing no competing unlabeled botrocetin, was determined. See Materials and Methods for additional details.

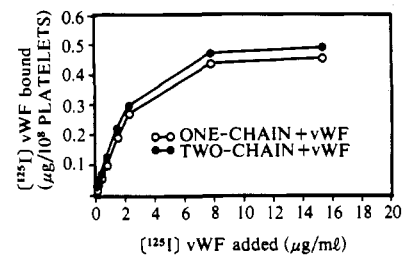

FIGURE 10: ^{125}I-vWF binding to platelets in the presence of one-chain or two-chain botrocetin. A constant amount of botrocetin (30 μg/mL for one-chain and 1 μg/mL for two-chain) was mixed with various concentrations of ^{125}I-vWF, as indicated, and the mixtures were incubated for 30 min at 22–25 °C. Then, formalin-fixed platelets were added to give a final concentration of 1×10^8/mL and incubated for additional 30 min. Separation of platelet-bound radioactivity from free ligand was achieved by centrifugation through 20% sucrose at 13000g, and the amount of vWF bound to the platelets was then calculated.

To determine whether iodination altered the functional characteristics of two-chain botrocetin, platelet aggregation studies mediated by ^{125}I-labeled and unlabeled two-chain botrocetin were performed using PRP from normal individuals. The values for minimal concentration to induce platelet aggregation (threshold) in both instances were compared with each other. There was no significant difference between these values, indicating that ^{125}I-labeled botrocetin and unlabeled two-chain botrocetin have similar vWF-dependent platelet aggregation activities (figure not shown).

^{125}I-Labeled two-chain botrocetin and vWF formed a complex in solution, as shown by the observation that an anti-vWF monoclonal antibody could bind botrocetin only when vWF was present (Figure 9). The specific binding of ^{125}I-labeled two-chain botrocetin to vWF was competitively inhibited in a dose-dependent manner by both the one-chain and the two-chain botrocetin (Figure 9). The corresponding IC_{50} values (namely, the concentration necessary to inhibit binding

Table II: Binding of ^{125}I-vWF to Platelets in the Presence of Botrocetin[a]

	K_d (M)	B_{max} (M)
one-chain	1.06×10^{-8}	3.1×10^{-9}
two-chain	1.3×10^{-8}	3.1×10^{-9}

[a] The dissociation constant (K_d) and maximal binding at saturation (B_{max}) were calculated from the binding isotherms shown in Figure 10 using the computer-assisted program LIGAND (Munson & Rodbard, 1980), and assuming a molecular mass of 275 000 for the vWF subunit.

to vWF by 50%) were 55 μg/mL for the one-chain and 1.7 μg/mL for the two-chain species. Thus, the two-chain botrocetin has a 32-fold higher affinity for vWF than the one-chain species.

The parameters of ^{125}I-labeled vWF binding to platelets were determined in the presence of the one- or the two-chain botrocetin (Figure 10). Since the two-chain botrocetin is approximately 30-fold more active than the one-chain species in promoting vWF binding, the concentrations used for this experiment were 1 and 30 μg/mL, respectively, which represent maximally effective doses (Figure 9). In both instances, the dissociation constant (K_d) and the maximal binding at saturation (B_{max}), calculated with the computer-assisted program LIGAND (Munson & Rodbard, 1980), were almost identical (Table II).

Discussion

The present results demonstrate the existence of two structurally and functionally distinct forms of botrocetin. On the basis of their amino acid composition, these two molecules appear to be related, particularly with regard to a high content of potentially acidic residues, Glx and Asx. Nevertheless, compared to the one-chain species, the two-chain botrocetin contains half the amount of Cys residues and 3 times as much Phe residues and differs significantly also in the content of Ser, Gly, and Val residues. These discrepancies in chemical composition establish the fact that the two-chain botrocetin cannot be derived from the one-chain molecule as a result of one or more proteolytic cleavages. This conclusion is supported by the observation that incubation of the one-chain botrocetin with crude venom of the same manufacturer lot from which two-chain botrocetin had been purified did not result in generation of the two-chain species. Thus, it is unlikely that a protease present in the crude venom may be responsible for the appearance of the two-chain form of botrocetin. The distinct nature of the two botrocetins is also clearly indicated by the different amino-terminal sequences of the one-chain botrocetin and of the two components of the two-chain species. The reduced subunits of the latter differ in their apparent molecular mass and have similar but different amino termini that make the two-chain species a heterodimer.

One-chain botrocetin is a polypeptide of approximately 280 amino acid residues, while the two-chain species is composed of approximately 300 residues. Both have a high content of Cys residues, but neither displays free sulfhydryl groups. Thus, the one-chain botrocetin contains numerous intrachain disulfide bonds, while the two-chain form must have at least one interchain bond. This chemical composition may explain why they are resistant to proteolytic attack in spite of the very low carbohydrate content. Moreover, the disulfide bridges may be important for maintaining the conformation necessary to display function, since treatment with reducing agents results in loss of activity.

Functionally, the two botrocetins differ in their affinity for vWF. Indeed, in agreement with the previous results of Howard et al. (1984), Read et al. (1989), and Andrews et al. (1989), we have also obtained evidence, using a new experimental approach, that vWF and botrocetin form a soluble complex. The binding of ^{125}I-labeled two-chain botrocetin to vWF was clearly inhibited in a dose–response manner by one-chain botrocetin as well as by unlabeled two-chain counterpart. At present, we do not know whether two forms of botrocetin compete for the same binding site on vWF or two binding sites for botrocetin exist on vWF where the binding of one form affects the binding of the other in a noncompetitive manner. However, the former conclusion appears to be more supported by the observation that vWF complexed to either the one-chain or the two-chain botrocetin interacts with platelets with identical binding characteristics. The reasons why the botrocetin–vWF complex acquires the ability to bind to GP Ib on the platelet membrane remain unknown (Read et al., 1989; Andrews et al., 1989).

In contrast to the results of Howard et al. (1984), we found that the highly purified one-chain botrocetin was unable to induce aggregation of PRP from patients with Bernard–Soulier syndrome. Because of this, it seems likely that the only platelet binding site for the vWF–botrocetin complex is the GP Ib–IX complex. The aggregation observed in Bernard–Soulier patients may have been caused by a thrombin-like enzyme possibly present in the partially purified botrocetin preparation used by Howard et al. (1984). Indeed, platelet aggregation independent of vWF binding to GP Ib, but mediated by GP IIb–IIIa, can be induced by botrocetin preparations of intermediate purity. This effect seems to correlate with the presence of even a trace amount of fibrinogen clotting activity.

The functional results presented here differ considerably from those of Andrews et al. (1989), who studied the binding of the two-chain botrocetin to vWF. The species purified by these authors had essentially the same structural features of the two-chain botrocetin described here, including a very similar amino acid composition and amino-terminal sequence, although they failed to identify the residue in cycle 2 and found different residues in cycle 8. Nevertheless, the "dimeric" botrocetin purified by Andrews et al. (1988) displayed a much lower affinity for vWF than the two-chain botrocetin used in the present studies, as judged by the concentration necessary to induce maximal vWF binding to platelets. Since the assay systems employed in the two studies are comparable, the discrepant results may be explained either by inactivation of function during the purification procedure described by Andrews et al. (1989) or, perhaps, by the existence of yet a third form of botrocetin, with a two-chain structure but a functional activity similar to that of the one-chain molecule described here.

A question remains unanswered at present, namely, why different forms of botrocetin can be isolated by using the same, or similar, purification procedures starting from crude venom obtained from the same supplier. In addressing this question, it should be considered that the two different molecular species of botrocetin were obtained from different lots of crude venom, suggesting that the composition of the starting material may determine the nature of the final product. Variations in the storage and handling of the crude venom before purification are unlikely to be responsible for the different results obtained since, as discussed above, the two forms of purified botrocetin do not appear to be the proteolytic product of the other. Rather, since botrocetin-like activity has been found in the venom of several snakes of the *Bothrops* species (Read et al., 1978), it is possible that the starting preparations used in these studies were different with respect to their composition, even though all labeled as the species *jararaca*. In this regard, it

is important to note that the supplier (Sigma Chemical Co.) feels compelled to publish a disclaimer in its catalogue (page 1546 of the 1990 edition) to the effect that it is impossible to have strict quality control on the crude venom preparations. According to this hypothesis, the two distinct botrocetins characterized here may be typical of different snakes of the *Bothrops* genus. Alternatively, the venom of *Bothrops jararaca* may contain different forms of botrocetin, with similar behavior in the purification procedures utilized in the course of these studies, and their respective concentrations may depend on undefined aspects of the life cycle of the snake and, consequently, vary in different lots of the crude venom preparations.

Clarification of the structure of the two forms of botrocetin is now required to establish the degree of their homology, as well as to understand the mechanisms of complex formation with vWF and to define the structural features that make the complex a competent ligand for GP Ib–IX. This, in turn, may help understand the pathophysiological functions of vWF complexed to subendothelial components in the vessel wall.

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Registry No. vWF, 109319-16-6; botrocetin, 85537-36-6.

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