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Thrombocytin, a Serine Protease from *Bothrops atrox* Venom. 2. Interaction with Platelets and Plasma-Clotting Factors†

Stefan Niewiarowski,* Edward P. Kirby, Tomasz M. Brudzynski, and Kurt Stocker

ABSTRACT: Thrombocytin, a serine protease from *Bothrops atrox* venom, caused platelet aggregation and release of platelet constituents at a concentration of 10^{-7} M and clot retraction at a concentration of 2×10^{-9} M. Thrombocytin was slightly more active when tested on platelets in plasma than on washed platelets suspended in Tyrode–albumin solution. Thrombin was 5 times more active than thrombocytin when tested on platelets in plasma and 50 times more active when tested on washed platelets. The patterns of release induced by thrombocytin and thrombin were similar. Prostaglandin E_1 (10^{-5} M) produced complete inhibition of platelet release induced by thrombocytin and thrombin. Indomethacin (10^{-4} M) was without any effect. Antithrombin III, in the presence of heparin, inhibited the action of thrombocytin on platelets and on a synthetic peptide substrate (Tos-Gly-Pro-Arg-pNA·HCl). Formation of an antithrombin III–thrombocytin complex was demonstrated on $NaDodSO_4$–polyacrylamide gel electrophoresis. Hirudin and α_1-antitrypsin did not inactivate thrombocytin. Thrombocytin had a low fibrinogen-clotting activity (less than 0.06% that of thrombin). Thrombocytin also caused progressive degradation of the α chain of human fibrinogen, and it cleaved prothrombin, releasing products similar to intermediate 1 and fragment 1 produced by thrombin. Thrombocytin activated factor XIII by limited proteolysis and increased the procoagulant activity of factor VIII in a manner analogous to that of thrombin.

Thrombin is a serine protease which has several important physiological functions. It cleaves fibrinopeptides A and B from fibrinogen, leading to the formation of a fibrin clot (Blombäck et al., 1967), and it also interacts with platelets, causing platelet aggregation, clot retraction, and release of some platelet constituents (Niewiarowski & Thomas, 1966; Holmsen et al., 1969). In addition, thrombin activates factor XIII (Takagi & Doolittle, 1974), it modifies factors V (Colman, 1969) and VIII (Legaz et al., 1973) to increase their coagulant activities, it cleaves prothrombin to form fragment 1 and intermediate 1 (Magnusson et al., 1975; Walz et al., 1977), and it interacts with antithrombin III to form a stable complex (Rosenberg & Damus, 1973).

Bothrops atrox venom contains two distinct serine proteases which together mimic the activity of thrombin but individually have only a few of its activities. Batroxobin readily clots fibrinogen, but it does not affect platelets, does not activate factor XIII, and is not inhibited by heparin–antithrombin III (Stocker & Barlow, 1976). Thrombocytin, on the other hand, activates platelets and factor XIII but has very little clotting activity toward fibrinogen (Niewiarowski, et al., 1977a; Kirby et al., 1979).

The purpose of the present study was to examine the function and specificity of thrombocytin relative to thrombin. We have compared the effects of thrombocytin and thrombin on platelet aggregation, the platelet release reaction, and clot retraction. In addition we have studied the effects of thrombocytin on fibrinogen, on prothrombin, and on factors VIII, X, and XIII. Thrombocytin was readily inhibited by plasma antithrombin III in the presence of heparin, with the formation of a stable inactive complex.

Materials and Methods

Reagents. *Echis carinatus* venom, Russell's viper venom (RVV),[1] indomethacin, antimycin A, and deoxyglucose were from Sigma Chemical Co. (St. Louis, MO). Batroxobin was from Pentapharm (Basle, Switzerland). Prostaglandin E_1 was from Upjohn Co. (Kalamazoo, MI). Hirudin was obtained from Pentapharm and from Arzneimittel Werke, VEB, Dresden, East Germany. Fibrinogen used for electrophoretic

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* Dr. Kirby is the recipient of Research Career Development Award HL-00275 from the National Heart, Lung and Blood Institute.

[1] Abbreviations used: RVV, Russell's viper venom; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; Tcn, thrombocytin; AT III, antithrombin III; PRP, platelet-rich plasma; 5-HT, 5-hydroxytryptamine; LTU, light transmission units; pNA, *p*-nitroaniline; LA-PF_4, low-affinity platelet factor 4; PGE_1, prostaglandin E_1.

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studies was obtained from Imco (Stockholm, Sweden). Diisopropyl fluorophosphate (DFP) was obtained from Aldrich Chemical Co. (Milwaukee, WI) and stored at −30 °C as a 1 M solution in acetonitrile.

Thrombocytin was prepared as described in the previous paper (Kirby et al., 1979). Fibrinogen free of factor XIII was prepared from human fibrinogen (A. B. Kabi, Stockholm, Sweden) by ammonium sulfate precipitation and chromatography on Sepharose 4B (Pharmacia, Piscatway, NJ) as described by Niewiarowski et al. (1977b). Bovine prothrombin, factor VIII, and factor X were purified as described by Mann (1976), Legaz et al. (1975), and Fujikawa et al. (1972), respectively. Human plasmin (Michigan State Department of Health, Detroit, MI) was kindly provided by Dr. D. L. Aronson, Division of Biologics Standards, Bethesda, MD.

Antithrombin III samples, prepared by the methods of Rosenberg & Damus (1973) and Miller-Andersson et al. (1974), were gifts from Dr. R. Rosenberg (Harvard University, Boston, MA) and from Dr. D. P. Thomas (National Institute for Biological Standards and Control, London, England), respectively. α_1-Antitrypsin was kindly donated by Dr. Harold James (Temple University, Philadelphia, PA). It was prepared by the method of Cohen & James (1978). A highly purified preparation of high-affinity heparin (Hopwood et al., 1976) was a gift from Dr. U. Lindahl, Uppsala, Sweden. Human thrombin, prepared by the method of Fenton et al. (1977), was kindly supplied by Dr. J. Fenton (Albany, NY). Factor XIII (fibrin-stabilizing factor), prepared by the method of Lorand & Gotoh (1970), was kindly supplied by Dr. L. Lorand (Evanston, IL).

Buffers. Tris–saline buffer contained 0.01 M Trizma base, 0.15 M NaCl, and 0.01% sodium azide and was adjusted to pH 7.4 with HCl. Phosphate–saline buffer contained 0.01 M NaH_2PO_4, 0.15 M NaCl, and 0.01% sodium azide and was adjusted to pH 6.5 with NaOH. Buffers used for platelet studies did not contain sodium azide.

Polyacrylamide gel electrophoresis, measurement of protein concentration, and assay of amidolytic and factor XIII activating activities were all performed as described in the previous paper (Kirby et al., 1979).

Factor VIII procoagulant activity was measured by a modification of the one-stage activated partial thromboplastin time as described by Proctor & Rapaport (1961). Thrombin was assayed as described by Fenton et al. (1977). Activated factor X was assayed after dilution in Tris–saline buffer by adding 0.1 mL of sample to a mixture of 0.1 mL of factor X deficient plasma (Diagnostic Reagents, Ltd., Thame, Oxon, England) and 0.1 mL of 0.038% inosithin (Associated Concentrates, Woodside, NY). After a 2-min preincubation, 0.1 mL of 0.025 M $CaCl_2$ was added to initiate clotting.

Human Platelets. Human platelet-rich plasma (PRP) was prepared by collecting blood in 0.1 M citrate and centrifuging at 150*g* for 15 min. Washed platelet suspensions were prepared by the method of Mustard et al. (1972) from blood collected in acid citrate dextrose. The final platelet count in the suspension was (5×10^8)–10^9/mL. Platelet aggregation was studied in a Payton aggregometer (Payton Associates, Inc., Scarborough, Ontario), and results were expressed in arbitrary light transmission units (LTU) as described previously (Niewiarowski et al., 1972). Platelets were labeled with [^{14}C]-5-hydroxytryptamime (0.3 μCi/10^9 cells) by incubation for 10 min at 37 °C. Release of [^{14}C]-5-HT was measured as described previously (Niewiarowski et al., 1972). [^{14}C]-5-HT had a specific activity of 30–50 mCi/mmol, and it was supplied by Amersham/Searle Corp. (Arlington Heights, IL). Release of low-affinity platelet factor 4 (LA-PF_4) from platelets was measured by radioimmunoassay (Rucinski et al., 1979). β-*N*-Acetylglucosaminidase was determined by the method of Day et al. (1969). Maximum release was defined as that which was caused by disruption of platelets by 1% Triton X-100. In some experiments platelets were labeled with sodium chromate-51 as described previously (Brown et al., 1977). Release of ^{51}Cr radioactivity was determined after 2-min centrifugation (7500*g*) of the platelets in an Eppendorf centrifuge.

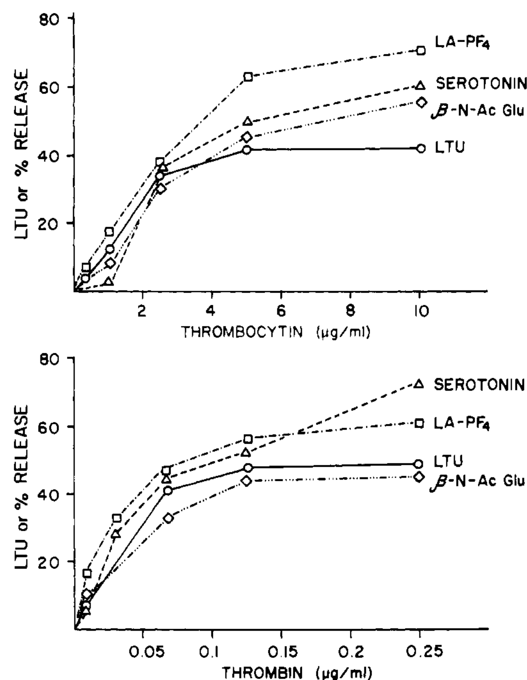

FIGURE 1: Effects of thrombocytin and thrombin on aggregation and release in washed platelets. The incubation mixture was composed of 0.9 mL of platelet suspension (10^9 platelets/mL) and 0.1 mL of thrombocytin or thrombin. Light transmission changes were measured in an aggregometer and expressed in arbitrary light transmission units (LTU). After a 3-min incubation in the aggregometer, samples were centrifuged in an Eppendorf centrifuge and LA-PF_4, [^{14}C]-5-HT radioactivity, and β-*N*-acetylglucosaminidase activity were determined in the supernatant. This experiment is a representative one from three similar experiments.

Clot retraction was measured at 37 °C in a system composed of 0.7 mL of platelet suspension (8×10^8 platelets/mL), 0.1 mL of the test sample, 0.1 mL of 0.5% fibrinogen, and 0.1 mL of batroxobin (20 units/mL). Alternatively, incubation mixtures were composed of 0.6 mL of human platelet-rich plasma (PRP), 0.2 mL of 0.9% NaCl, 0.1 mL of test sample, and 0.1 mL of batroxobin (20 units/mL). The clots were incubated for 30–60 min at 37 °C, and the volume of extruded fluid was measured after careful removal of the clot. Batroxobin alone did not cause any clot retraction during the 60-min incubation.

Results

Effect of Thrombocytin on Platelet Aggregation, the Platelet Release Reaction, and Clot Retraction. Purified thrombocytin caused extensive platelet aggregation and induced the platelet release reaction. On the other hand, incubation with thrombocytin (10 μg/mL) for 3 min did not cause platelets labeled with chromium-51 to release any radioactivity. Figure 1 shows the effect of various concentrations of thrombocytin and thrombin on platelet aggregation and on the release of LA-PF_4 antigen, [^{14}C]-5-HT and β-*N*-acetylglucosaminidase from washed platelets. Concentrations of

Table I: Effect of Thrombocytin and Thrombin on Clot Retraction[a]

enzyme (ng/mL)	volume of extruded fluid (%): platelet-rich plasma	volume of extruded fluid (%): washed platelet suspensions
	Thrombocytin	
0	5	5
15	10	5
30	35	35
60	35	50
125	63	95
500	95	95
	Thrombin	
0	5	5
50	5	95
100	55	95
400	95	95

[a] Samples were incubated for 30 min at 37 °C. These are the mean values from three experiments.

Table II: Effect of Inhibitors of Platelet Function on Platelet Aggregation Induced by Thrombin and Thrombocytin[a]

additive (inhibitor)	preincubn time of inhibitor with platelets (min)	platelet aggregation (LTU): thrombin	platelet aggregation (LTU): thrombocytin
NaCl (0.15 M)	10	64	58
	30	68	56
PGE_1 (10^{-5} M)	10	0	3
indomethacin (10^{-4} M)	10	60	57
deoxyglucose (2×10^{-3} M) and antimycin (5 μg/mL)	30	44	16
ethanol (0.17 M)	30	58	40

[a] Washed platelets (8×10^8 platelets in 0.8 mL of buffer) were preincubated with 0.1 mL of inhibitor for 10 or 30 min. Then 0.1 mL of thrombin (100 ng) or 0.1 mL of thrombocytin (2.5 μg) was added, and aggregation was recorded in an aggregometer for 3 min at 37 °C. The concentrations of inhibitors are the final ones. This experiment is representative of three similar experiments.

thrombocytin required to produce 50% release of platelet constituents varied from 5 to 7 μg/mL. Highly purified thrombin produced similar effects at concentrations that were approximately 50 times lower.

Platelets in fresh PRP were slightly more responsive to thrombocytin than washed platelets and gave 50% aggregation at about 2 μg/mL and 50% release at 4 μg/mL. In contrast, platelets in PRP were less responsive to thrombin than were washed platelets. Thrombin caused 50% aggregation of platelets in PRP at approximately 0.5 μg/mL and 50% release at 1 μg/mL. When platelets were incubated in PRP at room temperature for 1–4 h, their sensitivity to thrombocytin decreased.

Clot retraction appeared to be the most sensitive assay to detect thrombocytin. As little as 60 ng of thrombocytin caused 50% retraction in a washed platelet system or 35% retraction in PRP during a 30-min incubation at 37 °C (Table I).

Various inhibitors of platelet aggregation (Table II) exerted similar effects on aggregation induced by thrombocytin (2 μg/mL) and thrombin (0.1 μg/mL). PGE_1 is known to inhibit ADP-induced platelet aggregation at a concentration of 10^{-8} M (Mustard & Packham, 1970). PGE_1 at 10^{-5} M produced complete inhibition of aggregation induced by thrombin or thrombocytin. Indomethacin, which inhibits collagen-induced platelet aggregation in concentrations ranging between 2×10^{-6} and 10^{-5} M (Zucker & Paterson, 1970), was without any effect on either thrombin- or thrombocytin-induced aggregation. Partial inhibition occurred after incubation of the platelets with deoxyglucose (2×10^{-3} M) and antimycin (5 μg/mL) for 30 min. Part of this inhibition was probably caused by the ethanol used as the solvent for the antimycin (Table II).

Table III: Effects of Protease Inhibitors on the Amidolytic and Platelet-Aggregating Activity of Thrombocytin[a]

inhibitor	platelet aggregation in PRP (LTU)	amidolytic act. (μmol of pNA per min per μg)
buffer (control)	54	18.4
α_1-antitrypsin (5 mg/mL)	70	20.3
hirudin (12.5 units/mL)	41	19.2
antithrombin III (2.5 mg/mL)	50	18.4
heparin (100 units/mL)	0.0	27.2
antithrombin III (2.5 mg/mL) + heparin (10 units/mL)	0.0	4.3

[a] Thrombocytin (10 μg/mL) was incubated with the indicated concentrations of inhibitors for 10 min at 25 °C. Samples (0.2 mL) were then added to 1.0 mL of PRP at 37 °C for assay of the remaining aggregating activity. Samples (0.2 mL) were also assayed at 25 °C for amidolytic activity by using Chromozym TH as a substrate.

Effect of Protease Inhibitors on the Amidolytic and Platelet-Aggregating Activity of Thrombocytin. Thrombocytin is readily inhibited by DFP and by soybean trypsin inhibitor, which inactivate both its amidolytic and platelet-aggregating activities (Kirby et al., 1979). α_1-Antitrypsin and the specific thrombin inhibitor, hirudin, however, were without any significant effect on thrombocytin (Table III).

Heparin or antithrombin III alone did not inhibit the amidolytic activity of thrombocytin, but together they caused marked inhibition. Addition of heparin to the platelet-rich plasma assay system prevented aggregation by thrombocytin, presumably because of interaction of the thrombocytin with antithrombin III present in the plasma.

Thrombocytin can form a stable complex with heparin and antithrombin III, as shown in Figure 2. Thrombocytin incubated with antithrombin III alone did not form a complex during 10 min, but a small amount of complex was formed after 60 min of incubation. The presence of heparin in the incubation mixture considerably accelerated the formation of a complex which had an apparent molecular weight of approximately 80 000.

Effects of Thrombocytin on Fibrinogen. Samples of platelet-rich plasma aggregated with thrombocytin did not form any fibrin even after overnight incubation. However, concentrated preparations of thrombocytin were able to slowly clot purified fibrinogen. Purified thrombin, used as a standard in these experiments, contained about 2800 units/mg (Fasco & Fenton, 1973). Batroxobin contained 1900 units/mg fibrinogen-clotting activity. On the average, thrombocytin contained 1.7 units/mg fibrinogen-clotting activity; however, one preparation contained less than 0.3 unit/mg.

The fibrinogen-clotting activity of thrombocytin either could be due to contamination with batroxobin or could be an inherent property of thrombocytin. It is difficult to resolve these two possibilities because of the very low fibrinogen-clotting

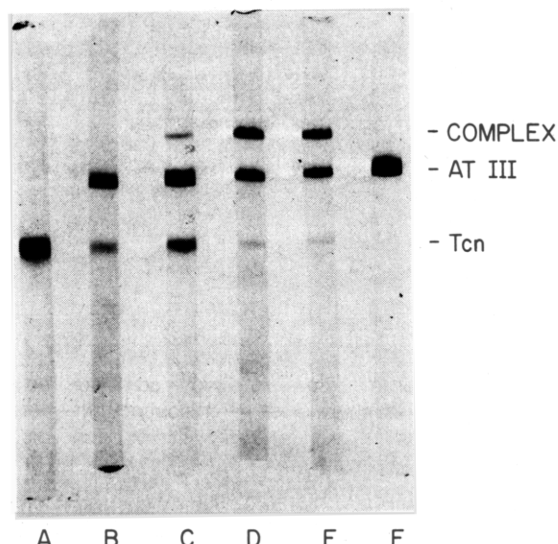

FIGURE 2: Formation of the complex between thrombocytin and antithrombin III. Samples were incubated at 25 °C, then $NaDodSO_4$ buffer was added, and they were electrophoresed without reduction. (A) Thrombocytin (10 μg); (B and C) thrombocytin (5 μg) incubated with antithrombin III (15 μg) for 10 and 60 min, respectively; (D and E) thrombocytin (5 μg) incubated with antithrombin III (15 μg) and high-affinity heparin (45 μg) for 10 and 60 min, respectively; (F) antithrombin III (10 μg).

activity of thrombocytin and because of its other activities on fibrinogen (see below).

Thrombocytin was free of fibrinolytic activity when tested by the fibrin plate method, as previously reported (Niewiarowski et al., 1977a). However, incubation of purified fibrinogen with high concentrations of thrombocytin resulted in a progressive degradation of the fibrinogen α chain (Figure 3). Upon very prolonged incubation (19 h), there was also degradation of the β and γ chains, but the action of traces of possible contaminating proteases cannot be ruled out under those conditions.

Digestion of the α chains of fibrinogen by thrombocytin seemed to be complete, and no α-chain fragments were observed which would be analogous to the 25 000 molecular weight derivatives seen with plasmin digestion (Budzynski et al., 1974; Harfenist & Canfield, 1975) or the 38 000 molecular weight derivatives seen with Ancrod digestion (Pizzo et al., 1972; Gaffney & Brasher, 1974). The fragment observed at 42 000 molecular weight in the 19-h thrombocytin digest probably corresponds to the 42 000 molecular weight plasmin fragment of the β chain (Ferguson et al., 1975).

Upon incubation of fibrinogen with 36 μg/mL thrombocytin, clotting was observed as a slight increase in the turbidity of the solution after 11 min. Figure 3 shows that the cleavage of the α chain which had occurred at this time was minor, suggesting that the clotting activity of thrombocytin is low because this is an intrinsic property of the enzyme and not because it degrades fibrinogen to an incoagulable form. When fibrin clots formed by the action of thrombocytin were washed and dissolved in $NaDodSO_4$–DTT, polyacrylamide gels showed the presence of little, if any, intact α chains.

Activation of Factor XIII by Thrombocytin. In agreement with our previous observations on partially purified thrombocytin (Niewiarowski et al., 1977a), we found that highly purified thrombocytin also activated factor XIII (Kirby et al., 1979). Fibrin formed by batroxobin is soluble in 2% acetic acid, but in the presence of factor XIII and either thrombocytin (10 ng) or thrombin (10 ng), the clot was rendered insoluble.

Thrombocytin activates factor XIII in a manner very similar to that seen with thrombin. Figure 4 shows the electrophoretic patterns of intact factor XIII and of factor XIII activated by thrombocytin or thrombin. It can be seen that the mobility of the a chain of factor XIII was increased upon treatment with thrombin or thrombocytin, corresponding to the cleavage of a peptide of approximately 4000 molecular weight.

Effect of Thrombocytin on Prothrombin. Incubation of purified prothrombin with thrombocytin did not lead to the development of any fibrinogen-clotting activity, although this prothrombin was readily activated by *E. carinatus* venom. Examination of $NaDodSO_4$ gel patterns (Figure 5), however, showed that thrombocytin did cleave prothrombin. The major products found had apparent molecular weights similar to intermediate 1 and fragment 1 (Magnusson et al., 1975; Walz et al., 1977) produced by thrombin.

Effect of Thrombocytin on Factor X. Thrombocytin preparations were able to activate factor X (Table IV), but the reaction was very slow and incomplete compared to that

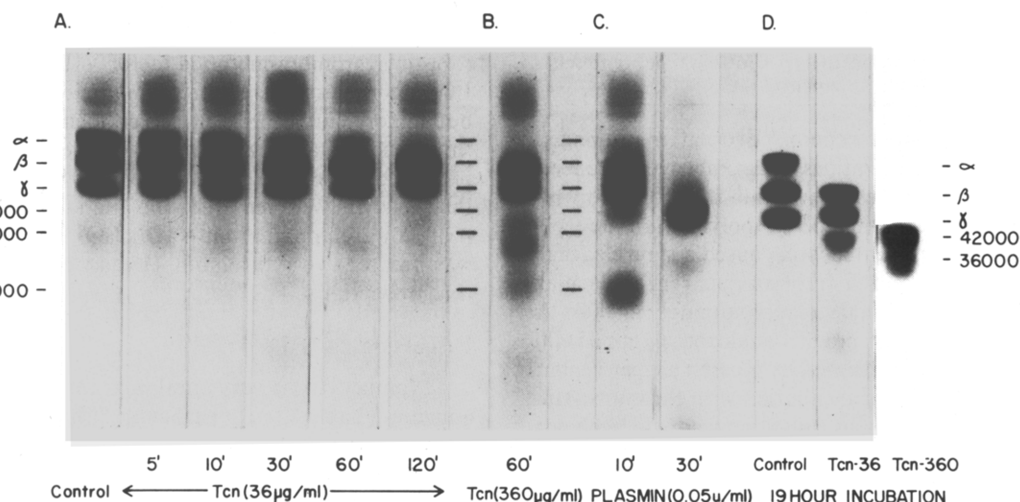

FIGURE 3: Degradation of fibrinogen by thrombocytin. Fibrinogen (20-μL samples of 1.0 mg/mL) was incubated at 37 °C with 20 μL of either thrombocytin or plasmin in small glass tubes. At the indicated times 50 μL of $NaDodSO_4$ buffer containing dithiothreitol (10 mg/mL) was added. The samples were incubated for 3 min at 100 °C and run on 5% polyacrylamide gels. (A) Thrombocytin, 36 μg/mL in the incubation mixture; (B) thrombocytin, 360 μg/mL in the incubation mixture; (C) plasmin, 0.05 CTA unit/mL in the incubation mixture; (D) fibrinogen incubated with buffer, thrombocytin (36 μg/mL), or thrombocytin (360 μg/mL) for 19 h at 37 °C (the bands with apparent molecular weights greater than 100 000 were artifacts seen only on this particular set of gels).

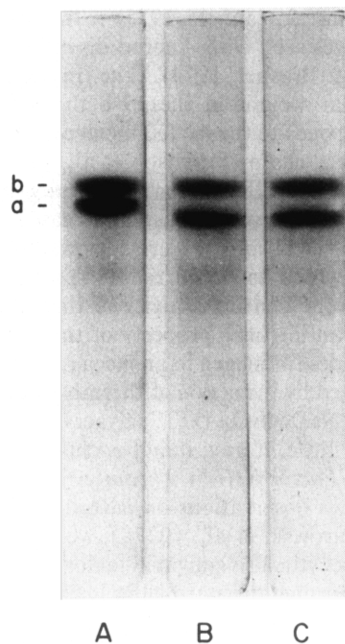

FIGURE 4: Activation of factor XIII by thrombin and thrombocytin. Human factor XIII (0.7 mg/mL) was incubated with either (A) buffer, (B) thrombin (10 μg/mL), or (C) thrombocytin (0.3 mg/mL) for 15 min at 37 °C. Samples were then diluted in NaDodSO$_4$–DTT, heated at 100 °C, and run on 7% polyacrylamide gels. Letters a and b refer to the subunit chains of factor XIII.

Table IV: Factor X Activation[a]

incubn time (min)	clotting time (s): activation with RVV, 1:100 000 dilution	activation with thrombocytin: 1:1000 dilution	activation with thrombocytin: 1:10 000 dilution
0	>100	>100	>100
10	32.2		
20	32.1		
30		51.4	85.7
60		31.7	76.4
120		26.0	58.0
180		24.9	49.7

[a] Factor X (0.7 mg/mL) was incubated with 8 mM CaCl$_2$ and either crude RVV (3.3 μg/mL) or purified thrombocytin (50 μg/mL) at 37 °C in Tris–saline buffer, pH 7.4. At the indicated times, samples were removed, diluted in Tris–saline buffer as indicated, and assayed for factor Xa activity. Clotting times represent mean values from duplicate measurements.

produced by Russell's viper venom. Even after prolonged incubation with high concentrations of thrombocytin, the level of factor Xa generated was less than 5% of that obtained with Russell's viper venom. NaDodSO$_4$–polyacrylamide gels (Figure 6) showed that thrombocytin caused a slow cleavage of the heavy chain of factor X from an apparent molecular weight of approximately 48 000[2] to approximately 44 000. A small amount of an activation product analogous to the 35 000 molecular weight fragment formed by Russell's viper venom was apparent after prolonged incubation with thrombocytin. Thrombin under these conditions caused neither activation nor cleavage of factor X.

[2] Apparent molecular weights estimated by our electrophoresis system differ only slightly from those proposed by other authors using similar techniques but differ greatly from molecular weights determined from sedimentation equilibrium studies, probably because of the difficulties in estimating the molecular weights of glycoproteins on NaDodSO$_4$–polyacrylamide gels (Weber & Osborn, 1975).

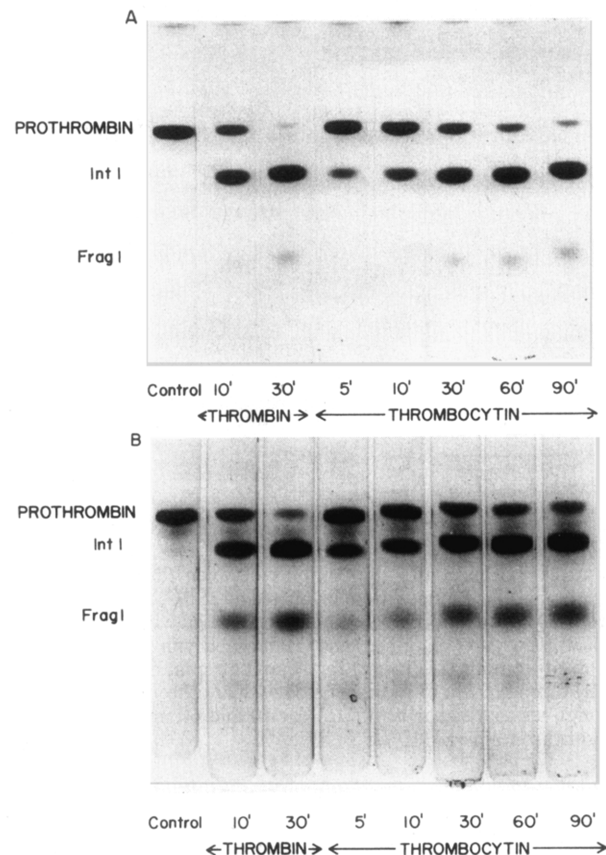

FIGURE 5: Effects of thrombin and thrombocytin on prothrombin. Samples of bovine prothrombin (0.5 mg/mL) were incubated with either thrombin (17 μg/mL) or thrombocytin (12 μg/mL) for the indicated times at 37 °C. Samples were taken into NaDodSO$_4$ buffer and heated at 100 °C in either (A) the absence or (B) the presence of dithiothreitol (10 mg/mL). Samples were run on 7% polyacrylamide gels.

Modification of Factor VIII Activity. Thrombin has been shown to increase the procoagulant activity of factor VIII by increasing its ability to function as a cofactor for factor IXa. In a one-stage assay system, this is observed as a (10–100)-fold increase in apparent activity (Legaz et al., 1973). When purified bovine factor VIII was incubated with thrombin, a 14-fold apparent increase in factor VIII procoagulant activity was observed at the earliest time point (Figure 7). The resulting thrombin-modified factor VIII rapidly lost activity. Lower concentrations of thrombin produced less of an increase but a more stable product. Treatment of factor VIII with thrombocytin also caused an increase in activity with the production of an unstable product, but high concentrations of thrombocytin were required. No alterations in the factor VIII protein were apparent on NaDodSO$_4$ gels when factor VIII was treated with thrombin (Legaz et al., 1975) or with thrombocytin.

Discussion

Thrombocytin is very similar to thrombin in many of its activities (Table V). In particular, it has a very strong ability to activate platelets and to induce their aggregation and release of storage granules. Thrombocytin caused platelet aggregation and the release reaction at concentrations of about 10^{-7} M (Figures 1 and 2), and significant effects on clot retraction were observed at 2×10^{-9} M (Table I). Thrombin was about 50 times more active than thrombocytin in aggregating platelets and in causing release in the washed platelet system. In PRP, however, thrombin was only 5 times more active than

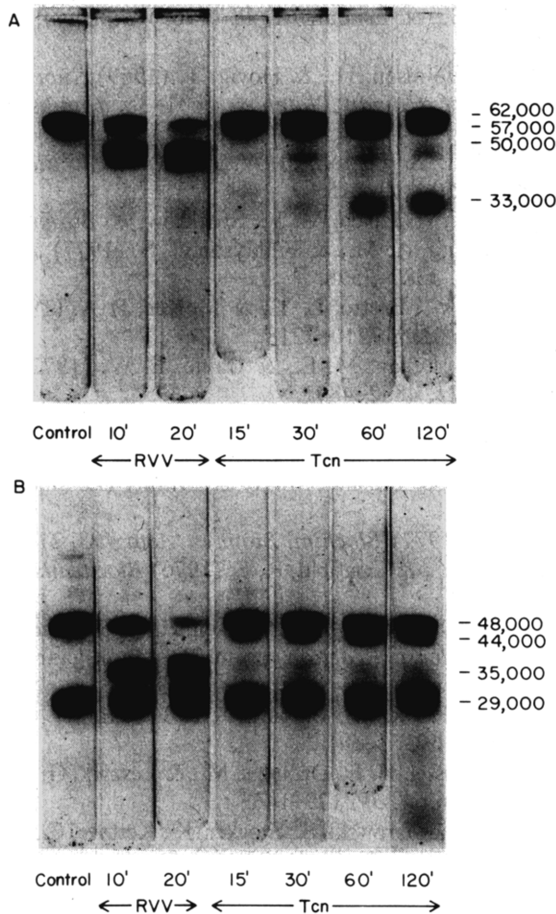

FIGURE 6: Effects of Russell's viper venom and thrombocytin on factor X. Samples from the experiment described in Table IV were taken into $NaDodSO_4$ buffer and heated at 100 °C in either (A) the absence or (B) the presence of dithiothreitol (10 mg/mL). Samples were run on 7% polyacrylamide gels.

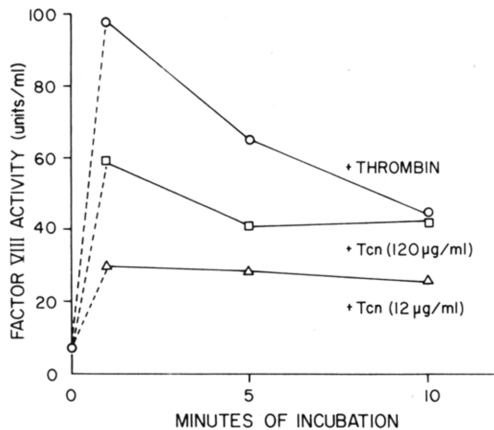

FIGURE 7: Modification of factor VIII by thrombin and thrombocytin. Samples of purified bovine factor VIII (36 μg/mL) were incubated with human thrombin (0.75 μg/mL) or thrombocytin (12 or 120 μg/mL) at 0 °C. Aliquots were taken at the indicated times, diluted 1:1000, and assayed for factor VIII procoagulant activity. The control (zero time) did not contain either thrombin or thrombocytin.

thrombocytin. These differences may be due to thrombin being more rapidly inactivated by the antithrombin III present in plasma than is thrombocytin. Fibrinogen is a much more sensitive thrombin substrate than are platelets (Kaplan et al., 1978), and it is also possible that fibrinogen may interfere with the action of thrombin on platelets in plasma. Citrate has also been shown to inhibit thrombin action on platelets (Shuman

Table V: Comparison of Activities

	thrombin[a]	thrombocytin	batroxobin[b]
platelet aggregation and release	+	+	−
clot retraction	+	+	−
clotting of fibrinogen	+	weak	+
activation of:			
factor XIII	+	+	−
factor VIII	+	weak	−
factor X	−	weak	−
factor V[c]	+	+	−
degradation of fibrinogen α chain	−	+	−
cleavage of prothrombin to intermediate 1	+	+	−
hydrolysis of arginine-*p*-nitroanilides	+	+	+
inhibition by:			
DFP	+	+	+
antithrombin III–heparin	+	+	−
hirudin	+	−	−
α_1-antitrypsin	+	−	−

[a] Lundblad et al. (1976); other literature data are reviewed in the introduction to this paper. [b] Stocker & Barlow (1976). [c] Rawala et al. (1978).

& Majerus, 1975). It is possible that other differences between plasma and the suspending medium for washed platelets may also contribute to the observed differences in sensitivity in the two systems.

A number of other proteolytic enzymes such as trypsin (Martin et al., 1975), papain, pronase (Davey & Lüscher, 1967), bovine pancreatic elastase (Kowalski et al., 1966), and plasmin (Niewiarowski et al., 1973) have been reported to cause platelet aggregation and the platelet release reaction; however, they all require much higher concentrations than does thrombin or thrombocytin. For instance, Martin et al. (1975) found that, for induction of platelet secretion, the optimum concentrations of thrombin, trypsin, and papain were 8.3×10^{-8}, 8.5×10^{-7}, and 6.5×10^{-6} M, respectively. Plasmin at concentrations greater than 10^{-6} M is also capable of causing platelet release (Niewiarowski et al., 1973). The action of trypsin and other broad spectrum proteases on platelets is not specific. These enzymes produce great alterations in platelet function by digesting several membrane glycoproteins (Phillips, 1972). This may render platelets resistant to stimulation by thrombin and may decrease thrombin binding to platelets (Ganguly, 1977).

Thrombocytin and thrombin may act on platelets by similar mechanisms. Both enzymes caused the release of serotonin, LA-PF_4, and β-*N*-acetylglucosaminidase, which are representative of the three types of platelet granules (Fukami et al., 1979). Moreover, the action of both thrombin and thrombocytin on platelets was inhibited completely by PGE_1 (10^{-5} M) and partially by antimycin–deoxyglucose. Indomethacin (10^{-4} M) did not inhibit release by either enzyme at the concentrations tested (Table II).

Thrombin and thrombocytin also seem to have similar mechanisms of action on some other macromolecular substrates. They both activate factor XIII, apparently by making very similar cleavages in the molecule. They both increase the procoagulant activity of factor VIII (although thrombocytin is much less active than thrombin in this system), and both cleave prothrombin in a similar manner and form a similar complex with antithrombin III. The affinity of thrombocytin for antithrombin III is much lower than the affinity of thrombin is, but in the presence of heparin this

affinity is greatly increased. Recently, Rawala et al. (1978) showed that thrombocytin can also activate factor V, although in a slightly different manner from thrombin. Thrombocytin, in contrast to thrombin, degrades the α chain of fibrinogen and causes a limited activation of factor X, in addition to another probably unrelated proteolytic cleavage in factor X. This factor X activating activity of thrombocytin is very weak, and the possibility cannot be excluded that it is due to a minor contamination with some other protease.

The most striking difference between thrombocytin and thrombin is that the specific fibrinogen-clotting activity of thrombocytin is less than 0.001 times that of thrombin. Concentrations of thrombocytin necessary to induce platelet aggregation and release do not clot fibrinogen during the few minutes required to study these platelet responses. This suggests that thrombocytin may be a very useful enzyme in studying thrombin-mediated responses of platelets in plasma or in a fibrinogen-containing system where the direct use of thrombin is precluded by the artifacts associated with fibrin generation.

Snake venoms contain a number of platelet-aggregating proteins. Davey & Lüscher (1965, 1967) suggested that the platelet-aggregating activity of certain snake venoms may result from the activation of factor X or prothrombin on the surface of platelets. Our unpublished experimental data suggest that this is the mechanism by which *E. carinatus* venom causes platelet aggregation and activates clot retraction. Davey & Esnouf (1969) isolated a high molecular weight protein–polysaccharide complex from the venoms of *Trimeresus okinavensis* and *Trimeresus purpureomaculata*. This complex was free of any enzymatic activity and it caused platelet aggregation. More recently, Schmaier et al. (1978) isolated a platelet-aggregating protein (M_r 83 000) from the venom of *Crotalus horridus* horridus. A number of the inhibitors of proteolytic enzymes did not interfere with the action of this protein on platelets. It appears that mechanisms of platelet activation by thrombocytin and thrombin are very similar but different from the mechanisms of platelet aggregation induced by other proteins from the snake venoms.

Acknowledgments

The authors thank Sha May Tang, Patricia Miller, Maxine Millman, Helena Yeh, and Tom Morinelli for their expert technical assistance and Dr. M. Fukami for determination of β-*N*-acetylglucosaminidase activity. Bovine blood for the purification of factors VIII and X and prothrombin was generously provided by the T. M. Landis Co., Mainland, PA.

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Purification of the Energy-Transducing Adenosine Triphosphatase Complex from *Rhodospirillum rubrum*†

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ABSTRACT: The oligomycin- and *N,N'*-dicyclohexylcarbodiimide-sensitive adenosine triphosphatase complex extracted with Triton X-100 from the chromatophores of *Rhodospirillum rubrum* was extensively purified. The purification procedure included (diethylamino)ethylcellulose chromatography and glycerol gradient centrifugation. The specific activity of Mg^{2+}-dependent ATP hydrolysis in the purified preparation increased about 11-fold, while that of Ca^{2+}-dependent ATP hydrolysis increased 50-fold as compared with chromatophores. The purified adenosine triphosphatase complex dissociated into a maximum of eight different polypeptides upon electrophoresis in the presence of sodium dodecyl sulfate. The estimated subunit molecular weights were as follows: 56 000 (α), 50 000 (β), 33 000 (γ), and those ranging from 17 000 to 9400 for the remaining smaller subunits. The purified preparation was incorporated into phospholipid vesicles by using the freeze–thaw technique. The reconstituted vesicles catalyzed [^{32}P]ATP exchange, which was almost completely inhibited by both oligomycin and *N,N'*-dicyclohexylcarbodiimide as well as by a protonophorous uncoupler, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

Proteolipid vesicles containing purified coupling factor ATPase complexes (CF_0–F_1)[1] seem to be very attractive models for studying mechanisms of oxidative- and photophosphorylation (Kagawa, 1972).

Numerous attempts have been undertaken in recent years to isolate and purify from various sources ATPase complexes capable of energy conversion.

Regarding mammalian mitochondria, a number of oligomycin-sensitive ATPase preparations containing 12–15 polypeptide bands on $NaDodSO_4$ gels have recently been described (Kagawa & Racker, 1966; Tzagoloff et al., 1968; Swanljung et al., 1973; Sadler et al., 1974; Serrano et al., 1976; Stigall et al., 1978; Berden & Voorn-Brouwer, 1978). Of those listed above, the preparation isolated by Serrano et al. (1976) seems to be most extensively studied with respect to energy-transfer activities. Vesicles, which catalyzed [^{32}P]ATP exchange and ATP-driven proton translocation, were reconstituted from the purified ATPase complex and phospholipids; in addition, site III phosphorylation was demonstrated when cytochrome oxidase was added to the reconstituted vesicles. However, these energy-transfer activities were assayed in the presence of purified F_1 and OSCP proteins added to the reaction mixtures.

More purified ATPase complexes, containing eight to ten polypeptides, were isolated from yeast mitochondria (Tzagoloff & Meagher, 1971; Ryrie, 1975a,b, 1977; Ryrie & Blackmore, 1976). One of these ATPase preparations (Ryrie, 1975b, 1977; Ryrie & Blackmore, 1976) was capable of energy-linked activities when incorporated into liposomes.

Highly purified DCCD-sensitive ATPase complexes have recently been isolated and extensively studied from aerobic thermophilic bacterium PS3 (Sone et al., 1975, 1977a,b) and from spinach chloroplasts (Pick & Racker, 1979). Both of these complexes dissociated into eight polypeptides on $NaDodSO_4$ gels and, after incorporation into phospholipid vesicles, were capable of ATP-driven proton translocation, [^{32}P]ATP exchange, and pH-jump phosphorylation. When bacteriorhodopsin was included in the vesicles, light-induced

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[1] Abbreviations used: F_0–F_1, energy-transducing ATPase complex sensitive to energy-transfer inhibitors, consists of water-soluble F_1 subunits and hydrophobic proton-translocating subunits (F_0); DCCD, *N,N'*-dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Tricine, tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; Triton X-100, octylphenoxypoly(ethoxyethanol); OSCP, oligomycin sensitivity conferring subunit of the ATPase complex.

0006-2960/79/0418-3577$01.00/0 © 1979 American Chemical Society