Studies on Human Platelet Proteins
II. Effect of Thrombin

By P. Ganguly

During the last ten years evidence has been presented from different laboratories that fibrinogen may not be essential for thrombin-mediated platelet aggregation. Although the presence, in platelets, of a thrombin-sensitive protein other than fibrinogen has been reported from this laboratory and also by other workers, there is no report of its isolation, structure, physicochemical properties, mechanism of action with thrombin or possible role in hemostasis. In this study, we report a high degree of resolution of platelet proteins by gel electrophoresis, evidence for more than one thrombin-labile protein other than fibrinogen, and some of the properties of one of these proteins.

Materials and Methods

Preparation of Platelet Extract

This has been described in detail earlier and was carried out at 0 to 4 C. Briefly, the platelets were washed twice with 1.0 per cent ammonium oxalate containing 0.1 per cent EDTA, pH 7.5, once with 0.2 M tris-HCl, pH 8.2, homogenized in a glass-Teflon homogenizer, and dialysed overnight against 0.2 M tris-HCl, pH 8.2. The homogenate was centrifuged in a Spinco model L centrifuge at 30,000 rpm. for 30 minutes to obtain the supernatant, which forms the platelet extract. The yield of protein in the extract, measured by the biuret method, was about 100 mg. per unit of blood.

Electrophoresis

Vertical starch gel electrophoresis was performed in borate buffer, pH 8.6 at a constant voltage of 220 v following the method of Smithies. The stains were amido black, sudan black, oil red 0 or iodine. Starch gel immunoelectrophoresis was performed as described by Poulak and disc electrophoresis according to the method of Davis. For immunoelectrophoresis and double diffusion studies, undiluted anti-sera (Behringwerke) were employed.

Thrombin Studies

Human thrombin, a gift from Dr. W. L. Westcott, Squibb Institute for Medical Research, New Brunswick, New Jersey, was dissolved in saline or saline buffered with 0.1 M tris-HCl, pH 7.5. The isolation and stability of this thrombin has been reported. It had no plasmin, plasminogen or plasminogen activator associated with it. Thrombin (0.05 ml; 50 units) or an equal volume of buffer was added to 1 ml. platelet extract. After one hour in the cold, the thrombin-treated extract showed a firm clot which was lightly squeezed with a smooth glass rod before centrifuging the tubes at 10,000 rpm. for 15 minutes. The supernatant solutions were collected and analysed. In gel electrophoresis, the same amount of sample was applied to each tube or slot.

From Blood Research Laboratory, The American National Red Cross, Bethesda, Maryland, 20014.
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P. Ganguly, Ph.D.: Research Scientist, Blood Research Laboratory, American Red Cross.
Isolation of the Thrombin-Sensitive Protein

The platelet extract was fractionated on a Sephadex G-200 column (2.5 × 90 cm.) equilibrated with 1.0 M NaCl buffered with 0.1 M tris-HCl, pH 7.6. The tubes were read at 280 mμ. Four partially resolved peaks were obtained. The four fractions, as shown in Figure 1, were assayed by electrophoretic and by immunologic methods before and after thrombin treatment. The thrombin-sensitive protein was in the second fraction, the tubes of which were pooled and the volume reduced approximately 10 fold by ultrafiltration. The concentrated fraction II was dialysed at 4 C. for 2 hours against distilled water and then against 0.1 M NaCl, pH 6.0. The dialysis bag was examined from time to time. After about 6 hours, the solution in the bag became cloudy. The bag was removed, the turbid material was centrifuged off and the pH was raised to 7.5 with NaOH. The solution was lyophilized. The freeze-dried material was reconstituted in a small volume of 0.2 M tris-HCl, pH 7.2 and fractionated on a narrow column (1.2 × 70 cm.) of Sephadex G-100. The thrombin-labile protein is excluded from this column and the impurities are included (Fig. 2). The excluded peak was pooled, dialysed overnight against distilled water and lyophilized.

Sedimentation Analysis

Sedimentation studies were carried out in a Spinco model E ultracentrifuge at 20 C. The position of the boundaries was measured directly from the photographic negatives. The values reported in Svedberg units are apparent and approximate values.

Tests for Coagulation Factors

Fibrin-stabilizing factor was assayed according to the method of Kiesselbach and Wagen16 except that, instead of bovine serum albumin, 0.5 per cent human serum albumin in isotonic saline containing 0.005 M sodium citrate was used as the diluent. The test system contained: 1.0 ml. of FSF-free fibrinogen at a concentration of 50 mg./100 ml.,
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Fig. 2.—Gel filtration pattern of fraction II after precipitation, in Sephadex G-100 eluted with 0.1 M tris-HCl plus 1.0 M NaCl, pH 7.5. Each fraction represents 1.3 ml.

0.2 ml. of the test material in serial dilutions, 0.1 ml. of 0.125 M CaCl₂, and 0.1 ml. of 100 u/ml. thrombin. After 30 minutes at 37 C., 1.4 ml. of 2 per cent monochboroacetic acid was added. The stability or dissolution of the clot indicated the presence or absence of FSF. Factor V was assayed as described by Denson, and platelet factor III by the method of Proctor and Rappaport.¹⁷,¹⁸

RESULTS

Figure 3 shows the starch gel electrophoretic pattern of the platelet extract before and after treatment with thrombin. Excellent resolution in the postalbumin region was achieved. Channels 1 and 8 contained thrombin and did not show any band at these concentrations. In the pattern for the platelet extract about 15 bands were observed, 3 of which migrated towards the cathode.

Comparison between the control and thrombin-treated platelet extracts revealed that three prominent bands (indicated by arrows at the top of the figure) disappeared from the platelet extract following addition of thrombin. At this pH, two of these bands migrated towards the anode while the other migrated towards the cathode. In starch gel immunoelectrophoresis, a precipitin line with anti-fibrinogen was obtained only at the origin. Fibrinogen could also be detected by immunodiffusion showing that although it was present in the platelet extract, it did not enter the gel. New bands also appeared following treatment of the platelet extract with thrombin. These bands are indicated by arrows at the bottom of the figure.

The starch gel electrophoretic pattern of the purified thrombin-sensitive protein showed primarily one component in the beta-globulin region. This band did not stain with sudan black, oil red 0 or iodine. Starch gel immunoelectrophoresis with anti-whole serum or anti-fibrinogen did not show any precipitin line corresponding to this band. The disc electrophoretic pattern of this protein before and after thrombin treatment is shown in Figure 4. The patterns show one prominent band with traces of impurities. Fibrinogen migrated only about one millimeter from the origin when run under the same conditions. Densitometer analysis (Figure 5) showed that thrombin produced
a considerable decrease in the amount of the main band, but hardly altered the height of the shoulder which followed it. Thrombin also reduced the amount of material near the origin; this may be fibrinogen. When the partially purified thrombin-labile protein was analysed in the ultracentrifuge, there was one prominent peak with an approximate sedimentation coefficient of 6.5s and a lighter peak due to impurities.

A stock solution of thrombin-sensitive protein (4 mg./ml.) was used for assay of the different clotting factors. Factor V assay was done at 5 × dilution with saline and gave negative results. Tests of the recalcified clotting time in the presence of kaolin in dilutions from 1:100 to 1:800 was performed. Using Bell and Alton phospholipid, the clotting time was 42 seconds, with all dilutions of the thrombin-labile material it was between 90 and 100 seconds. Assays for FSF were carried out at dilutions from 1:10 to 1:80 of the stock material. At all concentrations, even after cysteine inactivation, the clots lysed with monochloracetic acid while plasma samples did not.

**DISCUSSION**

The role of thrombin and platelets in hemostasis is well-established. Thrombin causes platelets to undergo profound changes—they aggregate, contract, and release serotonin, nucleotides, potassium and other materials. Although some workers believe that fibrinogen on the platelet membrane formed the
Fig. 4.—Disc electrophoretic pattern of the partially purified thrombin-sensitive protein. (A) Before thrombin treatment, (B) After thrombin treatment.

Fig. 5.—(A) Densitometer tracing of the disc electrophoresis pattern of the thrombin-sensitive protein shown in Figure 4(A).
substrate for the action of thrombin, recent evidence indicates that fibrinogen is not essential for thrombin-induced aggregation of platelets.

A considerable number of reports have appeared on the presence of a thrombin-labile protein, besides fibrinogen, unique to platelets. These studies report results which are not always consistent with each other. In gel electrophoresis, Salmon and Bounameaux noted a thrombin-labile protein, besides fibrinogen, in the α-globulin region while Davey and Luscher noted a reduction in the anodal mobility of the albumin-like fractions, inconstant alterations in the number and position of the anodal globulin-like fractions and one inconspicuous fraction on the cathodal side was sometimes not detected. In immunoelectrophoresis, the cathodal shift of a protein component when treated with thrombin has been observed. Nachman's immunodiffusion studies revealed a thrombin-sensitive protein which became thrombin-resistant in the congenital platelet defect thrombasthenia. Caen et al. and Weiss and Kochwa have failed to confirm this latter observation. A reduction in amount and an increase in the anodal mobility of the protein when treated with thrombin was noted. A thrombin-sensitive protein in human blood platelets has recently been identified as fibrin-stabilizing factor. The presence of a thrombin-sensitive protein has also been reported from this laboratory. Thus some workers observed complete disappearance of a band, some observed a diminution in amount concomitant with a cathodal or anodal shift in electrophoretic mobility.

The above studies were carried out on platelets from different sources, processed by different methods and studied by different technics. This may partly explain the variation in the data reported. In gel electrophoresis of different preparations of the platelet extract, before and after thrombin-
treatment, we have also noted some variation in the results obtained. In some cases, a single band disappeared completely,5 while in other cases several bands were affected with one prominent band showing a diminution in amount and a change in the electrophoretic mobility due to the effects of thrombin. The complexity in the results is obviously in part due to lack of a clear definition of “thrombin-sensitivity” of the platelet proteins. Davey and Luscher in their study on the action of coagulant and proteolytic enzymes on platelets observed that thrombin brings about platelet metamorphosis by acting on a substrate other than fibrinogen. In their own words “these results make it impossible to maintain that the enzyme action of thrombin on fibrinogen is also responsible for its effect on platelets.”4 It is known that thrombin is capable of hydrolysing peptide bonds like arginine-isoleucine, lysine-alanine or lysine-isoleucine besides arginine-glycine as in fibrinogen.23,24 Further, there are a considerable number of thrombin-sensitive coagulation factors present in platelets.19 Thus there are a large number of alternative possibilities by which thrombin may react and alter the characteristics of a protein in a complex mixture like the platelet extract. However, sensitivity to thrombin, which is a proteolytic enzyme, does not necessarily mean that that component is involved in hemostasis. The other interesting question is whether the non-fibrinogen thrombin-sensitive protein observed by different workers is one and the same. In view of the results of the present study, it appears that the thrombin-labile components observed by different workers might have been different.

Finally, the question arises about the identity of the thrombin-sensitive protein that have been partially purified in this study. Although FSF and factor V are thrombin-labile proteins present in platelets, the protein we have studied does not contain these clotting factors. The results obtained do not as yet throw light as to its possible identity or mechanism of action with thrombin. These questions must await further purification and structural studies of the protein.

Summary

The properties of proteins from blood platelets soluble in 0.2 M tris-HCl, pH 8.2 have been studied and more than one thrombin-sensitive protein detected by gel electrophoresis. The most prominent effect of thrombin is on a protein migrating in the beta-globulin region which shows a diminution in amount and a change in the electrophoretic mobility. This protein has been partially purified. The protein fails to produce a precipitin line against anti-whole serum on immunoelectrophoresis or against anti-fibrinogen in starch gel immunoelectrophoresis. It does not contain fibrin-stabilizing factor, factor V or platelet factor III.

SUMMARIO IN INTERLINGUA

Le proprietates de proteinas thrombocytic solubile in 0.2 M tris-HCl a pH 8.2 esseva studiate e plus que un thrombino-sensibile proteina esseva detegite per electrophorese a gel. Le plus promimente effecto de thrombina es a notar in un proteina que migra in le region beta-globulinic e que monstra un diminution in quantitate si ben como un alteration
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in le mobilitate electrophoretic. Iste proteina ha essite partially purificate. Le proteina non produce un linea de precipitina contra antisero integre in le immuno electrophorese o contra antifibrinogeno in immunoelectrophorese a gel de amyl. Illo non contine factor fibrino-stabilisatori, factor V, o factor plachettal III.

ACKNOWLEDGMENTS

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