Isolation and Properties of a Thrombin-sensitive Protein of Human Platelets*

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SUMMARY

A glycoprotein of human platelets is released into the incubation medium upon treatment of the intact cells with 1 unit per ml of thrombin for 2 min. The cellular location and properties of this protein differ from those of other thrombin-labile platelet proteins such as fibrin-stabilizing factor, fibrinogen, and the contractile protein thrombosthenin. It occurs only in the particulate fraction and is released only from intact cells. Thrombin concentrations of 100 units per ml do not release this thrombin-sensitive protein from sonicated platelets. As isolated from the incubation supernatant of thrombin-treated platelets the thrombin-sensitive protein is excluded from Sephadex G-200; it has an apparent molecular weight of 190,000 in sodium dodecyl sulfate polyacrylamide gels, from which it has been purified and partially characterized. No smaller subunits have been found. It has limited solubility in the absence of detergent, and is highly sensitive to proteolytic degradation in whole platelets and after release from the cells.

Membrane-bound and released forms of the thrombin-sensitive protein show no significant difference in molecular weight or amino acid and carbohydrate composition, suggesting that release does not involve proteolysis of the thrombin-sensitive protein itself by thrombin. This protein contains 3.9% sialic acid, 1.6% N-acetylgalactosamine, and 25% each of acidic and nonpolar residues. Although it contains carbohydrate the thrombin-sensitive protein does not represent the major membrane glycoprotein of platelets, for it contains only 5% of the total platelet sialic acid. The thrombin-sensitive protein accounts for approximately 5% of the total protein in the particulate fraction of human platelets and represents about 20 to 30% of the protein found in the incubation supernatant after thrombin treatment.

EXPERIMENTAL PROCEDURE

Materials

Sodium dodecyl sulfate obtained from Fisher Scientific was recrystallized by the method of Burgess (13). Human thrombin, 5000 units per mg, was obtained from Dr. Kent Miller and Dr.
David Aaronson and was assayed as described previously (14). It is free of plasmin, plasminogen, and Factor Xa activities.1 Canine reagents and apparatus were used for acrylamide gel electrophoresis. 1-Dimethylaminomorpholine, sodium fluoride (dansyl chloride, B grade) was from Calbiochem and 1-fluoro-2,4-dinitrofluorophosphoric acid (Dansyl chloride, B grade) from New England Nuclear. Eastman silicagel chromatograms or polyamide layer plates from Chen Chin Trading Co., Ltd., Taiwan, were used in thin layer chromatography. Phenethylmethyl sulfonyl fluoride, DFP, TLCK, and N-acetylneuraminic acid were obtained from Sigma.2 Erythrocyte-agglutinating phytohemagglutinin was isolated from Difco phytohemagglutinin P by the method of Weber et al. (15) and from lentil by the method of Howard and Sage (16).

Methods

Platelet Preparation—Platelet isolation and washing were carried out at room temperature. Fresh human platelet-rich plasma, prepared at room temperature by the American Red Cross, St. Louis Chapter, was centrifuged 25 min at 100 x g to sediment the platelets, which were washed twice in 10 to 15 volumes of pH 6.5 buffer containing 0.1% NaCl, 0.003% K3HPO4, 0.005% Na2HPO4, 0.032% NaH2PO4, and 0.0055% glucose. Platelets were incubated at 37° with shaking for 4 to 5 min in the absence or presence of 1 unit per ml (0.2 μg per ml) of human thrombin at 1.5 x 106 cells per ml. Incubation buffer contained 0.1% NaCl, 0.015% Triton, pH 7.4, and 0.005% glucose. The incubation mixtures were centrifuged for 10 min at 7,000 x g at 4°C, and the supernatant solutions were resuspended 1 min at 48,000 x g to remove residual platelets; then boiled 10 min and chilled immediately. Following overnight dialysis against distilled water the incubation supernatants were lyophillized. Platelet pellets from the 7,000 x g centrifugation were resuspended immediately in the above buffer at the original cell concentration, sonicated 15 to 25 sec at 70% intensity with a Biosonik sonifier, and centrifuged 30 min at 48,000 x g. The particulate fraction thus isolated was stored at -20°C.

SDS Solubilization and Gel Electrophoresis—The platelet particulate fractions were suspended by mild homogenization in a Potter-Elvehjem homogenizer in distilled water, assayed for protein, and the suspension immediately diluted with a phosphate buffer, SDS stock solution to a final concentration of 3% w/v SDS-0.1 M sodium phosphate, pH 7.4-1% 2-mercaptoethanol (17). The particulate fraction was solubilized at room temperature overnight. Glycerol, 20%, and N,N,N',N'-tetramethyl-ethylenediamine; dansyl chloride, 1-dimethylaminomorpholine, sodium fluoride; DNP, 2,4-dinitrophenol.

For preparative electrophoreses up to 1.2 mg of particulate fraction or the incubation supernatant protein from 0.7 to 1 x 108 platelets was applied to each gel, and the section of gel known to contain the desired protein was cut immediately after electrophoresis. The 8-mm slices were minced into smaller pieces, homogenized in a Potter-Elvehjem homogenizer in 1 ml of 3% SDS-0.1 M sodium phosphate, pH 7.4-1% 2-mercaptoethanol per four slices, and dialyzed against the same buffer overnight at room temperature. After centrifugation of the homogenate for 15 min at 20,000 x g at 20°C, the eluted protein was recovered in the supernatant. Recovery was quantitated by comparative densitometric scanning of SDS gels of the eluted protein and of incubation supernatant from a known number of thrombin-treated platelets.

Amino Acid and Amino Sugar Analyses—Thrombin-sensitive protein isolated by the above elution procedure was precipitated from the eluate by treating with an equal volume of 20% trichloroacetic acid for 30 min at room temperature, followed by freezing for several hours and rethawing. After centrifugation for 30 min at 20,000 x g at 20°C, the protein pellet was taken up in 1 to 2 ml of 0.5 M NaHCO3 and dialyzed against 2 liters of the same buffer to remove residual SDS, followed by dialysis against distilled water. The protein sample, 0.1 to 0.15 mg, was then lyophillized, hydrolyzed in vacuo at 105°C for 22 hours in 1 ml of constant boiling HCl, and the hydrolysate analyzed on a Beckman model 120 C amino acid analyzer. Methionine and cysteine were determined as methionine sulfoxone and cysteic acid after performic acid oxidation (18).

Stable Acid Assay—Stable acid was hydrolyzed from proteins by boiling samples 1 min in 1 N HCl and was assayed by a modification of the method of Warren (19), halving the reaction volume and extracting with 0.75 ml of cyclohexanone for 0 to 10 minutes and 1.5 ml for 10 to 70 minutes of stable acid. Eluted protein samples were dialyzed exhaustively against 0.5 M NaHCO3, followed by distilled water, to remove the bulk of SDS so that...
samples could be concentrated easily for the sialic acid assay. Trichloroacetic acid precipitation was not performed on samples taken for sialic acid assay to avoid potential hydrolysis. Residual SDS was assayed by the method of Reynolds and Tanford (20). An identical concentration of SDS was added to the sialic acid standards, since high levels of SDS raise the sample absorbance at 534 nm slightly.

**Amino-terminal Analysis—Dinitrophenylation of eluted particulate fraction thrombin-sensitive protein was done according to the method of Rosenberg and Guidotti (21), with 500 μCl of 1-fluoro-2,4-dinitrophenyl fluoride and 35 μl of unlabeled 1-fluoro-2,4-dinitrobenzene (100 μg per ml in absolute ethanol) added to 200 μg of protein in 0.2 ml of 1% SDS-0.02 M NaHCO₃. Dansylation of eluted particulate and released thrombin-sensitive protein was carried out by modification of the method of Gray (22), in which 100 to 300 μg of protein in 1 to 1.4 ml of 3% SDS-0.5 M NaHCO₃ was stirred overnight in the dark with 0.5 to 1 ml of danyl chloride, 20 mg per ml in acetone. The modified protein was dialyzed against 0.5 M NaHCO₃ and then distilled water to remove SDS; in some experiments it was precipitated out of the reaction mixture in 10% trichloroacetic acid before dialysis. After 16 to 18 hours of hydrolysis in vacuo at 105° in 5.5 M HCl, the dansyl or the ether-extractable DNP-amino acids were chromatographed by the method of Hartley (23), and of Brenner et al. (24), or Wang and Wang (25), respectively. Water-soluble DNP-amino acids were not examined.

**Column Chromatography—Crude incubation supernatant from thrombin-treated platelets was incubated with phenylmethyl sulfonyl fluoride (0.4 mM) or TLCK (40 pg per ml) at 4°, or with thrombin-treated platelets was incubated with phenylmethyl sulfonyl fluoride-treated supernatants from 13 to 25 x 10⁹ platelets. Dansylation of eluted particulate and released thrombin-sensitive protein was done according to the method of Rosenberg and Guidotti (21), with 500 PCi of 32P-orthophosphate, specific activity 2.5 x 10⁸ Ci/m mole, added to 2 ml of 3% SDS-0.5 M NaHCO₃ and then distilled water to remove SDS; in some experiments it was precipitated out of the reaction mixture in 10% trichloroacetic acid before dialysis. After 16 to 18 hours of hydrolysis in vacuo at 105° in 5.5 M HCl, the dansyl or the ether-extractable DNP-amino acids were chromatographed by the method of Hartley (23), and of Brenner et al. (24), or Wang and Wang (25), respectively. Water-soluble DNP-amino acids were not examined.

**Release of Platelet Protein by Thrombin—Our previous studies have shown that a major platelet protein with a molecular weight of 190,000, as analyzed by SDS-polyacrylamide gel electrophoresis, completely disappears from the cells within 15 sec to 2 min after the addition of 1 unit per ml of thrombin to whole platelets. Disrupted cells or the extracted protein itself are not affected by thrombin under these conditions, even after incubation times up to 30 min. We have referred to this protein as the thrombin-sensitive protein. It occurs exclusively in the particulate fraction of disrupted platelets, and remains in the 48,000 × g pellet through five washes of the particulate fraction. It is not found in the soluble fraction of disrupted platelets, nor in plasma (12). Further there is no protein contained in erythrocyte membranes corresponding to this protein. Electron micrographs of this 48,000 × g particulate fraction have shown it to consist of membrane vesicles, a few intact and many disrupted granules. This fraction contains all of the platelet adenylate cyclase activity, as well as other enzymes associated with cell surface membranes (11). Attempts to localize the thrombin-sensitive protein to the surface membrane fraction of platelets with the platelet membrane fractionation procedure of Barber and Jamieson (32) were unsuccessful due to extensive proteolysis of this protein which occurred during the fractionation procedure. In experiments where sonication of platelets for varying lengths of time were performed it was shown that when greater than 99% of β-glucuronidase was solubilized the thrombin-sensitive protein remained in the particulate fraction. Thus while it is not established that this protein is a component of the surface membrane it does appear to be tightly associated with some platelet memranous structure.

**SDS-acrylamide gel patterns of proteins appearing in the control particulate fraction and the incubation supernatants from control and thrombin-treated human platelets are shown in Fig. 1. Control incubation supernatants contain 0.03 to 0.1 mg of protein, and thrombin supernatants 0.25 to 0.4 mg of protein per 10⁹ platelets. No change in the electrophoretic mobility of the thrombin-sensitive protein in SDS gels is detectable upon release, so that this process is assumed not to involve any sig-
Note—Gels in all figures are lettered a, b, etc. from lower to upper.

**FIG. 1.** Release of platelet proteins into the incubation medium by thrombin. SDS-polyacrylamide gels contain a, 230 μg of platelet particulate protein, derived from 0.23 × 10⁹ platelets; b, incubation supernatant from 0.96 × 10⁹ control platelets; c, incubation supernatant from 0.23 × 10⁹ thrombin-treated platelets.

**FIG. 2.** SDS gels showing results of chromatography on Sephadex G-200 of incubation supernatant from thrombin-treated platelets. SDS gels contain a, crude incubation supernatant from 0.9 × 10⁹ thrombin-treated platelets; and b, protein from void volume peak from Sephadex G-200 chromatography of concentrated incubation supernatant from 10 × 10⁹ platelets. This gel contains 9% of the total protein peak and contains the thrombin-sensitive protein from an estimated 0.45 × 10⁹ platelets, indicating 39% recovery. No other bands were seen in the other broken half of Gel b. Note a faint band of degraded protein adjacent to the major band.

**FIG. 3.** Solubilization of platelet particulate fraction under various conditions. All preparations contained 3% SDS and 1% β-mercaptoethanol and a, 0.1 M sodium phosphate, pH 7.4; b, 0.05 M sodium phosphate, pH 7.4; c, no buffer; d, 0.1 M sodium phosphate, pH 7.4, and 4.3 M urea. SDS Gels a to c contained 230 μg of protein and Gel d, 280 μg. The arrow denotes the thrombin-sensitive protein.

**FIG. 4.** The effect of Na₂EDTA and storage in water on the SDS-polyacrylamide gel pattern of platelet particulate fraction. a, particulate fraction solubilized as under "Methods," 230 μg of protein; b, particulate fraction dialyzed 24 hours at 4° against 5 mM EDTA-5 mM β-mercaptoethanol, pH 7.0, then made 1% in SDS, boiled 3 min, and 230 μg applied directly to the gel; c, particulate fraction suspended in distilled water and stored overnight at 4°, then made 1% in SDS, boiled 3 min, and 230 μg applied directly to the gel.

**FIG. 5.** SDS gels showing degradation of the thrombin-sensitive protein under various conditions. a, incubation supernatant from 0.6 × 10⁹ thrombin-treated platelets, stored 2 days at room temperature under toluene, after addition of DFP to 1 mM final concentration; b, incubation supernatant from 0.5 × 10⁹ platelets, under the same conditions as Gel a, after 5 days at room temperature; c, incubation supernatant from 0.6 × 10⁹ platelets, untreated with DFP, stored 2 days at room temperature under toluene.

**FIG. 6.** Purification of thrombin-sensitive protein by elution from SDS-polyacrylamide gels. a, incubation supernatant from 0.17 × 10⁹ thrombin-treated platelets; b, eluted protein derived from the incubation supernatant of thrombin-treated platelets ("released" thrombin-sensitive protein); c, eluted thrombin-sensitive protein derived from the particulate fraction of control platelets. Gels b and c contain 2% and 1.3% of the respective total eluates. (20-cm gels have been cut in half for densitometry.)
significant change in molecular weight. However, a small molecular weight change occurring if the protein were cleaved from the platelet surface, leaving behind a peptide fragment, might not be detectable by this method.

In addition to the thrombin-sensitive protein, an SDS polyacrylamide gel of the incubation supernatant from thrombin-treated platelets contains another dense band at an apparent molecular weight of 68,000. This protein may be albumin, which Davey and Löscher (9) have previously found to be released from platelets by thrombin.

A number of other proteins bands are present in SDS gels of incubation supernatants from both control and thrombin-treated platelets. Among them are three bands at molecular weights of 60,000, 52,000, and 44,000. Other faint bands at molecular weights of 174,000, 156,000, 124,000, and 104,000 also occur in both supernatants. None of these proteins have been identified. A faint band in Gel b of Fig. 1, at the position of the thrombin-sensitive protein in the adjacent gels, indicates the release of some protein in a control incubation supernatant. The amount of this protein seen in control supernatants varies from none at all to about 5% of that seen in incubation medium from thrombin-treated platelets. Its presence may stem from the reported prothrombin activation on the surface of platelets, possibly during isolation and washing (33). Gel b in Fig. 1 contains the incubation supernatant from 3-fold more control platelets than Gel c does from thrombin-treated platelets. The quantity of thrombin-sensitive protein in Gel b is negligible compared to that of Gel c, and the other bands, with the exception of that at 68,000 molecular weight, appear of approximately the same density in both gels, suggesting that these proteins are increased 2- to 3-fold in a thrombin supernatant.

Size of Thrombin-sensitive Protein—When incubation supernatant from thrombin-treated platelets is applied to a Sephadex G-200 column in the absence of SDS, the thrombin-sensitive protein appears in the void volume (Fig. 2). In this state it probably consists of more than one 190,000 molecular weight unit, as a protein of one chain that size should be retarded on the column to some degree. When chromatography on G-200 is performed in SDS, this protein is retarded as shown below. Vigorous efforts to break down the apparent 190,000 molecular weight chain into smaller subunits have been unsuccessful. The following procedures have failed: (a) boiling the membrane fraction in the SDS solubilization medium, either before or after overnight solubilization at room temperature, and SDS solubilization (b) at lower ionic strength or (c) in the presence of 4.3 M urea. The thrombin-sensitive protein band is equally sharp in SDS gels of particulate fraction preparations solubilized in the regular buffer and at lower ionic strength (Fig. 3); smearing out of the band might be expected if the latter treatment were disaggregating the protein to subunits which then reaggregated in the higher ionic strength of gels and buffer during electrophoresis.

Because EDTA has been reported necessary for disaggregation of erythrocyte membrane proteins, the platelet particulate fraction has been solubilized by the method of Lenard (34). Overnight dialysis of particulate fraction against 5 mM Na2-EDTA-5 mM 2-mercaptoethanol at pH 7.0 prior to SDS-polyacrylamide gel electrophoresis results in disappearance of most of the thrombin-sensitive protein band. No new protein bands indicative of subunits are seen farther along the gel (Fig. 4). However, since overnight storage of the particulate fraction in distilled water at 4° prior to SDS-gel electrophoresis gives the same result as EDTA treatment (Fig. 4), and boiling the particulate fraction in distilled water for 10 min before the overnight dialysis against EDTA-2-mercaptoethanol results in little or no decrease of this protein in the SDS-gel pattern of the particulate fraction, the disappearance of thrombin sensitive protein from these preparations may be due to proteolysis rather than to EDTA-mediated disaggregation. We conclude from the foregoing experiments that the 190,000 molecular weight band on SDS gels represents either a single polypeptide chain or an aggregate held together by forces insensitive to the above disruptive techniques.

Sensitivity to Proteolysis—The thrombin-sensitive protein, both within the platelet and after release by thrombin, exhibits a marked sensitivity to proteolysis. When whole platelets are stored at 20°, little or none of this protein can be found in the subsequently prepared particulate fraction. A degradation product of the thrombin-sensitive protein appears in the incubation supernatant from thrombin-treated platelets with time. The experiment shown in Fig. 5 shows the degradation of the released protein as a function of storage conditions. Thrombin inhibitors such as TLCK, phenylmethyl sulfonyl fluoride, PMSF, and DFP decrease but do not completely halt this process. Boiling the incubation supernatant immediately after isolation prevents degradation of the protein. This breakdown may be due to thrombin or to some nontrombin proteolytic activity in whole platelets or in the incubation supernatant from thrombin-treated platelets. The protein appears to contain certain sites particularly susceptible to proteolysis, since it is first converted to a product of molecular weight 170,000.

Solubility and Purification—Thrombin-sensitive protein released by thrombin partially precipitates if the incubation supernatant is chilled or concentrated, or its ionic strength decreased; storing and handling it at room temperature causes less precipitation than at 4°. Attempts to purify this protein by standard methods of chromatography were abandoned due to the above difficulties. Elution from SDS-polyacrylamide gels (Fig. 6) proved to be a successful means of purifying this protein from the incubation supernatant of thrombin-treated platelets (“released” thrombin-sensitive protein) and that from the particulate fraction of control platelets (“particulate” thrombin-sensitive protein) in 25 to 40% yield, for purposes of comparing the two proteins. Caution was necessary in cutting the bands out of gels to avoid contamination with adjacent regions on gels, especially those in the particulate fraction moving directly ahead of the 190,000 molecular weight band, which stain poorly with Coomassie brilliant blue and contain much carbohydrate.

Glycoprotein Nature of Thrombin-sensitive Protein—Thrombin treatment of whole platelets releases 27% of the total platelet sialic acid into the incubation medium, averaging 6.5 mmoles of sialic acid released per 10° platelets. This incubation supernatant also contains a potent inhibitor of phytohemagglutinin-induced human erythrocyte agglutination, at a level of 1000 to 1200 inhibitory units per mg of protein by the assay of Kornfeld and Kornfeld (35). The phytohemagglutinin-inhibitory activity and a peak of sialic acid appear in the Sephadex G-200 void volume containing the thrombin-sensitive protein. Sialic acid determination on released and membrane protein eluted from SDS gels gave values of 22 residues per 190,000 g of protein for the former and 25 for the latter. Determination of amino sugar content indicated 16 residues of glucosamine per 190,000 g of protein for the released protein and 13 for the particulate protein. Identical recoveries of glucosamine were obtained from 4- and
Comparison of amino acid compositions of particulate and released thrombin-sensitive protein with other platelet proteins

The number of residues per 190,000 molecular weight chain were determined by calculating the total micrograms of amino acid in the hydrolysate and converting moles of each amino acid to micromoles per 190,000 micrograms. The contributions of tryptophan, sialic acid, and hexose to the total molecular weight were not included in the calculations.

| Amino acid | Thrombin-sensitive protein<sup>a</sup> | Human α-<sup>b</sup> | "Throm-<sup>c</sup> | Platelet Myosin<sup>d</sup> |
|------------|----------------------------------|----------------|----------------|------------------|
|            | Released<sup>e</sup> | Particulate<sup>f</sup> | Mucin<sup>g</sup>| Mucin<sup>h</sup> |
|            | residues/190,000 g protein | | | |
| Lysine     | 93 | 90 | 125 | 99 | 152 |
| Histidine  | 33 | 34 | 35 | 30 | 27 |
| Arginine   | 69 | 69 | 88 | 82 | 84 |
| Aspartic acid | 270 | 251 | 211 | 162 | 160 |
| Threonine  | 91 | 91 | 97 | 93 | 74 |
| Serine     | 185 | 185 | 122 | 127 | 44 |
| Glutamic acid | 230 | 227 | 188 | 184 | 342 |
| Proline    | 101 | 99 | 81 | 97 | 44 |
| Glycine    | 230 | 241 | 102 | 135 | 101 |
| Alanine    | 115 | 115 | 87 | 78 | 146 |
| Cystine    | 38 | 15 | 39 | 46 | 15 |
| Methionine | 15 | 16 | 39 | 30 | 42 |
| Valine     | 100 | 102 | 70 | 88 | 70 |
| Isoleucine | 50 | 59 | 68 | 66 | 65 |
| Leucine    | 91 | 94 | 97 | 110 | 182 |
| Phenylalanine | 41 | 52 | 54 | 46 | 34 |
| Tyrosine   | 32 | 44 | 57 | 46 | 50 |
| Glucosamine | 16 | 13 | | |

<sup>a</sup> Values averaged from two separate analyses.
<sup>b</sup> Data from McKee et al. (36).
<sup>c</sup> Data from Cohen et al. (30).
<sup>d</sup> Data from Booyse et al. (37).

22-hour hydrolysates of the protein in constant boiling HCl, so that separate 4-hour amino sugar hydrolyses were not used further and glycosamine content was determined from the 22-hour amino acid hydrolyses. There was insufficient material for determination of the neutral sugar content of the thrombin-sensitive protein.

Amino Acid Composition—Table I compares the amino acid compositions of released and particulate protein. The compositions of both proteins are very similar, the values for most residues agreeing within 11%. Differences outside this range, the discrepancy in tyrosine, cysteine, phenylalanine, and glucosamine content, may reflect differential losses during amino acid analysis of very small samples rather than true differences in composition of the protein from the two sources. Amide content was not determined. The thrombin sensitive protein is striking in its content of acidic residues, aspartic and glutamic acid constituting 27% of the total residues. Of the residues, 438 out of 1815 or 24% are nonpolar, including alanine, valine, methionine, isoleucine, leucine, and phenylalanine in the particulate protein, and 23% of the protein residues are nonpolar in the released protein.

No amino-terminal amino acid could be found in the protein. With techniques sensitive enough to detect 1 amino-terminal residue per mole of protein, no dansyl amino acid could be detected in either particulate or released protein, and dinitrophenylation of 1 mnmole of particulate protein with 500 µCi of 1-fluoro-2,4-dinitro[β14C]benzoate (26 µCi per mole) yield approximately 140 mCi of ether-soluble radioactivity which did not cochromatograph with any known ether-soluble DNP-amino acids. An amino acid which forms a water-soluble DNP derivative has not been excluded, although no dansyl amino acid corresponding to those forming water-soluble DNP derivatives was detected.

Isolation of Released Thrombin-sensitive Protein by Chromatography on Sephadex G-200 in SDS—While released and particulate thrombin-sensitive protein isolated by elution from SDS gels appeared to be homogeneous by SDS gels, no independent criteria of purity were available. Further, the amounts of material obtainable with this technique were very small. We thus attempted to isolate released thrombin-sensitive protein in larger quantities utilizing gel filtration on Sephadex G-200 in SDS as described under "Methods." With this method 106 mg of thrombin-released protein were fractionated as shown in Fig. 7A. While the major proteins of the post-thrombin supernatant are not as well separated here as on SDS gel, it is clear that thrombin-sensitive protein is separated from other glycoprotein material. The fractions containing this protein (75 to 94) (26 mg) were pooled, concentrated, and reapplied to the G-200 column (Fig. 7B). After this second chromatography on SDS Sephadex G-200, there was correspondence between sialic acid and thrombin-sensitive protein content with a constant ratio of sialic acid to thrombin-sensitive protein throughout the peak. This suggests that this protein is truly a glycoprotein, a conclusion that is further supported by experiments where fractions from both the front and rear of the protein peak were rechromatographed on SDS G-200 with identical positions of protein elution and sialic acid content. Amino acid analysis of protein isolated in this manner agreed within 10% with results presented in Table I, and the sialic acid content of the protein isolated by gel filtration ranged from 25 to 30 residues per mole which also agrees with the above data on protein eluted from gels. While this technique for protein isolation is quite similar in principle to purification on SDS gels, the apparent identity of the product isolated by the two methods further supports the hypothesis that the thrombin-sensitive protein is a single protein which is released relatively intact after thrombin treatment.

As an independent criterion of purity, an antibody to the protein purified by Sephadex chromatography in SDS was produced in rabbits. Immunodiffusion of this antibody against crude incubation supernatant from thrombin-treated platelets gave a single precipitin line, which was a line of identity with the purified antigen.

Relationship to Platelet Contractile Protein (Thrombosthenin)—Cohen et al. (30) have reported that thrombosthenin M, the myosin-like component of the platelet-contractile protein which contains Ca<sup>2+</sup>-activated ATPase activity, disappeared from whole equine platelets upon treatment with 10 units per ml of thrombin. The thrombin-sensitive protein was therefore examined for Ca<sup>2+</sup>-activated ATPase activity to ascertain whether it might in fact be thrombosthenin M. Assay of the incubation supernatant from thrombin-treated platelets revealed no Ca<sup>2+</sup>-dependent ATPase activity. Lack of activity was not due to inhibition by thrombin, as the addition of purified thrombosthenin to the incubation supernatant from thrombin-treated platelets did not affect the Ca<sup>2+</sup>-ATPase activity of the thrombosthenin. Assay of the post-thrombin supernatant concomi
trated 5-fold yielded Ca\textsuperscript{2+}-ATPase activity of less than 0.3
mmole of P\textsubscript{i} liberated per hour, compared with 6.5 mmoles of P\textsubscript{i}
liberated from ATP per hour in the particulate fraction of the
counterpart platelets, indicating that less than 5\% of the mem-
brane-associated Ca\textsuperscript{2+}-ATPase activity was released from the
cells. SDS-gel electrophoresis showed that 80\% of the platelet
thrombin-sensitive protein had been released into the incubation
supernatant assayed above. Thrombosthenin isolated from
both platelet membranes and the soluble cell supernatant showed
high specific activity Ca\textsuperscript{2+}-ATPase activity (1.9 and 4.03 pmol P\textsubscript{i}
liberated per mg of protein per hour, respectively). SDS-
polyacrylamide gel electrophoresis of both these preparations
showed no band corresponding to the thrombin-sensitive protein.

DISCUSSION

Davey and Lüscher (9) have previously described the types of
proteins released from intact platelets by thrombin, which were
examined by agarose gel electrophoresis and immunoelectropho-
resis. They found released proteins in the prealbumin, albumin,
\(\beta\)-lipoprotein, fibrinogen, and \(\gamma\)-globulin classes, as well as plate-
let-specific \(\alpha\)- and \(\beta\)-globulins. Enzyme studies have shown the
release of acid phosphatase and \(\beta\)-glucuronidase (9), and a
procoagulant activity believed to be either a lipoprotein (9) or
phospholipid micelles (38). In the studies of Davey and Lüscher
(9) most of the proteins and enzyme activities were present in
incubation supernatants from both control and thrombin-treated
platelets, with amounts in the latter supernatant 2- to 3-fold
higher than those of the former. Albumin was an exception,
being increased 7-fold in the supernatant from thrombin-treated
platelets. A \(\beta\)-globulin fraction containing a lipoprotein with
procoagulant activity comprised 37\% of the thrombin super-
natant.

We cannot correlate the protein bands on SDS-polyacrylamide
gel electrophoreses of control and thrombin supernatants with
the various proteins known to be released other than the band
at a molecular weight of 68,000, which is greatly increased in
thrombin over control supernatants and is probably albumin
protein (the molecular weight for human serum albumin is 66,000 (39)).
The other electrophoretic bands, excluding the thrombin-sensitive
protein, appear to be roughly 2- to 3-fold increased in throm-
bin supernatants. The procoagulant lipoprotein found by Davey
and Lüscher has properties similar to the thrombin-sensitive
protein and could well be the same protein, since the lipoprotein
seems from their data to be excluded from Sephadex G-200 and
the thrombin-sensitive protein comprises over 90\% of the protein
appearing in our G-200 void volumes. Davey and Lüscher found
the \(\beta\)-globulin fraction of the thrombin supernatant con-
taining the procoagulant lipoprotein to be increased only 2.6-fold
over that of the control supernatant, yet the 190,000 molecular
weight band on an SDS gel of a thrombin supernatant contains
far more than 3 times the amount of protein as the same band in
a control supernatant. However, the experimental conditions
used by these authors differed from ours; they incubated platelets
at an 8- to 20-fold higher concentrations than in our studies,
with a 10-fold greater thrombin concentration, and apparently
did not treat their supernatants with proteolytic inhibitors.
This may account for the discrepancy, since Davey and Lüscher
(9) would have lost much of the protein through precipitation
from an excessively concentrated solution and through proteo-
lytic degradation.

A number of investigators have reported thrombin-labile pro-
teins, different from fibrinogen, occurring in platelets. Such
proteins disappear from crude platelet extracts or from the solu-
ble supernatant fraction of the cell upon treatment with fairly
high thrombin concentrations (> 10 units per ml), as analyzed
by acrylamide and starch gel electrophoresis and immunodiffu-
sion. The protein which we are describing, in contrast to these
proteins, is located in the platelet particulate fraction and in in-
tact cells is sensitive to 0.1 to 1 unit per ml of thrombin. Its
location in the particulate fraction and its thrombin sensitivity
in intact cells, along with its insusceptibility to thrombin in son-
icates, distinguish it from the thrombin-labile proteins of Salmon
and Bounamaux (40) and Nachman (41), which disappear
from platelet sonicates, and the fibrin-stabilizing factor described
Thrombin-sensitive Protein of Human Platelets

The thrombin-sensitive protein bears little resemblance to human fibrinogen, which has been shown to be the same protein in plasma and in platelets. Although the molecular weight of the thrombin-sensitive protein would be compatible with an $\alpha + \beta + \gamma$ chain complex, no such species have been detected in SDS-gel electrophoresis of human fibrinogen (12). Fibrinogen's carbohydrate composition differs from that of the thrombin-sensitive protein in having 6 sialic and 20 hexosamine residues per 325,000 molecular weight, and it has alanine, tyrosine, and some aspartic acid detectable by amino-terminal analysis (44). Its amino acid composition (Table I) is also different from that of the thrombin-sensitive protein; only the values for histidine, threonine, and leucine agree within 10%, and the rest of the residues are similar only within 20% (arginine, aspartic, and glutamic acids, proline, and isoleucine) or are more widely disparate.

The thrombin-sensitive protein also differs from thrombosthenin M, that component of the platelet-contractile protein thrombosthenin which is assumed to be structurally and functionally similar to muscle myosin and has been reported thrombin-labile in intact equine platelets (30). Thrombosthenin has not been found in the incubation supernatant from thrombin-treated platelets (43), and we found such a supernatant to account for less than 5% of the platelet thrombosthenin Ca$^{2+}$-activated ATPase activity. The amino acid composition of thrombosthenin M (Table I) differs from that of the thrombin-sensitive protein, with the values for 5 residues (lysine, threonine, proline, phenylalanine, and tyrosine) agreeing within 10%, and the rest varying by 20% or more. Our purification of thrombosthenin M from human platelets by the method of Cohen et al. (30) yielded a preparation giving a single band on standard disc gel electrophoresis while SDS gels of this preparation disclosed the thrombin-sensitive protein and numerous other protein bands. Cohen et al. concluded that this single band seen on standard disc gel electrophoresis represented a homogeneous protein and noted that it disappeared from intact equine platelets upon thrombin treatment at 10 units per ml. Clearly their disappearing protein band must include the protein described here, but their purification procedure with human platelets results in an aggregate of the thrombin-sensitive protein with a number of other proteins, one of which is thrombosthenin. A preparation of the myosin-like protein from human platelets by Booyse et al. (37) also differs greatly from thrombin-sensitive protein in amino acid composition.

Whether the thrombin-sensitive protein resides in the platelet surface membrane or in the intracellular granules is not known. However, it shows a number of characteristics noted for other membrane proteins, such as size (46) and limited solubility; the precipitation and aggregation problems noted in attempts to purify the protein are like those encountered with other membrane proteins. Rosenberg and Guidotti (21) have reported two classes of erythrocyte membrane proteins, soluble in aqueous solvents, with amino acid compositions similar to that of the thrombin-sensitive protein; the thrombin-sensitive protein has a similar proportion of acidic residues (27 to 30%) and a lower content of nonpolar residues than these proteins (24% for the platelet protein versus 35% for the erythrocyte ghost proteins). A blocked amino-terminal has been observed by Winzler for the major erythrocyte membrane glycoprotein (47).

The absence of a detectable amino-terminal in either the particulate or released thrombin-sensitive protein could mean that release of the protein does not occur by thrombin's proteolytic action on the protein itself. Winzler suggests that the carboxy-terminal of the erythrocyte membrane glycoprotein is anchored in the membrane and the blocked amino-terminal is exposed to the outside. If the thrombin-sensitive protein were located in the platelet surface membrane in a similar manner, proteolytic release would not generate a new amino-terminal on the protein itself.

The carbohydrate composition of this protein indicates that it may contain more than one type of carbohydrate chain, since the sialic acid content exceeds that of amino sugar. No galactosamine was detected, so that if O-glycosidically linked short carbohydrate chains of the type described by Winzler (47) account for the excess of sialic acid, either they are linked through a different sugar or the thrombin-sensitive protein does contain galactosamine in quantities too small to measure in the limited amounts of protein available for these studies.

Reconstitution of released thrombin-sensitive protein by the technique of Weber and Kuter (48) after purification of the protein by Sephadex G-200 chromatography in SDS yields a soluble protein with potent haptene inhibitory capacity against agglutination of erythrocytes by kidney bean- and lentil-erythroagglutinating phytohemagglutinins. This further supports the hypothesis that it is a glycoprotein since the receptors for these lectins are all carbohydrate in nature. This result also indicates that the protein probably contains mannose residues since both kidney bean-erythroagglutinating phytohemagglutinin and lentil phytohemagglutinin receptors require mannose residues for activity (49).

Determining the true molecular weight of a carbohydrate-containing polypeptide chain in SDS-polyacrylamide gels poses a problem, for such polypeptides have an anomalous electrophoretic mobility, generally causing an overestimate of their molecular weight (50). Bretscher (51) has pointed out that the major erythrocyte membrane glycoprotein (molecular weight 30,000 to 50,000), which is two-thirds carbohydrate, coelectrophoreses with different ghost proteins of molecular weight 50,000 to 105,000 in different acrylamide gel concentrations. We do not know how far the thrombin-sensitive protein deviates from its true molecular weight on SDS gels; its maximum estimated carbohydrate content is 10%, and the true value may be lower.

Bretscher (51) and Fairbanks et al. (46) in studying erythrocyte membrane proteins have noted contamination of a membrane protein band in SDS gels by a coelectrophoresing glycoprotein nearly undetectable by Coomassie blue staining. The major glycoprotein in the platelet particulate fraction moves directly ahead of the thrombin-sensitive protein on SDS gels. This region stains poorly with Coomassie blue and contains glycoprotein material detected by periodic acid-Schiff staining and sialic acid determination. Since the released protein contains the same amounts of sialic acid and glucosamine as that from the particulate fraction it is unlikely that protein from the particulate fraction is contaminated with other glycoproteins from the adjacent region of the preparative SDS gel.

The exact cellular location of the thrombin-sensitive protein remains unknown since all of our experiments were performed...
with a crude particulate fraction. While it may occur on the platelet surface membrane it is also possible that it is contained within some membranous intracellular organelle whose contents are released during the thrombin-induced release reaction. This might explain why intact cells are required for release of the protein. If physiologically the protein is released from some intracellular structure by thrombin, once the cell's secretory apparatus is disrupted by sonication release might not occur.

The function of the thrombin-sensitive protein in platelet aggregation and hemostasis also remains unknown. It may be released secondary to thrombin action on another substrate on the platelet surface, rather than being itself the primary thrombin substrate in platelets. It may function as a cofactor in the coagulation sequence or may mediate the platelet surface changes which lead to platelet aggregation.

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