Formation of a Stable Complex of Thrombin and the Secreted Platelet Protein Glycoprotein G (Thrombin-Sensitive Protein, Thrombospondin) by Thiol–Disulfide Exchange†

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ABSTRACT: When ^{125}I-labeled thrombin was incubated with washed human platelets or with the supernatant solution of activated platelets, it formed a NaDodSO$_4$-stable complex of apparent mass greater than 450 000 daltons. Formation of the complex was temperature dependent; with 20 nM thrombin incubated with the supernatant solution of ionophore-activated platelets, the initial rate of formation of the stable complex was 1 nM thrombin/min at 37 °C, 50 times the rate at 22 °C. Thrombin with all free amino groups methylated was still reactive. Active-site-blocked thrombin formed the complex only slowly. The complex that formed with active thrombin was not dissociated by hydroxylamine in urea. Reduction with 2-mercaptoethanol dissociated the complex, and its formation was blocked by the sulfhydryl-blocking agents iodoacetamide and 4,4′-dithiodipyridine. The complex was thus unlike those of thrombin and α_2-macroglobulin or antithrombin III, but it had characteristics of a disulfide-linked complex. Of the secreted proteins, albumin and glycoprotein G adhered to an activated thiol-Sepharose column, indicating that they contained free thiol groups. Purified glycoprotein G and thrombin formed a complex similar to the complex formed when thrombin was incubated with the supernatant solution of activated platelets. The purified glycoprotein bound 2.6 mol of radioactive *N*-ethymaleimide/mol of protein, indicating three sulfhydryl groups per mole. After reacting with purified glycoprotein G, thrombin developed a new sulfhydryl group. It is concluded that glycoprotein G (thrombin-sensitive protein, thrombospondin) and thrombin form a dissociable complex that leads to a covalent complex by thiol–disulfide exchange of a thiol group on glycoprotein G and a disulfide on thrombin.

Thrombin, a serine protease, is a potent platelet agonist [for review, see Berndt & Phillips (1981)]. The mechanism by which thrombin activates platelets is not known. Platelets contain many proteins that might react with thrombin; these include cell-surface substrates (Phillips & Agin, 1977) and binding proteins (Okumura et al., 1978), secretable substrates (e.g., fibrinogen) and inhibitors (e.g., α_2-macroglobulin), and probably many as yet unidentified proteins.

In two lines of research aimed at establishing what actually happens to thrombin after addition to platelets, we observed the formation of a NaDodSO$_4$-stable[1] complex of labeled thrombin with a substance secreted by activated platelets. In the first of these studies (Danishefsky & Detwiler, 1984), incubation with thrombin derivatized with a photoactivatable cross-linking reagent led to the formation of a complex of thrombin with a released (supernatant) protein as well as with platelet-associated proteins. The supernatant complex was detected within 20 s after photoactivation, but after prolonged incubation (1 h), a NaDodSO$_4$-stable complex was observed even without photoactivation. In the second study (K.-T. Yeo and T. C. Detwiler, unpublished observations), the fate of labeled thrombin incubated with platelets for periods as long as 30 min was analyzed. In addition to changes in binding characteristics, a large NaDodSO$_4$-stable complex was formed, predominantly in the supernatant solution but partly tightly bound to the platelets.

In this paper we describe studies of a NaDodSO$_4$-stable complex between thrombin and a protein released from activated platelets. The complex formed spontaneously without use of a cross-linking reagent. We conclude that it is a complex formed by thiol–disulfide exchange between thrombin and the secreted α-granule protein glycoprotein G.[2]

Materials and Methods

Platelets. Platelets were prepared from blood obtained by venipuncture of volunteer donors as described by Detwiler & Feinman (1973). They were washed 3 times in a solution containing 138 mM NaCl, 3 mM KCl, 12 mM NaHCO$_3$, 5.5 mM glucose, 1 mM EDTA, and 0.36 mM sodium phosphate (pH 7.4), and they were suspended in the same buffer to a concentration of 8×10^9 platelets/mL.

Labeled Thrombin. Purified human α-thrombin was obtained from Dr. John W. Fenton II, Division of Laboratories and Research, New York State Department of Health. The thrombin was radioactively labeled with [^{125}I]iodine as described by Martin et al (1976). For some experiments thrombin was labeled by reaction with [^{14}C]formaldehyde and NaCNBH$_3$ in a procedure based on the study of Jentoft & Dearborn (1979). A total of 100 μL of 200 mM [^{14}C]form-

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[1] Abbreviations: NaDodSO$_4$, sodium dodecyl sulfate; GPG, glycoprotein G (thrombin-sensitive protein, thrombospondin); PAGE, polyacrylamide gel electrophoresis; DIP, diisopropylphosphoryl; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; Me$_2$SO, dimethyl sulfoxide.

[2] Several names have been used for this secreted glycoprotein. The initial name, thrombin-sensitive protein (TSP) (Baenziger et al., 1971), and the most recent name, thrombospondin (Lawler et al., 1978), were chosen with assumptions about properties or functions of the protein that have been shown to be incorrect. For example, it is secreted by platelets in response to secretogogues in general (not just thrombin), and it is not unique to platelets, so the prefix "thrombo" has no rational basis at this time. We use the name glycoprotein G (GPG) (George et al., 1978), for the major granule glycoprotein. This name implies no function or property that is not well established. A more precise name would include its apparent mass to avoid possible confusion with other secreted proteins, but we are reluctant to add yet another name to this protein, and in the context of this paper there can be no confusion.

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aldehyde, 24 mCi/mmol (Amersham, Arlington Heights, IL), was added to 825 μL of a cold solution of 60 μM thrombin, 1 M NaCl, 136 mM sodium phosphate, 80 mM benzamidine, and 2 mg/mL polyethylene glycol 6000, pH 7.0. $NaCNBH_3$ was added to give a final concentration of 20 mM. the reaction mixture was incubated for 24 h at 4 °C and then dialyzed against 750 mM NaCl and 25 mM sodium phosphate, pH 6.5. Thrombin radiolabeled by this procedure retained full amidolytic activity toward the synthetic substrate S2238 and had a specific radioactivity of 450 Ci/mol. Thrombin methylated at all its lysyl ε-amino groups with unlabeled formaldehyde was generously supplied by Dr. Dalton Wang. The methylation procedure was essentially the same as described above, but the final concentration of formaldehyde was 30 mM instead of 20 mM.

Supernatant Solution of A23187-Activated Platelets. The source of released platelet proteins for most of the work described here was the supernatant solution from A23187-activated platelets. Washed platelets were suspended to a concentration of 8×10^9/mL in a solution consisting of 138 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 1 mM EDTA, and 0.36 mM sodium phosphate (pH 7.4). After addition of 0.01 volume of 1 mM A23187 (Calbiochem, La-Jolla, CA) in dimethylsulfoxide, the suspension was allowed to stand at room temperature for 2 min before removal of platelets by centrifugation for 1 min at 10000*g*. The supernatant solution was subsequently centrifuged at 30000*g* for 45 min at 5 °C to remove any remaining platelets.

Antisera against GPG. Rabbit antisera to GPG were prepared essentially as described by Saglio & Slayter (1982). Two rabbits (New Zealand White, 4 kg) were each immunized with 1 mL of a 1:1 emulsion of 250 μg of GPG [purified as described by Alexander & Detwiler (1984)] in complete Freunds' adjuvant (Miles) injected into multiple sites in the footpads and back. Three identical injections were administered at 2-week intervals. The antisera used in this study were obtained 4 weeks after the final injection. The antisera were incubated at 56 °C for 30 min prior to use. When tested by double diffusion in 1% agar (Ouchterlony, 1953), these antisera displayed single precipitin lines against purified GPG and platelet extracts; these lines fused into lines of identity. To further test for monospecificity, protein A–Sepharose (Pharmacia) was mixed for 4–16 h at 4 °C with the antisera to adsorb immunoglobulins; after six washes the pellet was mixed with preparations of the antigens and then washed an additional 3 times. The sedimented material was analyzed by polyacrylamide gel electrophoresis in $NaDodSO_4$. By this method, the antisera immunoprecipitated GPG from the supernatant solution of A23187- or thrombin-activated platelets as well as purified GPG, but we detected no other Coomassie Blue stained material immunoprecipitated from platelet extracts or plasma.

While the antisera thus appeared to be monospecific, for the experiments described in this paper we took an added precaution to minimize the possibility of some antibody against fibrinogen, the most difficult contaminant to remove from GPG (Detwiler & Alexander, 1984) and a secreted platelet protein with properties consistent with the large thrombin complex. Prior to adsorption of immunoglobulins with protein A–Sepharose, 1 mL of each antiserum was mixed with 0.6 mL of human fibrinogen (Sigma, type I, 1.5 mg/mL), incubated for 2 h at 37 °C and overnight at 4 °C, and then centrifuged to remove any antigen–antibody complexes.

Electrophoresis. Electrophoretic analysis on polyacrylamide gels was by the method of Laemmli & Favre (1973). The acrylamide concentration of the 1.5 mm × 15 cm slab gels is given for each figure. A 1.5-cm 3% acrylamide stacking gel was used in most cases. Except where indicated, samples were electrophoresed without reduction of disulfide bonds. After electrophoresis at 8 mA for 16 h, the gels were stained with Coomassie Blue and destained in 10% methanol–10% acetic acid. They were soaked in 7% glycerol for 1 h prior to drying with heat and vacuum (Model SE 540 drying apparatus, Hoefer Scientific Instruments, San Francisco, CA). The dried gels were exposed for 36–48 h at −70 °C to Kodak X-O-Mat R film (Eastman Kodak, Rochester, NY) in X-ray cassettes containing Du Pont intensifying screens (Lightning Plus, Du Pont, Wilmington, DE). The films were developed in a Kodak X-O-Mat developer by standard procedures. Autoradiograms are shown here as contact prints.

Results

Time, Temperature, and Concentration Dependence of Complex Formation. Labeled thrombin incubated with the supernatant solution of activated platelets (10 μM A23187/0.2% Me_2SO) forms a $NaDodSO_4$-stable complex with a mass greater than 450000 daltons. The complex usually appears heterogeneous, with some radioactive material too large even to enter the 3% polyacrylamide stacking gel. As can be noted in the figures in this paper, there is considerable variability in the degree of this heterogeneity. The supernatant solution of control platelets (0.2% Me_2SO) had less than 10% as much radioactivity in the high molecular weight bands. The rate of stable complex formation was markedly dependent on temperature (Figure 1). At 37 °C, added thrombin was incorporated into the stable complex with an initial rate of about 5% (1 nM)/min, 50 times the rate at 22 °C. To assess the capacity of the supernatant protein(s) to form a stable complex with thrombin, the concentration of thrombin was varied (Figure 2). At low concentrations of thrombin (below 50 nM), 60–80% was incorporated into the complex. At higher concentrations of thrombin (1200 nM), as much as 400 nM was incorporated into the complex. The complex must therefore involve a major released protein or be composed of a high ratio of thrombin to released protein. (Note that Figure 2 is not a binding isotherm; it shows formation of a covalent complex that presumably follows an initial dissociable binding, as explained under Discussion.)

Test for Artifacts due to Iodination. In order to determine whether the ability of thrombin to produce a complex is a result of modification of the thrombin by iodination, the supernatant solution of the ionophore-activated platelets was incubated with thrombin labeled by reductive methylation with [^{14}C]formaldehyde and $NaBH_3CN$. After 30 min the reaction mixture was treated with $NaDodSO_4$ and electrophoresed in two dimensions (nonreduced/reduced). In the first (nonreduced) dimension, a labeled complex with an apparent mass of about 500000 daltons contained about 30% of the radioactivity; in the second (reduced) dimension, the radioactivity in this complex migrated identically to thrombin (data not shown). Thus, similar complexes form with iodinated or methylated thrombin.

Comparison with Known Covalent Thrombin Complexes. Thrombin, as well as other serine proteases, is known to form covalent complexes with protein inhibitors. We tested the possibility of known types of complexes by several experiments. Catalytically active thrombin formed the complex much more extensively than did thrombin with a covalently blocked active site (Figure 3). The poor ability of inhibited thrombin to form the complex could be because (i) proteolytic "activation" of the secreted protein is necessary [as in the thrombin–α_2-

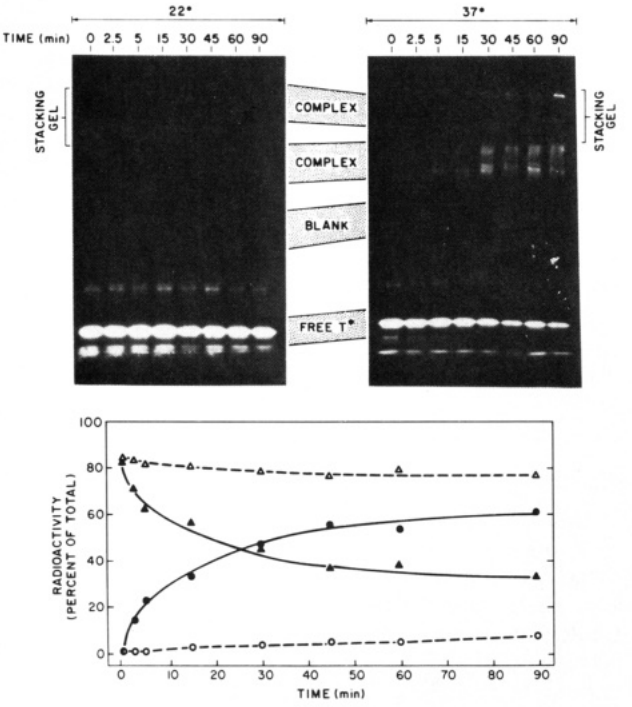


FIGURE 1: Time course of incorporation of ^{125}I-labeled thrombin into large complexes. Labeled thrombin (20 nM) was incubated at either 22 or 37 °C with the supernatant solution of A23187-activated platelets. At indicated times the reaction was stopped by boiling in $NaDodSO_4$ sample buffer. The samples were electrophoresed on 3.5–14% polyacrylamide gradient gels. After the gels were dried autoradiograms were made (2 days of exposure). Prints of these autoradiograms are shown. To quantify radioactivity, the gels were cut into individual lanes and then into bands as indicated for counting in a γ counter. Radioactivity in the band labeled "blank" was subtracted from all other counts. The corrected counts were summed for total radioactivity, the counts in the lower band were taken as "free thrombin", and the sum of the upper two bands plus the stacking gel was taken as "complex". Total radioactivity in each lane varied by less than 10%. Radioactivity in free thrombin (△, ▲) and in the complex (○, ●) are plotted as a percent of total radioactivity for 22 (open symbols) or 37 °C (closed symbols).

macroglobulin complex (Barrett & Starkey, 1973)], (ii) the complex is formed through an ester linkage to the thrombin active-site serine [as in the thrombin–antithrombin complex (Owen, 1975)], or (iii) the large inhibitor group sterically interferes with interaction of thrombin with the secreted protein. The first of these, the possible requirement of proteolytic activation of the secreted protein, as with α_2-macroglobulin, was investigated by including unlabeled active thrombin during incubation of labeled inhibited thrombin with the supernatant solution of ionophore-activated platelets (Figure 3b); this failed to enhance formation of labeled complex (compare lanes 4 and 6). The complex did form when all the free amino groups of thrombin were blocked by reductive methylation; in contrast, methylated thrombin does not form a covalent complex with α_2-macroglobulin (Wu et al., 1981). Unlike the thrombin–antithrombin complex (Owen, 1975), the complex was not dissociated even after denaturation in boiling 1% $NaDodSO_4$ and subsequent incubation in 2 M hydroxylamine for 4 h. The complex formed equally well in the presence of 50 mM EGTA, ruling out a transglutaminase-catalyzed reaction, which would require calcium (Folk & Finlayson, 1977). We conclude that the covalent complex between thrombin and a protein released by activated platelets is not like that of other reported thrombin complexes.

Evidence for Formation of a Complex through Thiol–Disulfide Exchange. Reduction of disulfide bonds with 2-mercaptoethanol caused the complex to dissociate (Figure 4).

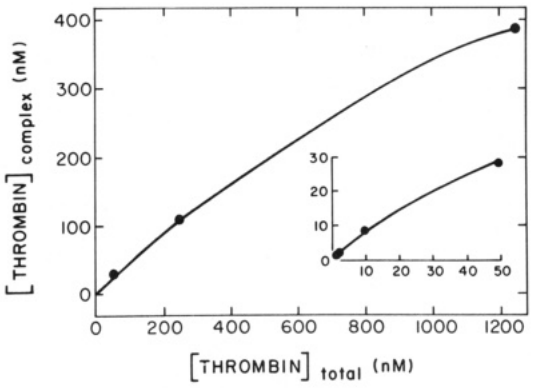


FIGURE 2: Incorporation of ^{125}I-labeled thrombin into the high molecular weight complex as a function of thrombin concentration. Labeled thrombin was incubated at 37 °C with the supernatant solution from A23187-activated platelets for 90 min. For the wide range of thrombin concentrations used, a constant specific radioactivity was not suitable, so mixtures of labeled and unlabeled thrombins were used. After 90 min, the reactions were stopped by addition of $NaDodSO_4$ and heating, and samples were electrophoresed on 3.5–10% polyacrylamide gradient gels. The gels were dried, and the autoradiogram was developed after 2 days of exposure. The gel was cut into lanes, and bands were cut out and counted as described for Figure 1. From the total radioactivity, total thrombin, and radioactivity in the complex, the amount of complexed thrombin in each lane was calculated. Formation of the complex was not a linear function of thrombin concentration, but it did not saturate. The fraction of thrombin complexed was as high as 80% at the lower concentrations, whereas at the highest thrombin concentration 30% was complexed.

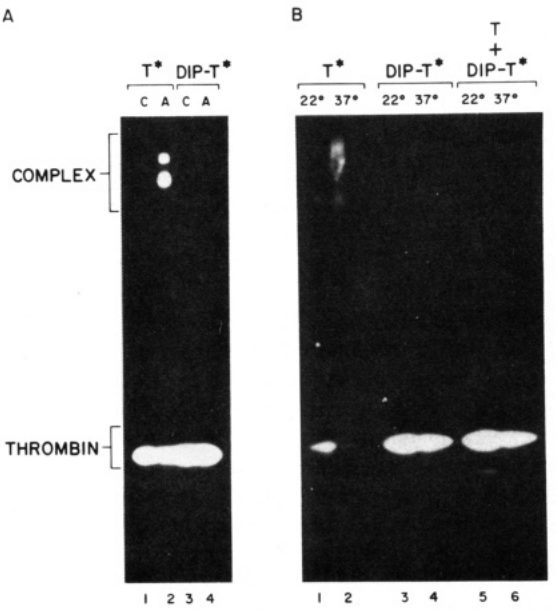


FIGURE 3: Complex formation is inhibited by blocking the thrombin active site with diisopropyl fluorophosphate. Labeled thrombin was incubated with platelet supernatant solutions before preparation of samples for electrophoresis on 4–16% polyacrylamide gradients. Autoradiograms of dried gels are shown for two separate experiments. For panel A, 10 nM ^{125}I-labeled thrombin was incubated at 37 °C for 30 min in platelet wash buffer (control, C) or the supernatant solution of A23187-activated platelets (A). The labeled thrombin was catalytically active (T*) or inhibited with diisopropyl fluorophosphate (DIP-T*). For panel B, 10 nM ^{125}I-labeled active thrombin (T*) or ^{125}I-labeled DFP-inhibited thrombin (DIP-T*) was incubated with the supernatant solution from A23187-activated platelets for 90 min at either 22 or 37 °C. For lanes 5 and 6, 10 nM unlabeled active thrombin was mixed with 10 nM labeled inhibited thrombin.

The two-dimensional electrophoretic analysis shown in Figure 4, panel B, demonstrates that after reduction nearly all of the radioactivity originally in the complex migrated as free thrombin. A possible explanation for dissociation of the label

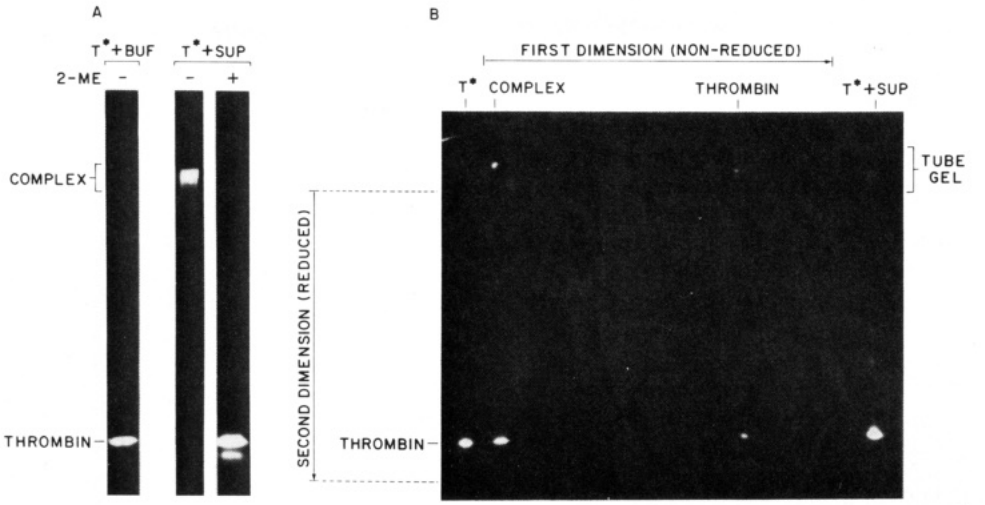


FIGURE 4: Dissociation of the high molecular weight complex by a disulfide-reducing agent. The complex was formed by incubation of 10 nM ^{125}I-labeled thrombin with the supernatnat solution of A23187-activated platelets at 37 °C, and the samples were analyzed by electrophoresis and autoradiography. Two separate experiments are shown. For panel A, samples for electrophoresis on a 3–16% acrylamide gradient gel were prepared after a 90-min incubation of labeled thrombin (T*) with buffer or with the supernatant solution (SUP) of activated platelets. The samples were electrophoresed in the absence (–) or presence (+) of 5% 2-mercaptoethanol (2-ME). For panel B, labeled thrombin was incubated with the supernatant solution for 60 min. The sample was electrophoresed on a tube gel, which was subsequently incubated at 60 °C for 45 min in $NaDodSO_4$ sample buffer containing 10% 2-mercaptoethanol. This tube gel was then loaded on top of a slab gel for electrophoresis in the second dimension. For the second-dimension electrophoresis, a sample of labeled thrombin alone (T*) was applied to the left of the first-dimension tube gel, and a sample of the initial reaction mixture (T* + SUP) was applied to the right. After electrophoresis in the second dimension, a second tube gel (run at the same time as the first) was placed on the slab gel, and the combination was dried for autoradiography. Resolution of the first dimension is very poor because of distortion of the thick tube gel during drying.

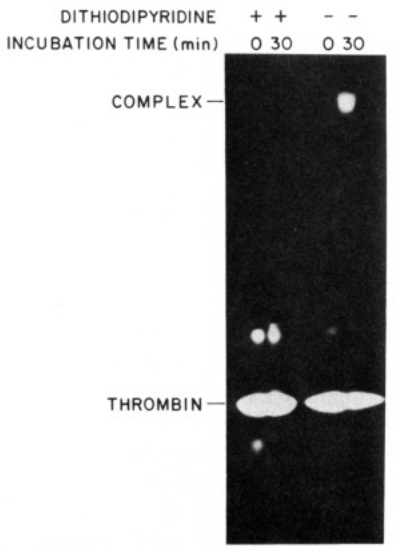


FIGURE 5: Effect of a thiol-blocking agent on complex formation. A supernatant solution of A23187-activated platelets was incubated at room temperature for 5 min with no addition (–) or with 1 mM 4,4′-dithiodipyridine (+); 10 nM ^{125}I-labeled thrombin was added, and samples were prepared for electrophoresis either immediately (0) or after 30 min at 37 °C (30). After electrophoresis on a 3.5–16% gradient of polyacrylamide, an autoradiogram was prepared.

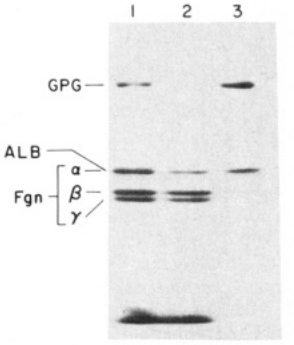


FIGURE 6: Chromatography of secreted platelet proteins on a column of activated thiol-Sepharose. One milliliter of the supernatant solution from A23187-activated platelets was made 8 M with urea and applied to a 0.5 × 5 cm column of activated thiol-Sepharose. The column was developed with 150 mM NaCl, 8 M urea, 1 mM EDTA, and 50 mM sodium phosphate (pH 7.4). After 5 mL of this buffer, the column was washed with the same buffer containing 2% 2-mercaptoethanol. The eluted fractions were made 1% with $NaDodSO_4$ and 5% with 2-mercaptoethanol and heated at 100 °C before electrophoresis on a 7% polyacrylamide gel. The samples were the following: lane 1, the supernatant solution of activated platelets; lane 2, the flow through from the activated thiol-Sepharose; lane 3, the fraction that eluted from the activated thiol-Sepharose after addition of 2-mercaptoethanol. Similiar results were obtained with native proteins (no urea).

by a disulfide-reducing reagent is that the complex is the result of thiol–disulfide exchange between thrombin and the released protein. If this is the case, complex formation should be inhibited by thiol-blocking agents, since disulfide exchange involves the insertion of a free sulfide with displacement of a sulfide in an existing disulfide bond (Torchinskii, 1974). Figure 5 shows that formation of the complex was indeed prevented when 4,4′-dithiodipyridine was included in the reaction mixture. Identical results were also obtained with iodoacetamide.

Thrombin has six cystines but no free sulhydryls (Elion et al., 1977). Therefore, any protein that undergoes thiol–disulfide exchange with thrombin should contain a free sulfhydryl, at least transiently. In order to determine which of the proteins released by activated platelets contain free sulfhydryls, the supernatant solution of ionophore-activated platelets was passed through an activated thiol-Sepharose column. Figure 6 demonstrates that GPG and albumin were retained on the column and could be removed with 2-mercaptoethanol.

Evidence That the Complex Includes GPG. Since the reported mass of GPG, 450,000 daltons (Lawler et al., 1978), is consistent with the observed complex, and since our previous studies with the photoactivated crosslinking reagent suggested a thrombin-GPG complex (Danishefsky & Detwiler, 1984), we purified GPG (Figure 7, top) in order to quantitate the number of thiols and to determine whether it reacts with

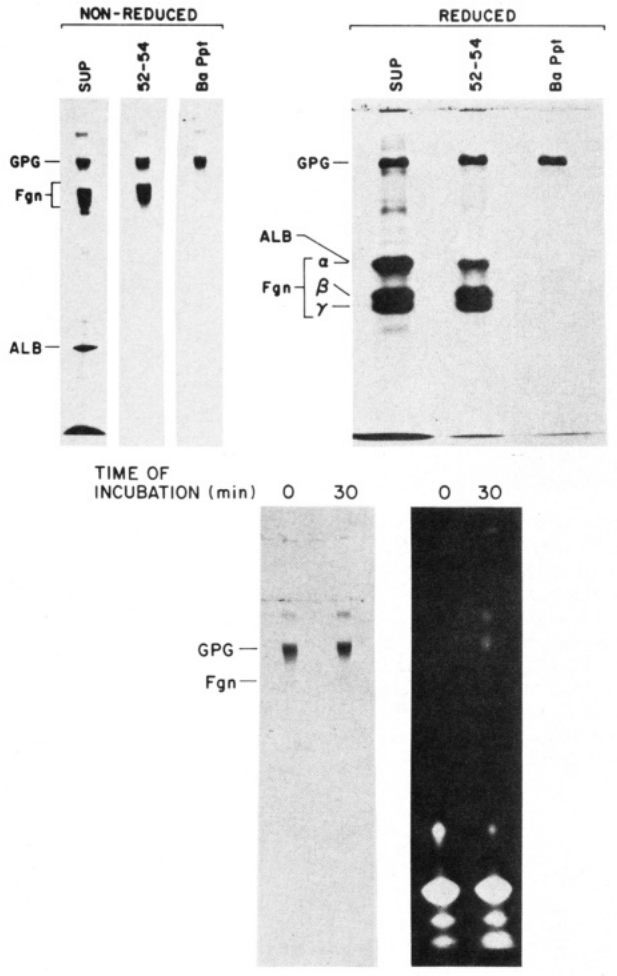


FIGURE 7: Formation of an $NaDodSO_4$-stable complex on incubation of labeled thrombin with purified GPG. Five milliliters of supernatant solution (SUP) from a suspension of 4 units of platelets after activation with 10 μM A23187 was concentrated to 1 mL in dialysis tubing placed in dry Sephadex G-200 at 4 °C. The solution was made 500 mM with NaCl and chromatographed on a Sephacryl S-300 column (155 × 0.8 cm) developed with 500 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 1 mM EDTA, and 0.36 mM sodium phosphate, pH 7.4. Fractions of 1.0 mL were collected at a rate of 5 mL/h. The elution pattern was monitored by absorbance at 280 nm. The three highest absorbing fractions (52–54) from the first peak were pooled. A barium citrate precipitate (Alexander & Detwiler, 1984) was formed by first adding 0.2 volume of 0.12 M trisodium citrate and then slowly adding 0.12 volume of 1.0 M $BaCl_2$. The supernatant solution was discarded, and the precipitate was suspended in 1 mL of platelet wash buffer containing 5 mM EDTA and dialyzed against the same buffer in order to remove the barium ions. Gel electrophoretic analysis of the product is shown in the top panel for nonreduced and reduced samples after staining with Coomassie Blue. Stained bands are identified as fibrinogen (Fgn) and albumin (ALB). The interaction of ^{125}I-labeled thrombin with the purified GPG is analyzed in the bottom panel. Labeled thrombin (20 nM) was added to the solution of purifed GPG, and samples were prepared for electrophoresis either immediately (0) or after incubation at 37 °C for 30 min (30). The samples were electrophoresed on 3.5–14% polyacrylamide gradient gels that were analyzed by Coomassie Blue staining (left) and autoradiography (right).

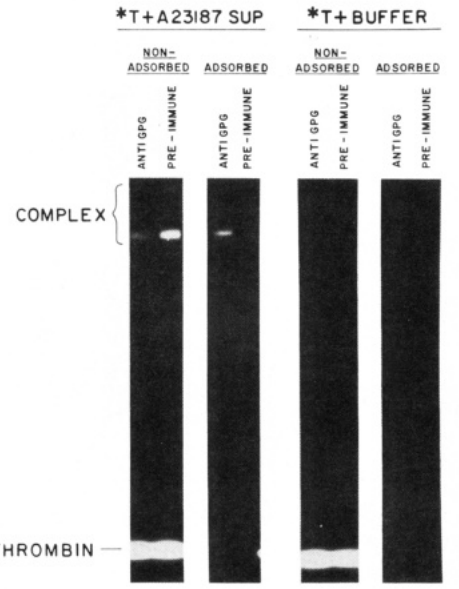


FIGURE 8: Immunoadsorption of the labeled $NaDodSO_4$-stable complex. ^{125}I-Labeled thrombin (20 nM) was incubated for 45 min at 37 °C with the supernatant solution of A23187-activated platelets or with buffer alone. A total of 120 μL of each of these solutions was then incubated for 90 min at 4 °C with protein A–Sepharose to which was bound immunoglobulins from the serum of a rabbit immunized with GPG or from the preimmune serum of the same rabbit. Adsorption of immunoglobulins is described under Materials and Methods. The samples were centrifuged; the supernatant solutions (containing proteins that were not immunoadsorbed) and the pellets after three washes (containing immunoadsorbed proteins) were analyzed by electrophoresis on 6% polyacrylamide gels with a 3.9% stacking gel in $NaDodSO_4$. An autoradiogram of the electrophoresis is shown. It demonstrates that adsorption of radioactive material was specific for the complex (free thrombin was not adsorbed) and specific for the immune serum (no complex was adsorbed by the preimmune serum). Identical results were obtained with the antiserum of a second rabbit or with a monoclonal antibody (TSP-I-1) to GPG.

thrombin. The number of thiols was determined by denaturation of the purified protein in 8 M urea followed by incubation with 10 μM [^{14}C]NEM at room temperature under nitrogen for 10 min. The solution was then dialyzed until the dialysate was free of radioactivity. From the retained radioactivity, the specific radioactivity of the NEM, and the amount of GPG (determined from the reported $A_{280}^{1\%}$ of 8.85 and M_r 450000), we calculated that there were 2.6 mol of NEM/mol of GPG or nearly one NEM per polypeptide chain. Figure 7 (bottom) shows that purified GPG did indeed form a complex with labeled thrombin. Furthermore, the complex that formed in the supernatant solution of activated platelets was immunoadsorbed by a monospecific antiserum against GPG (Figure 8); similar results were obtained with a monoclonal antibody against GPG (TSP I-1) supplied by Drs. Mark Ginsberg and Edward Plow (data not shown). These data are all consistent with formation of a complex of thrombin with GPG by thiol–disulfide exchange.

If formation of the complex were indeed due to insertion of a thiol from GPG into an intrathrombin disulfide bond, complex formation should be accompanied by generation of a new sulfhydryl group on thrombin. To test this, purified GPG (0.4 μM) in platelet wash buffer adjusted to pH 7.0 was incubated with thrombin (0.1 μM) at 37 °C for 30 min. Diisopropyl fluorophosphate (100 μM) was then added, and the mixture was incubated for 20 min at room temperature to prevent proteolysis in subsequent steps. Solid urea was then added to give a final concentration of 6 M, increasing the volume 1.3 times. The solution was deaerated, and [^{14}C]NEM was added to a final concentration of 2.5 μM. The reaction mixture was incubated under a nitrogen atmosphere for 10 min at room temperature. A control consisting of thrombin in the absence of GPG was treated in the same way. The solutions were then dialyzed until the dialysis buffer was no longer radioactive. The dialyzed samples were boiled in 1% $NaDodSO_4$, reduced with 5% mercaptoethanol, and electrophoresed through a 7.5% polyacrylamide gel. The bands corresponding to free thrombin were cut out, and the radioactivity was measured. Thrombin that had been incubated

with GPG had 1224 cpm/mL of dailyzed reaction mixture, corresponding to 0.4 mol of NEM/mol of initial thrombin, whereas the control sample had only 61 cmp/mL or 0.015 mol of NEM/mol of thrombin.[3] These results show that, by reacting with GPG, thrombin developed a NEM-reactive moiety, as predicted for a mechanism involving disulfide exchange between a thiol group on GPG and a disulfide on thrombin.

Discussion

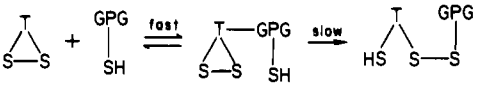
From this study we conclude that thrombin and a secreted platelet protein, glycoprotein G, form a $NaDodSO_4$-stable complex by thiol–disulfide exchange. This is a rather surprising result, because both thrombin (Elion et al., 1977) and GPG (Lawler & Slayter, 1981) have been reported to contain no free sulfhydryl group, which must be present, at least transiently, for disulfide exchange. Our evidence for the formation of a disulfide-linked complex of thrombin and GPG follows.

We conclude that the complex involves the secreted platelet protein GPG because (i) the electrophoretic migration of the complex on $NaDodSO_4$–PAGE suggests a complex slightly larger than the 450 000-dalton GPG, (ii) the migration of the complex formed with a cross-linking reagent migrates with an apparent mass slightly larger than that of a polypeptide chain of GPG on $NaDodSO_4$–PAGE after reduction of disulfide bonds (Danishefsky & Detwiler, 1984), (iii) complexes formed by addition of thrombin to either platelet suspensions or to the supernatant solution of activated platelets are similar to complexes formed with purified GPG, and (iv) the complex can be adsorbed with antibodies against GPG.

We deduce that thiol–disulfide exchange is the mechanism from several lines of evidence. The fact that reduction of disulfide bonds appears to dissociate the complex (Figure 4) suggests a disulfide-linked complex, but it is not proof; reduction could have only dissociated the labeled B chain of thrombin, leaving an intact but unlabeled complex between GPG and the unlabeled A chain of thrombin. Inhibition of complex formation by thiol-blocking reagents (Figure 5) is predicted for thiol–disulfide exchange, but this could be an indirect effect due, for example, to a change in protein conformation. The demonstration that GPG has a free thiol (Figure 6) is essential but by itself does not constitute proof of this proposed mechanism. In combination with this suggestive and consistent evidence, however, the demonstration that, on incubation with GPG, thrombin developed a new NEM-reactive group makes it very likely that the complex arose from an exchange between a thiol group on GPG and a disulfide on thrombin.

Lawler & Slayter (1981) concluded that there are no sulfhydryl groups on GPG, but they used a bulky reagent (fluorescein mercuric acetate) that might fail to react with thiols that were sterically blocked.

The thiol–disulfide exchange of GPG and thrombin most likely follows some specific association of these proteins to position the thiol and disulfide groups properly. Our interpretation is based in part on our independent studies from which we deduced a $NaDodSO_4$-dissociable complex of apparently high affinity. Thus, when labeled thrombin that contained photoactivatable cross-linkers was mixed with the supernatant solution of activated platelets and immediately irradiated for 20 s, the thrombin became cross-linked to GPG. The cross-linking was blocked by hirudin. The efficiency of such cross-linking reagents is very low, so the extent of association of thrombin with GPG could not be determined. It is however, significant that there was no observed cross-linking to fibrinogen, a major thrombin substrate present in these supernatant solutions, suggesting that thrombin binds with considerably greater affinity to GPG than to fibrinogen. We thus propose a reaction (eq 1) in which there is a rapid,



(1)

noncovalent association, blocked by hirudin, followed by a slow exchange of a thiol group on GPG with a disulfide group on thrombin. The initial quick association is deduced from experiments with the cross-linking reagent, and formation of the slow, disulfide-linked complex is concluded from the studies described in this paper. We have no direct evidence to establish a sequential transition from the inferred dissociable complex to the disulfide-linked complex; they may be unrelated.

Thiol–disulfide exchange is a nucleophilic displacement reaction by the mercaptide ion (Torchinskii, 1974). The pK_{SH} of cysteine is about 8.5, so that there would be little mercaptide at neutral pH, and therefore, little thiol–disulfide exchange would be expected. On the other hand, the pK is influenced by local environment and neighboring groups in proteins, where pK_{SH} of cysteine has been reported to range from 7.3 to 10.5 (Barns & Keech, 1968; Donovan, 1964). Thus, if the initial thrombin–GPG complex positions a disulfide bond on thrombin close to a thiol on GPG, and if the pK of the thiol is lower than average, a rapid thiol–disulfide exchange is reasonable. There appeared to be less complex formed with purified GPG (Figure 7, bottom) than with the supernatant solution, suggesting a requirement for rather precise conformation or other factors.

There are several published studies that may bear on interpretation of our results. It has been reported that GPG is a substrate for thrombin, which catalyzes limited degradation to discrete products (Lawler & Slayter, 1981; Lawler et al., 1982). We observed this limited proteolysis, but, as reported, it is seen only with a considerably higher concentration of thrombin and longer incubations than necessary for complex formation, so it cannot be essential for complex formation. The disulfide cross-linking could, of course, depend on a proteolytic cleavage that caused no detectable change in electrophoretic mobility.

GPG has also been shown to be sensitive to calcium (Lawler et al., 1982). A calcium-induced conformational change was used to estimate the cooperative binding of calcium with an average apparent dissociation constant of 120 μM (Lawler & Simons, 1983). While the effect of calcium on the formation of the thrombin–GPG complex described here remains to be analyzed in detail, it is apparent from the data presented here that the disulfide-linked complex can form in the presence or absence of free calcium ions. Thus, most of the studies described here (e.g., Figures 1–5) were with the supernatant solution obtained after activation of platelets suspended in a solution containing 1 mM EDTA to prevent aggregation prior to activation. The activated platelets would secrete enough calcium to bring the final extracellular concentration to about 3 mM[4] (Detwiler & Feinman, 1973). Thus, most of these

[3] The ratio NEM:thrombin of 0.4 is for total thrombin in the reaction mixture. Since only about half of the added thrombin would have formed the stable complex under these conditions, the ratio NEM:complexed thrombin was presumably higher.

experiments demonstrate complex formation in the presence of much more than a saturating concentration of calcium. On the other hand, it is clear that calcium is not essential for complex formation, since (i) the complex formed when the supernatant solution was made 50 mM with EGTA, (ii) the complex formed (but to a lesser extent) with GPG purified in the presence of EDTA, and (iii) the initial observations of a photoactivatable cross-linking of thrombin to GPG were with high concentrations (20 mM) of EDTA.

There has been another report of a thrombin–GPG interaction. Hagen et al. (1983), who analyzed thrombin-binding proteins of platelets by crossed immunoelectrophoresis, reported coelectrophoresis of GPG and thrombin, indicating a complex stable in Triton X-100.

There are too many unknowns to permit an assessment of the significance of this study. Does the thiol–disulfide exchange take place because the thiol on GPG is especially reactive, because the disulfide on thrombin is especially sensitive, or because there is a highly specific complex of thrombin and GPG? Does thrombin in the complex retain catalytic activity? Without answers to these questions, it is not clear whether we have learned more about GPG, about thrombin, or about the interaction of these two proteins. GPG is considered to be an "adhesion" protein in the way that fibronectin is considred an adhesion factor. GPG is released by cultured endothelial cells (McPherson et al., 1981; Mosher et al., 1982) and fibroblasts (Jaffe et al., 1983), and it can be seen with fluorescent antibodies to form matrices similar to those observed with fluorescent antibodies against fibronectin (Jaffe et al., 1983). It is possible that GPG anchors thrombin (or other proteases) at sites of exposure of subcellular matrices, or it may be that the thiol on GPG readily exchanges with a disulfide on other proteins, thereby acting as a large cross-linking reagent.

Acknowledgments

We are grateful to Dr. John W. Fenton II for the generous supply of purified human thrombin and for his stimulating discussions and to Dr. Mark Ginsberg and Dr. Edward Plow for a monoclonal antibody against glycoprotein G.

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[4] It has been reported (Phillips et al., 1980) that, in the presence of calcium, secreted GPG is almost entirely bound to the platelets, so that if there were, in fact, calcium present, no GPG would be expected in the supernatant solution. We have not successfully reproduced the observations of calcium-dependent binding of most secreted GPG. In our hands most of the secreted GPG is recovered in the supernatant solution whether the medium contains an excess of EDTA or 2 mM Ca^{2+} and 0.5 mM Mg^{2+}, whether the platelets are activated with thrombin or A23187, and whether incubation after activation is for 2 or 30 min at 22 or 37 °C. The reported calcium-dependent binding of secreted GPG also seems inconsistent with widely used procedures for the isolation of GPG; platelets are activated in media containing calcium (Lawler et al., 1982) or low enough concentrations of citrate (Lawler et al., 1978) that secreted calcium is sufficient to allow platelet aggregation, but GPG is obtained in high yield in the supernatant solution.