Platelet Protein Organization: Analysis by Treatment With Membrane-Permeable Cross-Linking Reagents

By Gregg E. Davies and Jiri Palek

We have examined platelet protein organization by treatment of intact resting or thrombin-activated platelets with two cross-linking reagents, diamide or dithiobisis(succinimidypropionate) (DTSP). Cross-linked complexes were separated by polyacrylamide gel electrophoresis in the absence of reducing agent and their composition determined after reductive cleavage and analysis in a second-dimensional gel. The most prominent cross-linked species produced by diamide treatment of resting platelets are (A) cytoskeletal protein homopolymers, such as myosin heavy chain dimer and actin oligomers, and (B) high molecular weight material consisting of homo- or heteropolymers of cytoskeletal proteins and 230,000, 170,000, 100,000, 55,000, and 52,000 dalton proteins. DTSP treatment forms similar complexes and also cross-links membrane glycoproteins IIb and III into high molecular weight material. Thrombin activation of platelets before treatment with diamide or DTSP results in increased cross-linking of myosin and increased incorporation of several proteins, particularly myosin and glycoproteins IIb and III, into high molecular weight material. The results provide evidence for reorganization of cytoskeletal and membrane proteins during platelet function.

When platelets are activated by contact with damaged blood vessel walls or soluble aggregating agents, there is a complex reorganization of membrane and internal structure leading to shape change, aggregation, and release of granule contents. Recent evidence indicates that platelet activation is associated with reorganization of cytoplasmic contractile and structural proteins (the cytoskeleton). It is believed that the cytoskeleton is mostly disassembled in resting platelets, since very few filamentous cytoplasmic structures are seen by transmission electron microscopy of platelets prepared under conditions that minimize activation. In contrast, electron microscopy of activated platelets reveals abundant actin-containing microfilaments filling the cytoplasm. As measured by a direct assay using deoxyribonuclease I inhibition, polymerization of actin occurs during platelet activation. Studies using the nonionic detergent Triton X-100 are also consistent with assembly of cytoskeletal proteins upon activation. Treatment of intact platelets with this detergent solubilizes most cellular constituents, leaving assembled cytoskeletal proteins behind as a precipitate. Triton extraction of resting platelets yields only a small amount of precipitate, but platelet activation by thrombin or the ionophore A23187 results in a significant increase in the amount of precipitate. Reorganization of platelet membrane proteins may also occur during activation, as suggested by exposure of fibrinogen binding sites, altered labeling of surface-exposed glycoproteins, and increase in number of reactive membrane sulfhydryl groups. Much of the aforementioned data has been obtained only after fixation or disruption of the platelet. New approaches are needed for study of protein organization in intact platelets before and after activation. As discussed in recent reviews, cross-linking reagents have been successfully used to study protein organization of membranes and, to a limited extent, whole cells. Cross-linking studies have significantly contributed to the current model of red cell membrane and cytoskeletal structure. A variety of reagents are available for the formation of cleavable protein-protein linkages, and several of these reagents have been shown to penetrate cell membranes. We have found that treatment of intact platelets with diamide or dithiobisis(succinimidypropionate) (DTSP) forms many cross-linked complexes of functionally important platelet proteins. Diamide, a sulfhydryl-specific reagent, readily forms cross-links involving the major platelet cytoskeletal proteins. DTSP, an aminospecific reagent, cross-links additional proteins such as several surface-exposed membrane glycoproteins. In this article, we describe the identification of the cross-linked complexes by two-dimensional SDS-polyacrylamide gel electrophoresis and compare the cross-linking patterns of resting and thrombin-activated platelets.

MATERIALS AND METHODS

Reagents were obtained from the following sources: diamide (diazinedicarboxylic acid bis(N,N-dimethylamide)), Calbiochem (La Jolla, Ca.); DTSP, Pierce (Rockford, Ill.); prostaglandin E, tetracaine, and Triton X-100, Sigma, St. Louis, Mo.; human plasma fibrinogen, AB Kabi, Stockholm; lactoperoxidase, P-L Biochemicals (Milwaukee, Wis.); dimethylsulfoxide, Fisher (Pittsburgh, Pa.); bovine thrombin, Parke-Davis (Detroit, Mi.); NaCl (carrier-free, 006-4971/82/5903-0007$01.00/0

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PLATELET PROTEIN ORGANIZATION

pH 8–10), New England Nuclear, Boston, Mass.; 14C-serotonin creatinine sulfate (58 Ci/mole), Amersham-Searle, Arlington Heights, Ill.; and Na235CrO4 (91 Ci/g), Squibb (Princeton, N.J.). Rabbit skeletal muscle actin and myosin were kindly donated by Dr. Carl Cohen, St. Elizabeth's Hospital (Boston, Mass.). G-actin was prepared from freshly isolated F-actin by dialysis against 2 mM Hepes, 0.02 mM CaCl2, 0.1 mM ATP, pH 7.4, for 3 days at 4°C, followed by centrifugation at 150,000 g for 2 hr. Withdrawal of blood from normal volunteers was approved by an Institutional Human Investigation Committee. Platelet concentrates, less than 24 hr old, in citrate-phosphate-dextrose anticoagulant were obtained from the American National Red Cross Blood Program, Northeast Region, Boston.

The following buffered solutions were used: (A) Rossi's buffer,15 containing 140 mM NaCl, 5 mM KCl, 5 mM glucose, 7 mM citric acid, pH 6.5, with 50 ng/ml prostaglandin E2, added, and (B) Hepes buffer,16 containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5.6 mM glucose, 3.3 mM Na2HPO4, 3.8 mM Hepes, pH 7.35, with bovine serum albumin omitted.

Platelet Preparation

From 50 to 100 ml of fresh blood were mixed with 2.2% trisodium citrate, 1.2% citric acid at a blood:anticoagulant ratio of 8.5:1.5. The platelet suspension from normal volunteers was approved by an Institutional Human Investigation Committee. Platelet concentrates, less than 24 hr old, in citrate-phosphate-dextrose anticoagulant were obtained from the American National Red Cross Blood Program, Northeast Region, Boston.

The following buffered solutions were used: (A) Rossi's buffer,15 containing 140 mM NaCl, 5 mM KCl, 5 mM glucose, 7 mM citric acid, pH 6.5, with 50 ng/ml prostaglandin E2, added, and (B) Hepes buffer,16 containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5.6 mM glucose, 3.3 mM Na2HPO4, 3.8 mM Hepes, pH 7.35, with bovine serum albumin omitted.

Platelet Preparation

From 50 to 100 ml of fresh blood were mixed with 2.2% trisodium citrate, 1.2% citric acid at a blood:anticoagulant ratio of 8.5:1.5. Platelet-rich plasma was obtained by centrifugation at 160 g for 10 min and was recentrifuged once to remove residual red cells. Platelets were collected by centrifugation at 1500 g for 10 min and washed twice with Rossi's buffer warmed to 37°C. The platelets were resuspended in a minimum volume of Rossi's buffer and diluted with Hepes buffer to 0.5–1.0 × 1010 platelets/ml. For some experiments, 1–4 platelet concentrates were brought to pH 6.5 by addition of 2.2% trisodium citrate, 1.2% citric acid. The platelet suspension was centrifuged 5 times at 2000 g (20 sec each time) to remove red cells, and the platelets were collected and washed as noted above. Platelets for aggregation and secretion studies were prepared by gel filtration on Sepharose 2B gel equilibrated with Hepes buffer.16

125I Labeling

The buffer used in all steps contained 137 mM NaCl, 3.3 mM Na2HPO4, 3.8 mM Hepes, 1 mM Na2EDTA, pH 7.35. Platelet surface glycoproteins were 125I-labeled by a modification of a published method.17 A quantity of 0.25–0.5 mCi of Na125I and 2.5 μl of tetracaine and thrombin treatment

Washed platelets were incubated with 1.5 mM tetracaine and 0.75% dimethylsulfoxide or with 0.1–0.6 U/ml thrombin and 2 mM EDTA for 10 min at 37°C. EDTA is included in activated samples to prevent aggregation, since it is possible that cross-linking reagents would penetrate poorly into an aggregate. In several experiments, platelet-rich plasma was incubated with 1.5 mM tetracaine and 0.75% dimethylsulfoxide for 5 min at 37°C, and the platelets were isolated by gel filtration on Sepharose 2B gel equilibrated with Hepes buffer containing tetracaine and dimethylsulfoxide at the same concentrations. An aliquot of each sample was fixed by addition of 1/4 volume of 10% glutaraldehyde, 140 mM NaCl, 3.8 mM Hepes, pH 7.4, for examination by phase contrast microscopy, and the remainder was treated with a cross-linking reagent.

Cross-Linking

Stock solutions of cross-linking reagents were prepared as follows: (A) 15 mM diamide in Hepes buffer, and (B) 200 mM DTSP in N,N-dimethylformamide, diluted with Hepes buffer to a concentration of 1 mM just before use. An aliquot was added to the platelet suspension to yield the indicated reagent concentrations. Each experiment included a control sample treated with an equivalent volume of buffer or 0.5% N,N-dimethylformamide in buffer. The platelet suspension was gently mixed for 15 sec and then left at 37°C for 2–5 min. Where diamide was used, the reaction was stopped by addition of 1/80 part of 200 mM N-ethylmaleimide, and the platelets were left at 37°C for 30 min. For concentrations of platelet proteins of 2.5 mg/ml or less, this amount of N-ethylmaleimide alkylates more than 98% of sulphydryl groups in intact platelets and thereby prevents spontaneous oxidative cross-linking of platelet proteins at later steps.17 Where DTSP was used, 1/40 part of 0.2 M glycine, pH 7.4, was added, followed by incubation at 37°C for 10 min and then N-ethylmaleimide treatment. Platelets were collected by centrifugation at 1000 g for 10 min, washed once with Rossi's buffer, and dissolved in a solution containing 3% SDS, 0.25 M sucrose, 50 mM Tris hydrochloride, 2 mM Na2EDTA, 0.005% pyronin Y, pH 7.4. A control experiment showed that the procedure prevented continuation of cross-linking after platelet disruption by SDS. Non-cross-linked alkylated platelets were dissolved in the gel solution and treated with up to 1.5 mM diamide or 0.25 mM DTSP, without cross-linked complexes found upon subsequent electrophoretic analysis.

SDS-Polyacrylamide Gel Electrophoresis

One-dimensional cylindrical gel electrophoresis was performed in a 0.6 × 8 cm 0.3% agarose-2.5% polyacrylamide gel,26 followed by staining with Coomassie blue. For two-dimensional gel electrophoresis, platelet proteins were first separated in a cylindrical agarose-polyacrylamide gel. The gel was removed from the tube and embedded in agarose over a 6-mm thick slab gel consisting of a Laemmli 5.5% polyacrylamide system over which a dithiothreitol-containing agarose layer had been poured.19 In experiments using labeled proteins, the agarose-polyacrylamide gels were left at −20°C for 30–45 min, trimmed to 3 mm in thickness, thawed, and embedded over 3-mm thick slab gels for separation in the second dimension. Stained gels containing 125I-labeled proteins were soaked for 6–12 hr in a solution containing 10% acetic acid, 2% glycerol, and dried using a Bio-Rad gel drier. Autoradiograms were obtained using Kodak XRP:1 X-ray film exposed for 1–14 days in Dupont x-ray cassettes with high-speed intensifier screens.

Protein concentrations in samples for gels were determined by Lowry's method,19 with bovine serum albumin standards containing appropriate amounts of the SDS-containing solutions used to dissolve platelet proteins. Then 50–100 μg (one-dimensional gels) or 250–350 μg (two-dimensional gels) of protein were applied. Equal amounts of protein were applied to gels run in parallel. Some gels were scanned using a densitometer (Helena Laboratories) with a 570-nm filter.

Platelet protein molecular weights were estimated on gels using standard plots and the following marker proteins and molecular weights: skeletal muscle myosin heavy chain, 200,000; bovine serum albumin monomer, 68,000, and dimer, 136,000; and red cell membrane proteins, band 1, 240,000, band 2, 215,000, band 3, 88,000 (diffuse band), band 4, 78,000, band 4.2, 72,000, and band 5, 43,000. For additional high molecular weight markers, muscle myosin was cross-linked with dimethylsulfoxide by a published method.26

Platelet proteins were identified by apparent molecular weight,
comparison to standards such as muscle actin and myosin, and reference to published data.21 Coomassie blue staining glycoproteins were identified by comparison with the autoradiographic pattern of
\[ ^{125} \text{Tl} \]-labeled platelet proteins.27 Identification of cross-linked complexes by apparent nonreduced molecular weight and two-dimensional gel pattern is based on studies of Wang and Richards.22

**Triton Precipitate**

In a slight modification of the method of Phillips et al.,8 washed platelets in Hepes buffer were treated with 0.5 U/ml thrombin and 2 mM EDTA for 10 min at 37°C, followed by one wash and addition of one volume of 2% Triton X-100, 10 mM EDTA, 0.1 M Tris hydrochloride, pH 7.4. After 15 min at 4°C, the precipitate was obtained by centrifugation at 1500 g for 10 min and dissolved in 3% SDS, 0.25 M sucrose, 50 mM Tris hydrochloride, 2 mM EDTA, 0.005% pyronin Y, 40 mM dithiothreitol, pH 7.4, for electrophoresis.

**Assay of Platelet Aggregation, Secretion, and Disruption**

Aggregation was studied by the method of Born and Cross.23 14C-serotonin secretion was assayed according to Kaplan and Nachman.24 For assay of platelet disruption under cross-linking conditions, platelets were labeled by addition of 50 μCi of Na2CrO4 to the first wash with Rossi's buffer, followed by incubation for 30 min at 37°C. After 2 more washes and suspension in Hepes buffer, the platelets were divided into aliquots. Treatment with cross-linking reagents or N-ethylmaleimide, and assayed for 51CrO4 release by a method analogous to that for 14C-serotonin release.

**RESULTS**

**Cross-Linking of Resting Platelets**

We first analyzed freshly washed platelets on cylindrical agarose-polyacrylamide gels in the absence of reducing agent (Fig. 1). Before cross-linking (gel A), the most prominent proteins are thrombospondin, actin-binding protein, a 230,000-dalton protein, myosin heavy chain, and actin. Treatment of platelets with 0.6 mM diamide for 5 min (gel B) forms a prominent new 400,000–dalton complex and material that remains at the top of the gel. As in cross-linking studies of red cell proteins, we refer to this latter material as a "high molecular weight complex." Treatment with 1.5 mM diamide (gel C) extensively cross-links platelet proteins into the high molecular weight complex. Treatment with 0.13 mM DTSP for 2 min (gel D) produces a cross-linking pattern similar to that obtained with 0.6 mM diamide, except that several minor 600,000–800,000-dalton components are visible. Treatment with 0.25 mM DTSP (gel E) forms a large amount of high molecular weight complex.

We next determined the composition of the cross-linked complexes by electrophoresis in the two-dimensional gel system (Fig. 2). Analysis of non-cross-linked platelets is shown in gel A. A diagonal pattern of various platelet proteins is seen, with thrombospondin and fibrinogen subunits migrating faster after reduction due to cleavage of intersubunit disulfide bonds. In most experiments, the Aα subunit of fibrinogen was partially degraded. Three membrane glycoproteins are detected by Coomassie blue staining, termed Ib, IIb, and III in the nomenclature of Phillips and Agin.17 Glycoproteins Ib and IIb are known to migrate faster after reduction due to dissociation of small, disulfide-linked subunits. In contrast to data with a two-dimensional gel system of equivalent gel composition in each dimension,17 our gel system also produces a shift of glycoprotein III slightly to the left of the diagonal pattern. This effect is probably due to retarded mobility of membrane glycoproteins in the agarose-polyacrylamide gels, since a similar off-diagonal location of all red cell membrane glycoproteins in this gel system has been described.18 Analysis of platelets treated with a low concentration of diamide (gel B)
shows that the 400,000-dalton complex seen in one-dimensional gels is a dimer of myosin heavy chain. Other complexes are actin-binding protein dimer; dimer of the 230,000-dalton protein; myosin trimer and tetramer; actin multimers; and small amounts of unidentified complexes, many of which are dimers of various 40,000–120,000-dalton platelet proteins. Two-dimensional electrophoresis of platelets treated with DTSP (gel C) shows an array of cross-linked complexes resembling that obtained with diamide but including several additional complexes (fibrinogen dimer, thrombospondin multimers, and others). Also, there are minor quantitative differences between the results with diamide and DTSP, such as increased yields of myosin trimer and tetramer relative to dimer after DTSP treatment.

Fig. 2. Two-dimensional SDS-polyacrylamide gel electrophoresis of platelet proteins before and after treatment with diamide or DTSP (2-min incubations). Abbreviations are as in Fig. 1, plus Fib, fibrinogen; and GP, glycoprotein. GP IIb and IIII merge with nearby unidentified proteins of molecular weight 120,000 and 100,000, respectively, due to overloading of protein for demonstration of faint bands. A schematic diagram is shown below each gel pattern.

Fig. 3. Two-dimensional gel electrophoresis of platelet proteins extensively cross-linked by diamide treatment (2-min incubation). The 170,000, 55,000, and 52,000 dalton proteins cross-linked into the high molecular weight complex are denoted by arrows (see text).
The High Molecular Weight Complex

To better illustrate the high molecular weight complex, we analyzed heavily cross-linked platelets on two-dimensional gels (Fig. 3). For this pair of gels, platelets from a concentrate in citrate-phosphate-dextrose were treated with diamide. Platelets from concentrates are consistently more susceptible to oxidative cross-linking of proteins by diamide, probably because of depletion of reduced glutathione known to occur during storage; however, treatment of fresh platelets with 1.5 mM diamide for 5 min produces a similar two-dimensional gel pattern (data not shown). The complex contains actin-binding protein, a 230,000-dalton protein, myosin, actin, and proteins with apparent reduced molecular weights of 170,000, 100,000, 70,000, 55,000, and 52,000. Minor constituents with reduced molecular weights of 120,000, 85,000, and 40,000 are also inconsistently found in the complex. The 170,000, 55,000, and 52,000 dalton proteins in the high molecular weight complex have apparent reduced molecular weights similar to those of thrombospondin, fibrinogen Bβ, and fibrinogen γ chains, respectively; however, comparison of the gel patterns in Fig. 3 shows that thrombospondin and fibrinogen are not significantly cross-linked by diamide treatment. The 170,000, 55,000, and 52,000 dalton constituents of the high molecular weight complex are derived from other proteins that, before cross-linking, lie on the diagonal portion of the gel pattern, as denoted by arrows. The 55,000 and 52,000 dalton proteins may be the α- and β-subunits of platelet tubulin, based on identical electrophoretic mobilities (data not shown). The high molecular weight complex produced by treatment of fresh or stored platelets with 0.25 mM DTSP is similar in composition to that produced by diamide treatment, except that small amounts of a number of additional proteins, including thrombospondin and fibrinogen, appear in the complex.

Tetracaine-Treated Platelets

Platelets washed by the standard method are discoid, but examination by phase microscopy showed that 97%-100% of these platelets had extended one or more filopodia, indicating partial cytoskeletal assembly. To further examine whether the cross-linking results are representative of completely nonactivated platelets, we compared the cross-linking patterns before and after tetracaine treatment. Exposure to tetracaine is known to inhibit platelet function, suppress filopodia extension, and convert the platelet to a nearly spherical form with an amorphous, microfilament-free cytoplasm. Incubation of washed platelets with 1.5 mM tetracaine and 0.75% dimethylsulfoxide for 10 min at 37°C completely suppressed filopodia formation, and cross-linking with 1 mM diamide before and after tetracaine treatment produced identical one- and two-dimensional gel patterns (data not shown).

Platelet Membrane Glycoproteins

The membrane glycoproteins are not heavily enough stained by Coomassie blue to easily evaluate after cross-linking; therefore, we treated 125I-labeled platelets with diamide or DTSP and analyzed the products by two-dimensional electrophoresis and autoradiography.
phy. Treatment with 0.25 mM DTSP (Fig. 4) forms oligomers of glycoproteins Ib and III and also causes a significant amount of these glycoproteins to appear in the high molecular weight complex. Two complexes containing glycoproteins Ia and IIa are tentatively identified as dimers of these glycoproteins. In contrast to DTSP, treatment with 0.6-1.5 mM diamide forms no complexes containing labeled glycoproteins (data not shown).

**Summary of the Cross-Linked Complexes**

In Table 1 we list the cross-linked complexes that have been found using various diamide and DTSP concentrations. Since quantitation of the results of

<table>
<thead>
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<th>Protein Band</th>
<th>Apparent Nonreduced mol wt Before Cross-linking (× 10^3)</th>
<th>Apparent Nonreduced mol wt of Cross-linked Complexes (× 10^3)</th>
<th>Presumptive Identity of the Complexes</th>
<th>Yield of Cross-linked Complexes*</th>
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<td>(1) Cytoskeletal proteins</td>
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<tr>
<td>Actin-binding protein (ABP)</td>
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<td>800</td>
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<td>Actin (A)</td>
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<td>(2) Membrane glycoproteins</td>
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<td>GP IV</td>
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<td>(3) Other proteins</td>
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<td>HMWC</td>
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*Estimates of yield of cross-linked complexes: +, faint bands seen only on optimal gels, and ++, medium to heavy bands seen in most experiments.
†High molecular weight complex.
two-dimensional electrophoresis is difficult, we have used a rough scale to indicate the approximate yields of complexes.

Cross-Linking of Activated Platelets

We compared the two-dimensional electrophoretic patterns of platelets treated with diamide before and after incubation with thrombin-EDTA. In experiments at various concentrations of thrombin (0.1–0.5 U/ml) and diamide (0.3–1.5 mM), two consistent differences were found. First, there is an increased yield of cross-linked myosin heavy chain dimers after activation, as shown in Fig. 5. This difference was confirmed by densitometric scanning in six experiments, with increased yields of myosin dimer of 130–200% (range of all values). Second, there is increased incorporation of myosin and the 230,000, 55,000, and 52,000 dalton proteins into the high molecular weight complex, as shown in Fig. 6. Due to overlapping bands, this change in composition of the high molecular weight complex is difficult to quantitate; however, the cross-linking patterns depicted in Fig. 6 were noted in more than 10 experiments. Similar results were obtained with DTSP cross-linking (data not shown).

125I-labeled platelets were thrombin-activated, treated with DTSP, and analyzed on two-dimensional gels (Fig. 7). In comparison to a sample of resting platelets, there is increased incorporation of glycoproteins IIb and III and possibly several faintly labeled glycoproteins into the high molecular weight complex. As for resting platelets, no 125I-labeled proteins in activated platelets are cross-linked by 0.6–1.5 mM diamide (data not shown).

Comparison of the High Molecular Weight Complex With a Triton Precipitate

The cross-linked high molecular weight complex (shown in Figs. 3 and 6) consists largely of cytoskeletal proteins. This was further examined by comparison of the protein compositions of whole platelets (Fig. 8, gel A), the Triton precipitate of activated platelets (gel B), and the high molecular weight complex produced by diamide treatment of activated platelets (gel C). The major components of the Triton precipitate are actin-binding protein, myosin, actin, and proteins with apparent reduced molecular weights of 170,000, 100,000, 55,000, and 52,000. Using 5%–20% polyacrylamide gradient gels, Phillips et al. describe a very similar composition of the Triton precipitate, except for the appearance of a single 56,000-dalton protein rather than the doublet resolved by our gels. Except for minor quantitative differences, the high molecular weight complex contains the same proteins as the Triton precipitate, plus a 230,000-dalton protein.

Cross-Linking of Skeletal Muscle Actin

The preceding data show relatively little cross-linking of actin in resting platelets, in which actin is principally nonpolymerized, and in thrombin-activated platelets, known to contain a large amount of F-actin. We have compared these results with the cross-linking pattern of more readily obtainable skeletal muscle actin, which is similar in many properties to purified platelet actin. Muscle G-actin in 2 mM Hepes, 0.02 mM CaCl₂, 0.1 mM ATP, pH 7.4, was polymerized by addition of NaCl and MgCl₂ to 100 mM and 2 mM, respectively, and incubation for 30 min at room temperature. Samples of G- and F-actin were treated with diamide or DTSP by the standard cross-linking procedure (final actin concentration of 5.9 mg/ml) and analyzed on agarose-acrylamide gels. Untreated actin...
Platelet Aggregation, Secretion, and Disruption Under Cross-linking Conditions

To determine whether cross-linking significantly activates or disrupts platelets, we have measured aggregation, $^{14}$C-serotonin secretion, and $^{51}$CrO$_4^{2-}$ release during cross-linking. As shown in Table 2, diamide or DTSP treatment of gel-filtered platelets causes slight aggregation. Diamide-treated platelets spontaneously disaggregate as reported previously for nonwashed platelets. Cross-linking reagents cause no significant secretion of $^{14}$C-serotonin. Treatment with 1 mM diamide or 0.15 mM DTSP releases 1.5% or 0%, respectively, of $^{51}$CrO$_4^{2-}$ from labeled platelets, indicating that the reagents do not significantly disrupt platelets. Subsequent alkylation with 2.5 mM N-ethylmaleimide causes 8% $^{51}$CrO$_4^{2-}$ release. For several reasons, it is felt unlikely that protein cross-linking in platelets disrupted by manipulation or N-ethylmaleimide contributes to the complexes identified by two-dimensional electrophoresis: (A) platelets washed by Rossi's method are only minimally damaged by ultrastructural and functional criteria; (B) in experiments using DTSP, excess glycine is added to consume extracellular reagent before addition of N-ethylmaleimide, and (C) in several experiments, cross-linked platelets were washed before alkylation and compared to platelets treated by the standard method, without differences found in the two-dimensional electrophoretic pattern (data not shown).

DISCUSSION

We have shown that treatment of intact platelets with diamide or DTSP forms a variety of cross-linked protein complexes, about half of which involve identified, functionally important platelet proteins. The
major complexes after diamide treatment are myosin heavy chain dimers and high molecular weight material remaining at the origin of agarose-acrylamide gels. DTSP treatment also forms homopolymers and high molecular weight material containing membrane glycoproteins IIb and III. Differences in cross-linking patterns before and after thrombin activation may reflect several significant aspects of platelet protein organization.

The high yield of myosin heavy chain dimers, with minimal formation of larger oligomers, upon treatment of resting platelets with cross-linking reagents may indicate that myosin is mostly nonfilamentous before platelet activation. This conclusion is based on the known cross-linking pattern of skeletal muscle myosin. A high yield of cross-linked heavy chain dimers is produced by treatment of muscle myosin with DTSP or dimethyl dithiobis(propionimidate) in a high ionic strength solution in which the myosin is nonfilamentous. In contrast, treatment of muscle myosin thick filaments with dimethyl suberimidate or dimethyl dithiobis(propionimidate) forms higher oligomers by cross-linking between myosin heads and the thick filament surface. Our results are also consistent with electron microscopy of resting platelets, in which thick filaments have not been consistently seen, although Niederman and Pollard have calculated that the low concentration of myosin in the platelet would make visualization of thick filaments very difficult. Additional evidence for nonfilamentous myosin in resting platelets is of considerable interest since there is so little information from other methods concerning the state of platelet myosin in vivo.

By analogy with muscle myosin, increased yield of cross-linked myosin heavy chain dimers in activated

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**Table 2. Aggregation and Secretion of Gel-Filtered Platelets Under Cross-linking Conditions**

<table>
<thead>
<tr>
<th>Additives</th>
<th>Maximum Aggregation (%)</th>
<th>Serotonin Secretion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 U/ml thrombin</td>
<td>87</td>
<td>99</td>
</tr>
<tr>
<td>1.5 mg/ml fibrinogen and 10 μM ADP</td>
<td>55</td>
<td>28</td>
</tr>
<tr>
<td>0.45 mM diamide</td>
<td>7.5*</td>
<td>0.1</td>
</tr>
<tr>
<td>1 mM diamide</td>
<td>12*</td>
<td>0</td>
</tr>
<tr>
<td>0.15 mM DTSP</td>
<td>3.7</td>
<td>1.1</td>
</tr>
<tr>
<td>0.3 mM DTSP</td>
<td>5.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Spontaneous disaggregation occurred within 3 min of diamide addition.

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**Fig. 8.** Protein composition of whole platelets (A), a Triton X-100 precipitate of platelets activated with 0.5 U/ml thrombin–2 mM EDTA (B), and the high molecular weight complex of platelets activated with 0.5 U/ml thrombin–2 mM EDTA and then treated with 1.5 mM diamide for 5 min (C).
platelets is probably due to altered accessibility or proximity of intramolecular reactive groups. For example, ATP hydrolysis by muscle myosin is associated with changes in reactivity of two essential thiols in the myosin heads and of nonessential thiols probably located at the flexible junction of the head and rod.34

The cross-linked high molecular weight complex is not a random aggregate of proteins. Rather, it is enriched in cytoskeletal proteins, as indicated by comparison with a Triton precipitate of whole platelets. The 230,000-dalton protein found in the high molecular weight complex, but not the Triton precipitate, is a previously described platelet protein of unknown function.21 As for similar complexes produced by cross-linking of red cells,12,13,18,22 it is not clear at present whether the platelet high molecular weight complex is homogeneous (consisting of a single large polymer of covalently attached proteins) or heterogeneous (consisting of various high molecular weight polymers, such as actin or myosin homopolymers). If the complex is homogeneous, its formation upon cross-linking of resting or tetracaine-treated platelets may indicate that platelets contain some assembled cytoskeletal proteins even before activation. It appears more likely that at least part of the cross-linked high molecular weight complex is heterogeneous, in view of the extensive formation of platelet protein homopolymers by diamide or DTSP treatment. Also, incorporation of thrombospondin and fibrinogen into the high molecular weight complex upon DTSP treatment probably is an example of heterogeneity. Except for possible residual surface-bound plasma fibrinogen, these two proteins are contained in granules15,36 and thus are most likely cross-linked by DTSP into large homopolymers, rather than cross-linked to cytoplasmic proteins.

A major change in the high molecular weight complex upon cross-linking of thrombin-activated platelets is increased myosin incorporation. This may reflect (A) myosin assembly, (B) increased association of myosin with other cytoskeletal proteins, or (C) changes in protein associations within myosin filaments. The cross-linking results do not distinguish among these possibilities but, from data for muscle myosin, are most consistent with the first two. Changes in associations within filaments are least likely, since no differences have been found in the cross-linking patterns of muscle myosin thick filaments or glycinated myofibrils under resting or rigor conditions.31 There is some ultrastructural evidence for myosin assembly upon platelet activation, since structures that may be thick filaments have been seen in retracted clots, but not in resting platelets.31 Also, platelet activation is associated with myosin phosphorylation,37 and there is recent evidence that phosphorylation of platelet myosin in vitro induces assembly.38 Our results correlate well with data of Gonnella and Nachmias3 and Jennings et al.9 who describe a marked increase of myosin incorporation into Triton precipitates of activated platelets.

At present, it is difficult to interpret the increased incorporation of the 230,000, 55,000, and 52,000 dalton proteins into the high molecular weight complex after activation. The 230,000-dalton protein is not present in the Triton precipitate of activated platelets (Fig. 8) and, therefore, presumably does not become associated with the cytoskeleton. It is possible that activation is associated with other changes of the 230,000-dalton protein, such as state of polymerization or accessibility of reactive groups, resulting in increased cross-linking. Based on possible identity of the 55,000 and 52,000 dalton proteins and platelet tubulin, the cross-linking results may indicate closer association of tubulin with cytoskeletal proteins upon activation. The data probably do not reflect tubulin polymerization, since tubulin is already 57% polymerized in resting platelets and transiently depolymerizes upon activation.39

Actin is only minimally cross-linked into homopolymers and high molecular weight material, even after thrombin activation. This result is surprising in view of studies showing that several bifunctional sulphydryl- or amino-specific reagents can cross-link F-actin.40,41 We have shown that purified muscle G- and F-actin are resistant to cross-linking by diamide. We conclude that the results with intact platelets may be explained by inefficient cross-linking of actin. Formation of some platelet actin homopolymers by diamide treatment (Figs. 2 and 3) may be due to intermolecular cross-linking of G-actin, which is maintained at a very high intraplatelet concentration (10–40 mg/ml) by interaction with profilin.42 Poor cross-linking of intraplatelet actin by DTSP is not fully explained but may involve unknown factors, such as inability of the reagent to penetrate to intracellular sites of greatest actin concentration.

Cross-linking of activated, in comparison to resting, platelets with DTSP causes a larger amount of glycoproteins IIb and III to appear in the high molecular weight complex. This result may reflect increased intramembrane associations of these glycoproteins, analogous to ligand-induced surface receptor redistribution shown in lymphocytes and other cells.43 This possibility is consistent with the recent finding that glycoproteins IIb and III associate in vitro into a high molecular weight complex in the presence of calcium.44 The cross-linking results could also indicate increased associations of glycoproteins IIb and III with the cytoskeleton at the inner membrane face. Ligand-
induced membrane-cytoskeletal interactions are believed to occur in many cell types, of which lymphocytes are a well known example. Immunofluorescent studies show that actin and myosin accumulate IIb-III antibody.

There is some preliminary evidence for similar membrane-cytoskeletal interactions in platelets, since actin and glycoproteins IIb and III can be coeluted after application of Triton-solubilized platelet membranes to an affinity column containing an antiglycoprotein IIb-III antibody.

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Platelet protein organization: analysis by treatment with membrane-permeable cross-linking reagents

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