Disulfide-Linked and Transglutaminase-Catalyzed Protein Assemblies in Platelets

By Isaac Cohen, Chang T. Lim, David R. Kahn, Tova Glaser, Jonathan M. Gerrard, and James G. White

Energy depletion induces the formation of disulfide-linked and transglutaminase-catalyzed protein assemblies in platelets. The disulfide type polymers, formed following incubation at 37 °C in the absence of adenosine triphosphate (ATP)-generating precursors, are composed of cytoskeletal proteins and are associated with a decrease of reduced glutathione levels accompanying ATP depletion. The maintenance of ATP and reduced glutathione levels to, respectively, 34% and 47% of their original values is sufficient to prevent the formation of both polymer types. The transglutaminase-type cross-links are formed in the presence of calcium in either "energy-depleted" or thrombin-stimulated platelets. 125I-surface-labeled membrane proteins, presumably transmembrane proteins, are incorporated into the transglutaminase-catalyzed cross-linked polymer of thrombin-stimulated platelets. Glycoproteins Iib and Ilia are not essential to the polymer formation, since thrombosthenic platelets treated with thrombin exhibit the same type of labeled polymer. The transglutaminase-catalyzed polymer formation following thrombin stimulation of platelets is inhibited by a calcium channel blocker, an intracellular calcium antagonist, as well as other inhibitors such as indomethacin, dibutyryl cyclic AMP, and prostaglandin E1. Although the evidence points to the formation of transglutaminase-catalyzed cross-linking in the cytoplasmic compartment, additional cross-linking of extruded components cannot be excluded.

THE MAINTENANCE of a normal shape in cells such as erythrocytes or platelets has been known for more than a decade to depend on the ability of cells to maintain a steady state level of adenosine triphosphate (ATP). 1-3 Calcium uptake has also been implicated in preservation of cell form because it causes the loss of red cell membrane deformability. 4,5 Following ATP depletion, high mol wt polymeric assemblies of the disulfide type accompany spherocytocyte formation, and the changes in biochemistry and shape are reversed upon restoration of a normal ATP level. 1,4 Lowering of reduced glutathione, generally associated with depletion of ATP, was found to correlate with appearance of the disulfide type polymers. 6,7 When polymers were induced in platelets with sulfhydryl and amino-specific bifunctional reagents, they involved cytoskeletal proteins with a predominance of myosin and actin. 8

Irreversible types of polymers have been obtained in both red blood cells and platelets. When the cytoplasmic calcium concentration was substantially increased in red cells with the aid of the ionophore A23187, irreversible polymers of the (γ-glutamyl) lysine type resulting from the Ca2+-activation of erythrocyte transglutaminase were formed. 7,9 They were associated with the formation of stable echinocytes. 2,10 We demonstrated the formation of similar irreversible polymers in platelets, cross-linked with (γ-glutamyl) lysyl bridges upon ionophore-induced increase of the cytoplasmic calcium concentration. 11

In this study, our aim was to elucidate the type of protein–protein interactions that may potentially occur in platelets. We describe here the formation of two types of polymers, a disulfide-reversible type and an (γ-glutamyl) lysine-stable type that may have functional relevance in platelets.

MATERIALS AND METHODS

Platelet isolation. Blood was obtained from normal adult donors who gave a written informed consent and was mixed with acid-citrate-dextrose (ACD) (93 mmol/L sodium citrate, 7 mmol/L citric acid, 140 mmol/L dextrose) in a ratio of nine parts of blood to one part of the anticoagulant. All the steps for platelet isolation were carried out at room temperature. Platelet-rich plasma was obtained by centrifuging the citrated whole blood at 120 g for ten minutes. Contaminating red and white cells were sedimented by centrifuging the platelet-rich plasma at 120 g for five minutes. Occasionally, plateletpheresis was carried out using the Model 30 Cell Separator Blood Processor (Haemonetics Corp, Braintree, Mass). Six cycles (500 ml of blood each) were usually performed for collecting 1.2 × 1010 platelets. The platelet-rich plasma was adjusted to pH 7.4 and once in 0.1540 mol/L NaCl, 5.5000 mmol/L glucose, 6.6000 mmol/L EDTA, and 0.0154 mol/L HEPES pH 7.4 and once in 0.1540 mol/L NaCl, 1 mmol/L EDTA, 0.0154 mol/L HEPES pH 7.4. Platelets were sedimented by centrifugation at 1,000 g for 15 minutes. When necessary, the platelet suspension was centrifuged at room temperature at 120 g for five minutes to sediment contaminating red and white cells. The final pellet, consisting of nearly pure platelets, was then suspended in 5 mmol/L KCl, 2 mmol/L MgCl2, 0.15 mol/L NaCl, and 50 mmol/L glycylglycine pH 7.4. Platelets prepared in this fashion did not have the morphological alterations reported to occur in EDTA-collected blood incubated at 37 °C. 12

Gel filtration was performed according to Lages et al. 13 Fifty-milliliter polypropylene syringes were used as columns and fitted with 25-mm diameter polypropylene porous discs and o rings. Ca2+-free Tyrode buffer with 0.1% dextrose and 0.2% bovine serum albumin was used for equilibration and dilution. The last eluting platelet fractions were discarded in order to prevent plasma contamination. The gel-filtered platelets required the addition of fibrinogen for adenosine diphosphate (ADP)- or epinephrine-induced platelet aggregation. The platelet count averaged 2 × 108 per milliliter.

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When 125I-surface-labeled platelets were used, the columns were equilibrated and eluted with 0.15 mol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA pH 7.4.

**Energy depletion of platelets.** Washed platelets, in a final volume of 2 mL (3 x 10^10 platelets per milliliter) were incubated at 37 °C with 10 μL of a mixture containing 100 units penicillin, 10 μg streptomycin, 0.25 μg fungizone (GIBCO, Grand Island, NY). Where indicated, 2 mmol/L sodium phosphate buffer pH 7.0, 11 mmol/L glucose, 0.5 mmol/L adenosine, 12.7 mmol/L inosine, 1 mmol/L CaCl_2_, and 0.5 mmol/L leupeptin were also included.

**Platelet aggregation.** Human fibrinogen (0.32 mg/mL, IMCO, Stockholm) was added to gel-filtered platelets only when ADP (10 to 100 μmol/L, Sigma Chemical Corp, St Louis) or epinephrine (16.5 to 165.0 μmol/L) was used. α-Thrombin (2,000 U/mL) used at concentrations ranging from 0.05 to 0.25 U/mL was kindly provided by Dr John Fenton (Albany, NY). Collagen (0.5 to 5.0 μg/mL) was prepared from human skin. When the calcium ionophore A23187 (5 μmol/L, Calbiochem, San Diego) was used, the control experiments contained an equivalent volume of its solvent, dimethyl sulfoxide. Where indicated, CaCl_2_, leupeptin (Peptide Research Institute, Osaka, Japan), ([14C]-histamine (59.7 Ci/mol), indomethacin (Merck, West Point, Pa), dibutyl cyclic adenosine monophosphate (AMP) (Sigma), prostaglandin E1, verapamil, or 8-(N,N-diethylamino)-octyl 3,4,5 trimethoxybenzoate (TMB-8) were also included prior to the platelet agonists. The concentrations of the platelet activators indicated were final in gel-filtered platelets. Activation proceeded in polypropylene tubes with stirring at 37 °C for three minutes and followed by incubation at 37 °C without stirring for the specific incubation times. Following aggregation, the platelets were washed twice in 0.15 mol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA pH 7.6 and dissolved in the electrophoresis sample buffer.

**Platelet release reaction.** The platelet release reaction was assessed by measuring the release of platelet [14C]-serotonin as described by Jerushalmy and Zucker, modified by including 12.5 μmol/L imipramine to prevent the reuptake of the released serotonin.

**Platelet surface labeling.** The 125I-surface-labeling procedure using lactoperoxidase and glucose oxide, a modification of the original lactoperoxidase-catalyzed iodination using H2O2, appeared to be mild enough to allow a normal response of platelets to aggregating agents. The gel-filtered platelets were centrifuged, washed once in 0.15 mol/L NaCl, 0.01 mol/L Na phosphate-buffered saline pH 7.4 (PBS), and resuspended in PBS containing 20 mmol/L dextrose. The freshly prepared PBS-dextrose was kept overnight at room temperature before use to allow the dextrose to aggregate agents. The gel-filtered platelets were centrifuged, the platelets were washed twice in 0.15 mol/L NaCl, 20 μmol/L Tris, 1 mmol/L EDTA pH 7.6 and dissolved in the electrophoresis sample buffer.

**Gel electrophoresis.** SDS-polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed in a Bio-Rad Laboratories (La Jolla, Calif), Model 220 apparatus, using Laemmli’s discontinuous system for platelet proteins with an exponential gradient of acrylamide from either 6% to 15% or 8% to 15%. The stacking gel contained 3% of acrylamide, and the thickness of the gel was 1.5 mm. Prior to electrophoresis, the washed platelet pellets were boiled for five minutes in a sample buffer mixture of 3% SDS, 5% glycerol, 1 mmol/L EDTA, 5 mmol/L N-ethylmaleimide, 62.5 mmol/L Tris pH 6.8. Then 8 mol/L urea was added to the sample buffer mixture but not to the gel, to permit quantitative solubilization of the thrombin-stimulated platelets. The samples were centrifuged at 12,000 g in an Eppendorf microcentrifuge (model 5412; Eppendorf, Brinkman, Westbury, NY), and the supernatants were diluted ten times in 0.005 mol/L EDTA, 0.1 mol/L Na phosphate buffer pH 8.0. Samples were deproteinized with 0.2 mL of 25% metaphosphoric acid and left for ten minutes in ice. Following sonication for ten seconds at power 3 with a standard microtip (Heat Systems Ultrasonics, Plainview, NY, Sonicator model W-225R), the samples were centrifuged for six minutes at 12,000 g in an Eppendorf microcentrifuge (model 5412; Eppendorf, Brinkman, Westbury, NY), and the supernatants were diluted ten times in 0.005 mol/L EDTA, 0.1 mol/L Na phosphate buffer pH 8.0. Then 0.1 mL of o-phenthaldehyde reagent was added to 1.9 mL of the diluted supernatant, and fluorescence at 420 nm was determined with activation at 350 nm in an Amino Bowman Spectrophotofluorimeter (Urbana, III). Known concentrations of reduced glutathione (GSH) (Boehringer, Mannheim, NY) were assayed in the same conditions. The concentration of the standard GSH solution was determined by the 5,5'-Dithiobis (2-nitrobenzoic acid) procedure using a molar extinction coefficient of 13,600 in the conditions specified by Beutler.

**Leakage of platelet factor XIII.** The method used to measure leakage of the cytosolic transglutaminase from platelets proved to be more sensitive than the determination of lactic dehydrogenase activities usually carried out for probing platelet lysate. The leakage of the cytosolic transglutaminase from supernatants of platelets was determined by its ability to catalyze the [14C]-histamine incorporation into dimethyl casein in a filter paper assay. To determine the percentage of the transglutaminase leaked, a reference standard was obtained by sonicating platelets for 15 seconds at maximal amplitude (Heat Systems Ultrasonics Sonicator, model W-225R) and designating the lysate as having 100% activity.
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There was no appreciable change in pH (7.4 to 7.3) during incubation for 18 hours at 37 °C under our experimental conditions. A polymer was detected under nonreduced conditions only after 18-hour incubation at 37 °C and was associated with a diminution in intensity of most monomeric protein bands (Fig 1, gel 2, left panel), probably contributing to the polymer formation. Polymer formation and disappearance of monomeric protein bands were prevented by the presence of glucose, adenine, and inosine (Fig 1, gel 3, left panel). Under reduced and alkylated conditions, no polymer was apparent (Fig 1, right panel), defining, therefore, the disulfide-type linkage of the polymer in nonreduced conditions. The staining pattern of monomeric bands in the presence of glucose, adenine, and inosine in nonreduced conditions and in the presence or absence of these ATP-generating precursors in reduced and alkylated conditions is similar to that of the control nonincubated platelets. This indicates that the diminution in intensity of the monomeric bands seen in nonreduced conditions after 18-hour incubation is mainly due to their contribution to the disulfide-linked polymer. Following platelet incubation at 37 °C in the absence of glucose, adenine, and inosine, the concentrations of total ATP, ADP, and reduced glutathione (GSH) in the incubation mixture were gradually decreased, reaching after 18 hours respective levels of 7%, 16% and 8% of the normal values in nonincubated platelets. Under the same conditions, but in the presence of glucose, adenine, and inosine, the concentrations of ATP, ADP, and reduced glutathione reached after 18 hours respective levels of 34%, 60%, and 47% of the normal values (Table 1). Noteworthy is the diminished release of serotonin in the presence of ATP-generating precursors.

The disulfide-linked polymers were isolated in Sepharose CL-4B in an SDS-containing buffer and under nonreduced conditions (Fig 2). The electrophoretic profile of the eluted fractions (Fig 2, insert) indicated that at least two sizes of polymeric assemblies were separated in the first asymmetrical peak (Fig 2, fractions 30 to 47): An apparent mass of above 10^6 daltons at the top of the stacking gel, defined as A, and corresponding to the major polymer of fractions 30 to 36 and an apparent mass of about 5 x 10^5 daltons near the top of the running gel, defined as B, and corresponding to the major polymer of fractions 42 to 47. Following the elution of the polymers, the other fractions were eluted in order of decreasing mol wt. Fractions containing each of the protein entities, A and B, were pooled, concentrated, and analyzed by SDS-

RESULTS

Disulfide-linked protein assemblies. Platelets incubated in a synthetic medium devoid of ATP-generating precursors reveal the formation of disulfide-linked polymers. The protein assemblies were analyzed by SDS gel electrophoresis after three, six, nine, and 18 hours' incubation at 37 °C.

![Fig 1. SDS-PAGE of incubated platelets in the absence of calcium. Platelets were incubated at 37 °C for 0 hours (control, gel 1), 18 hours (gel 2), and 18 hours in the presence of 2 mmol/L phosphate, 11 mmol/L dextrorotatory, 0.5 mmol/L adenine and 12.7 mmol/L inosine (gel 3). Left panel, nonreduced conditions, a polymer fraction (P) is present in gel 2; right panel, reduced and alkylated conditions, no polymeric band was apparent.](http://www.bloodjournal.org)<br>

<table>
<thead>
<tr>
<th>Incubation Time, Hours</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>18</th>
</tr>
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<tbody>
<tr>
<td>G, A, I Addition*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>ATP (μmol · 10^11 platelets)</th>
<th>4.86</th>
<th>5.08</th>
<th>2.89</th>
<th>5.23</th>
<th>1.78</th>
<th>3.61</th>
<th>0.33</th>
<th>1.73</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (μmol · 10^11 platelets)</td>
<td>4.78</td>
<td>5.04</td>
<td>3.58</td>
<td>3.52</td>
<td>2.80</td>
<td>3.88</td>
<td>0.77</td>
<td>3.05</td>
</tr>
<tr>
<td>GSH (nmol · 10^11 platelets)</td>
<td>19.64</td>
<td>19.62</td>
<td>15.60</td>
<td>18.04</td>
<td>14.27</td>
<td>16.69</td>
<td>1.63</td>
<td>9.25</td>
</tr>
<tr>
<td>[14C]-serotonin release (%)</td>
<td>9.40</td>
<td>4.60</td>
<td>14.90</td>
<td>9.30</td>
<td>47.70</td>
<td>19.50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results are representative of three experiments.

*G, glucose, 11.0 mmol/L; A, adenine, 0.5 mmol/L; I, inosine, 12.7 mmol/L.
Isolation of disulfide-type polymers. Platelets were incubated for 18 hours at 37 °C in the absence of Ca\(^{2+}\), phosphate, glucose, adenine, and inosine and processed for gel filtration on Sepharose CL-4B in the presence of sodium dodecyl sulfate and under nonreduced conditions (Materials and Methods). The insert corresponds to the SDS-PAGE pattern of fractions eluted from the Sepharose CL-4B column. Electrophoresis was conducted under nonreduced conditions. (A) >10^6-dalton polymer; (B) 5 × 10^4-dalton polymer.

PAGE under nonreduced (nr) and reduced (r) conditions (Fig 3). Each pooled polymer fraction was contaminated with a minor portion of the other. The polymer A fraction was dissociated under reduced conditions into several monomeric components with a predominance of bands corresponding in mol wt to myosin, thrombospondin, and actin. The polymer B fraction, on the other hand, was dissociated under reduced conditions into several monomeric bands with a predominance of myosin, actin, and a 130,000-dalton band (band 5). The different distribution of the polymer components was confirmed in a two-dimensional nonreduced/reduced and alkylated SDS-PAGE of platelets incubated in the absence of ATP-generating precursors (Fig 4). Table 2 shows the presumptive identity of the polymer components and their different distribution in the two sizes of assembly. The autoradiographic pattern of [\(^{14}\)C]-surface-labeled platelets on a nonreduced/reduced and alkylated two dimensional gel, does not reveal labeled disulfide polymers induced by energy depletion.

Calcium-induced formation of irreversible protein assemblies. Calcium and the protease inhibitor leupeptin, added at respective concentrations of 1 mmol/L and 0.5 mmol/L to the platelet incubation medium affected neither the levels of ATP and reduced glutathione reported in Table 1 nor the disulfide-type polymerization pattern. When platelets were incubated for 18 hours at 37 °C, electrophoresis under nonreduced conditions showed polymers A and B in all samples devoid of glucose, adenine, and inosine (Fig 5, gels 2, 4, 6, 8, upper panel). Under reduced and alkylated conditions, the presence of an undissociated polymer was apparent mainly in samples containing calcium and only in the absence of glucose, adenine, and inosine (Fig 6, gels 4 and 8, upper panel). In order to determine whether the formation of the undissociated polymer was transglutaminase-dependent, platelets were incubated in the presence of tracer concentrations of [\(^{14}\)C]-histamine, a pseudodonor amine known to be enzymatically incorporated into glutamine acceptor sites. The stained protein pattern was not affected by [\(^{14}\)C]-histamine. Under nonreduced conditions, the fluorographic pattern reveals the presence of a strongly labeled polymer in the presence of Ca\(^{2+}\), provided glucose, adenine, and inosine are absent (Fig 5, gels 4 and 8, lower panel). Under reduced and alkylated conditions, [\(^{14}\)C]-histamine was similarly incorporated into the undissociated polymers A and B (Fig 6, gels 4 and 8, lower panel). When gels were overloaded, the incorporation of [\(^{14}\)C]-histamine was also apparent in bands corresponding to the relative electrophoretic mobility of actin-binding protein, myosin, thrombospondin, actin and other nonidentified bands. This indicates that substrates for the platelet transglutaminase are associated with transglutaminase-catalyzed bonds as well as with disulfide bonds. The transglutaminase-type polymer formation was prevented by the presence of 5 mmol/L histamine.

Since platelet stimulating agents induce an active consumption of metabolic ATP associated with Ca\(^{2+}\)-uptake, their role in the formation of transglutaminase-catalyzed cross-links in the presence of Ca\(^{2+}\) was investigated. When gel-filtered platelets were aggregated with \(\alpha\)-thrombin in the
presence of CaCl\textsubscript{2} and \textsuperscript{14}C-histamine, polymer bands separated by SDS-PAGE were found under reduced and alkylated conditions on top of the stacking and separating gels and were visualized on fluorograms by the incorporation of the radioactive label into the polymer bands. When α-thrombin was used at a concentration of 0.05 U/mL gel-filtered platelets, a polymer was apparent within 15 minutes of incubation at 37 °C following aggregation. Figure 7 (right panel) shows the increase in the labeled polymer formation as α-thrombin concentrations increased from 0.025 to 0.250 U/mL. The calcium concentration used was 1 mmol/L. Stirring, which induced the formation of large platelet aggregates, was not essential for polymer formation. The increase in density of three bands of 105,000, 72,000, and 59,000 mol wt following staining with Coomassie brilliant blue (Fig 7, left panel) suggests that a proteolytic process or low mol wt polymer formation accompanies the high mol wt protein assemblies. These bands also incorporate \textsuperscript{14}C-histamine (Fig 7, right panel). No polymer was formed in the absence of either Ca\textsuperscript{2+} or thrombin. A minimum of 0.05 to 0.10 mmol/L Ca\textsuperscript{2+} was found necessary for polymer formation. ADP and epinephrine used at concentrations up to 165 μmol/L under stirring conditions and in the presence of 1 mmol/L Ca\textsuperscript{2+} did not induce detectable polymer formation following platelet aggregation and showed minimal release of serotonin. Collagen at a concentration of 5 μg/mL gel-filtered platelets showed a faint polymer band after two-hour incubation at 37 °C.

In order to ascertain whether membrane proteins exposed at the platelet surface contribute to the transglutaminase-catalyzed polymer formation, surface \textsuperscript{125}I-radiolabeled platelets were aggregated with α-thrombin in the presence of 1 mmol/L Ca\textsuperscript{2+}. Figure 8 (gel 2, right panel) shows an \textsuperscript{125}I-labeled polymer in the thrombin-aggregated platelets. A fivefold increase in the labeling of the polymer area was observed when compared to the equivalent area of the control nonaggregated platelets. The same pattern was obtained in platelets from thrombasthenic patients activated with thrombin (gel 4, right panel). These results suggest that although membrane proteins contribute to the transglutaminase-catalyzed polymer formation, glycoproteins IIb and IIa are not essential for this process. Non-surface-labeled thrombasthenic platelets treated with thrombin in the presence of Ca\textsuperscript{2+} and \textsuperscript{14}C-histamine generated a labeled polymer similarly to normal platelets.

### Table 2. Identification of Disulfide-Type Polymers Formed in Incubated Platelets

<table>
<thead>
<tr>
<th>Mol Wt of Reduced Components (x 10\textsuperscript{-1})</th>
<th>Presumptive Identity</th>
<th>Location in S–S Polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (&gt;10\textsuperscript{6} Mol Wt)</td>
<td>B (5 x 10\textsuperscript{6} Mol Wt)</td>
</tr>
<tr>
<td>1. 250</td>
<td>Actin-binding protein</td>
<td>+</td>
</tr>
<tr>
<td>2. 200</td>
<td>Myosin</td>
<td>+</td>
</tr>
<tr>
<td>3. 160</td>
<td>Thrombospondin</td>
<td>+</td>
</tr>
<tr>
<td>4. 150</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>5. 130</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>6. 100</td>
<td>GP IIa and/or α-actinin</td>
<td>±</td>
</tr>
<tr>
<td>7. 70</td>
<td>Fibrinogen α</td>
<td>±</td>
</tr>
<tr>
<td>8. 58 and 53</td>
<td>Fibrinogen β and γ (and/or tubulin)</td>
<td>±</td>
</tr>
<tr>
<td>9. 43</td>
<td>Actin</td>
<td>+ + +</td>
</tr>
</tbody>
</table>
Fig 5. SDS-PAGE pattern of incubated platelets under nonreduced conditions: effect of Ca\(^{2+}\), glucose (G), adenine (A), inosine (I) and leupeptin (LEU). Samples containing 11.0 mmol/L G, 0.5 mmol/L A, and 12.7 mmol/L I also contained 2 mmol/L phosphate. Gel 1, control nonincubated platelets. G, A, and I were present in samples 3, 5, 7, and 9. Ca\(^{2+}\) (1 mmol/L) was present in samples 4, 5, 8, and 9. LEU (0.5 mmol/L) was present in samples 6 to 9. (A) >10^5-Dalton area; (B) 5 x 10^5-dalton area. Under panel, protein staining pattern; lower panel, fluorography. Same experimental conditions with the addition of 0.08 mmol/L \[^{14}C\]-histamine to all samples.

mmol/L, and 0.028 mmol/L inhibited thrombin-induced polymer formation by 65%, 81%, and 92%, respectively (Table 3). Polymer formation was also inhibited by 35% with 0.05 mmol/L of the Ca\(^{2+}\)-channel blocker verapamil and by 69% with 100 μmol/L of the Ca\(^{2+}\)-antagonist TMB-8 and was dependent on the Ca\(^{2+}\) concentration in the incubation mixture. Whereas inhibition was achieved using 0.1 mmol/L Ca\(^{2+}\) and concentrations of verapamil and TMB-8, respectively, of 50 μmol/L and 100 μmol/L, it could be overcome with higher calcium concentrations. At the concentrations used, indomethacin and verapamil do not affect platelet aggregation or release, whereas dibutyl cyclic AMP, PGE, and TMB-8 inhibit thrombin-induced platelet aggregation and release.

Leakage of factor XIII in thrombin-stimulated platelets. In order to determine whether the presumed factor XIII-dependent polymer formation occurs intracellularly, factor XIII activity was determined in the supernatant of platelets aggregated with various α-thrombin concentrations ranging between 0.05 to 0.25 U/mL. The percentage of factor XIII leakage varied between 1% and 3% with all thrombin concentrations. In order to determine whether this amount of leaked factor XIII could cross-link surface proteins of intact platelets, 2 mL of gel-filtered platelets were incubated for two hours at 37 °C with 2 mL of supernatant originating from thrombin aggregated platelets (0.15 U α-thrombin/mL) in the presence of 1 mmol/L Ca\(^{2+}\) and 0.08 mmol/L \[^{14}C\]-histamine. Before mixing with the gel-filtered platelets, the thrombin activity of the supernatant was quenched with two inhibitory equivalents of hirudin. No polymer was formed as revealed by the absence of \[^{14}C\]-label in the gels, suggesting that the amount of factor XIII leaked from thrombin-aggregated platelet did not induce detectable cross-linked surface proteins.

DISCUSSION

The formation of disulfide-linked polymers in energy-depleted platelets is associated with reduced levels of ATP and reduced glutathione. Proteolysis was not a factor in polymer formation since leupeptin, a potent inhibitor of calcium-dependent proteolysis\[^1\] that enters cells,\[^29\] had no effect on polymer production. The two sizes of polymers characterized on the basis of their different apparent masses were isolated by gel filtration. However, due to the limits of resolution of this procedure, the presence of a mixture of different aggregates in each polymeric size cannot be ruled out. The disulfide-mediated associations of proteins may be responsible for the deterioration of platelet discoid shape, which is accompanied by an extensive vacuolization in aged platelets. These anomalies are prevented if adequate levels of ATP and reduced glutathione are maintained in the presence of glucose, adenine, and inosine (unpublished observations, March 1983).

When calcium is present in the platelet incubation mixture, the polymer that forms under nonreduced conditions is not dissociated under reduced and alkylated conditions. This polymer forms if platelets are either incubated at 37 °C in the absence of an ATP-regenerative system or stimulated with strong platelet agonists such as thrombin. The incorporation of \[^{14}C\]-histamine into the nondissociated polymer indicates the transglutaminase-catalyzed nature of its formation. Histamine, which is freely taken up by the platelet cytosol,\[^30\] serves as a pseudodonor amine and as such inhibits transglutaminase-catalyzed cross-linking. When used at a tracer concentration of 0.08 mmol/L, however, histamine is incorporated into the free glutamine sites of the polymer.
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Fig 7. SDS-PAGE pattern of thrombin-induced platelet aggregates in the presence of 1 mmol/L Ca\textsuperscript{2+} and 0.08 mmol/L \[^{3}C\]-histamine as a function of thrombin concentration. Platelets were incubated for 15 min at 37 °C. The washed platelet aggregates were quantitatively dissolved in an SDS mixture, provided that it contained 8 mol/L urea. Samples were reduced and alkylated. Urea was not present in the acrylamide gel. The mol wt determinations were performed by comparison with the relative electrophoretic mobility of mol wt standards run in the absence of 8 mol/L urea. Due to the presence of 8 mol/L urea in the sample mixture, anomalous migration was observed with some fractions. Left panel, protein staining; right panel, fluorographic pattern.

and enhances its detection. We have recently shown in platelets that the increase in the cytosolic calcium concentration by the ionophore A23187 contributes to the activation of a platelet transglutaminase. This resulted in the formation of \(\gamma\)-lysyl bridges\textsuperscript{11} cross-linking cytoskeletal and membrane proteins. We speculated that the cleavage of platelet factor XIII, necessary for the Ca\textsuperscript{2+}-induced exposure of the reactive sulfhydryl group, is catalyzed by a cytosolic protease. In our present studies, the depletion of ATP may contribute to the weakening of the calcium pump, resulting in the leakage of extracellular calcium, when present, into the platelet cytosol and the subsequent activation of factor XIII. The maintenance of ATP and reduced glutathione levels—at 34% and 47% of their original values, respectively, brought about by the presence of glucose, adenine, and inosine—prevented the formation of nondissociated polymers in incubated platelets. This may be due either to the effectiveness of the calcium pump and/or to the prevention of transglutaminase-catalyzed cross-linking by ATP.\textsuperscript{31} The strengthening of monomeric electrophoretic bands in thrombin-stimulated platelets in association with the polymer formation confirms the observation of Ca\textsuperscript{2+}-dependent proteolysis in thrombin-stimulated platelets.\textsuperscript{32} Membrane proteins, presumably transmembrane proteins, appear to be a component of the cross-linked polymer, since the latter bears the same radioactive label as that of surface-labeled platelet glycoproteins. Thrombasthenic platelets activated by thrombin show the same amount of labeled polymer as normal platelets, whether \[^{14}C\]-histamine was included in the reaction mixture or \(^{125}\)I-surface-labeled platelets were

Fig 8. Thrombin-induced polymer formation in \(^{125}\)I-surface-labeled platelets of a normal control and a thrombasthenic patient. Samples 1 and 3, no thrombin added, respectively, in normal and thrombasthenic samples; samples 2 and 4, thrombin (0.25 U/mL)-treatment of, respectively, normal and thrombasthenic platelets. Platelets were incubated for 15 min at 37 °C and 1 mmol/L Ca\textsuperscript{2+} was present in all samples. Area of polymer (P) of dried gels counted in a \(\gamma\)-counter and indicated on top of right panel. Eight-molar urea present in the sample mixture. Reduced and alkylated conditions. Note that glycoprotein Ila coelectrophoreses with IIb, due to the presence of 8 mol/L urea in the sample.\textsuperscript{28} Left panel, protein staining; right panel, autoradiography.
used. Glycoproteins IIb and IIIa are therefore not an essential component of the cross-linked polymer formed.

The thrombin-induced polymer formation in platelets must be associated with the uptake of Ca\(^{2+}\) occurring already at the early stages of the shape change\(^{11}\) and through the release reaction.\(^{34,38}\) In order to test this hypothesis, verapamil, a calcium channel blocker and TMB-8 (8-[N,N-diethylamino] octyl-3,4,5-trimethoxybenzoate), an intracellular calcium antagonist\(^{37}\) were included in the platelet mixture before the addition of thrombin. The inhibition of the polymer formation induced by these two agents is suggestive of cross-linking being mainly an intracellular event. The inhibition of the thrombin-induced polymer formation by various agents does not necessarily follow inhibition of platelet aggregation and secretion. Indeed, whereas indomethacin and verapamil do not affect thrombin-induced platelet aggregation and secretion, they inhibit the polymer formation to a certain extent. Dibutyryl cAMP, PGE\(_1\), and the Ca\(^{2+}\)-antagonist TMB-8 are strong inhibitors of platelet aggregation, secretion, and polymer formation.

### Table 3. Inhibition of Polymer Formation

<table>
<thead>
<tr>
<th>Inhibitors, mmol/L</th>
<th>Polymer % Label ± SD (Mean of Three Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin, 0.050</td>
<td>36 ± 5.0</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP, 1.000</td>
<td>19 ± 2.5</td>
</tr>
<tr>
<td>Prostaglandin E, 0.028</td>
<td>8 ± 1.5</td>
</tr>
<tr>
<td>Verapamil, 0.050</td>
<td>65 ± 5.0</td>
</tr>
<tr>
<td>TMB-8, 0.100</td>
<td>31 ± 3.5</td>
</tr>
</tbody>
</table>

Gel-filtered platelets were stimulated with 0.25 U/mL α-thrombin in the absence or in the presence of inhibitors and incubated for one hour at 37 °C. Ca\(^{2+}\) was present at a concentration of 0.1 mmol/L when verapamil and TMB-8 were included and 1 mmol/L for the other inhibitors in both experiments and controls. All samples contained 0.08 mmol/L. \(^{14}C\)-hisfamine. Platelets were then washed, electrophoresed, stained, cut, and counted as described in Materials and Methods. Results are given in the percentage of label relative to the control reaction in the absence of inhibitors.

The percentage of transglutaminase leaked following thrombin-stimulation in the presence of Ca\(^{2+}\) confirms the extent of platelet lysis reported with thrombin used as a stimulating agent.\(^{34}\) The low concentrations of factor XIII leaked were unable to catalyze the formation of detectable polymers in nonactivated gel-filtered platelets. This finding, coupled with the formation of an \(^{125}\)I-labeled polymer following aggregation of surface radioiodinated platelets with thrombin suggests that cross-linking occurs in the cytoplasmic compartment. The possibility of an additional extracellular cross-linking involving released platelet components cannot be ruled out. Leakage of 1% to 3% cytosolic components observed in our experimental conditions is a possible in vivo event, since membrane gaps have been observed in platelet clumps associated with human skin wounds.\(^{9,42}\)

Both disulfide- and \((\gamma\text{-glutamyl})\) lysine-linked proteins generate high mol wt assemblies in platelets upon incubation or platelet activation. The components of the disulfide-type polymers involve actin, myosin, thrombospondin, and glycoproteins. Those of the transglutaminase-type cross-links are yet to be determined. The two types of protein associations should be considered stable in vivo as suggested by Matacic and Loewy,\(^{41}\) the biological role of cross-links could be to Vulcanize structural proteins as well as membrane proteins. Above a threshold concentration, these cross-links may stabilize the shape change, preventing further platelet participation in hemostatic processes. The occurrence of high mol wt assemblies involving cytoskeletal and membrane components may explain the irreversibility of "old" platelet aggregates when subjected to strong disaggregating agents such as prostacyclin.\(^{42,43}\) While "young" platelet thrombi, generated by weak inducers such as ADP, disaggregate easily upon infusion of prostacyclin,\(^{44}\) "old" thrombi, especially if generated by strong inducers such as thrombin, cannot disaggregate.

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