Reversibility of the Association of Fibrinogen With Rabbit Platelets Exposed to ADP

By Elizabeth J. Harfenist, Marian A. Packham, and J. Fraser Mustard

Fibrinogen enhances the adenosine diphosphate (ADP) induced aggregation of washed rabbit platelets and has previously been shown to associate with platelets upon the addition of ADP. This association has been investigated further by measuring the binding of 125I-fibrinogen to platelets exposed to ADP under physiologic conditions of aggregation and deaggregation. To determine the extent of 125I-fibrinogen binding, the platelets were removed by rapid centrifugation and the radioactivity in the platelet pellet was measured; trivalent chromium-51 was used as a space marker. If fibrinogen is present before ADP is added, the fibrinogen binds maximally to the platelets as they change shape in response to ADP. When ADP is not removed from the platelet suspension, deaggregation is slow, and fibrinogen also binds when added shortly after the ADP, but the amount that binds is less. The ability of the platelets to bind fibrinogen upon exposure to ADP is lost rapidly if an enzyme that removes the ADP is present in the suspension. A large portion of the 125I-fibrinogen bound to platelets during ADP stimulation, can be displaced by unlabeled fibrinogen added shortly after the ADP. Bound fibrinogen dissociates from platelets when they deaggregate upon removal of the ADP stimulus, and characterization of this fibrinogen by SDS-PAGE did not show any change resulting from its reactions with platelets. If the platelets are allowed to recover and then are stimulated a second time with ADP, they aggregate and bind fibrinogen to the same extent as platelets not previously aggregated with ADP. Thus, fibrinogen binding sites become unavailable when platelets are exposed to ADP, become unavailable within a short time, and, if conditions are such that the platelets can recover, fibrinogen binding sites may become available again upon subsequent addition of ADP. It appears that neither the fibrinogen nor the fibrinogen binding site is permanently altered during the processes of aggregation and deaggregation.

Although adenosine diphosphate (ADP) plays an important role in platelet aggregation, its mode of action is not fully understood. It has been known for many years that fibrinogen is required to support ADP-induced aggregation of human platelets and must be added to suspensions of washed human platelets for aggregation to occur in response to ADP. Washed rabbit platelets, on the other hand, aggregate in response to ADP without added fibrinogen, but their aggregation is enhanced by the addition of fibrinogen. Mustard et al. have shown, by using 125I-fibrinogen, that fibrinogen becomes associated with human or rabbit platelets during ADP-induced shape change and aggregation under conditions that do not cause release of granule contents. This association takes place as the platelets change shape immediately after the addition of ADP, and when apyrase is present in the suspension to degrade the ADP, the radioactive material is lost rapidly from the platelets as they deaggregate. More recently, several investigators have studied the reaction of fibrinogen with human platelets and have agreed with the suggestion of the presence of fibrinogen binding sites or receptors on the surface of platelets stimulated with ADP. However, few of them have recognized the transient nature of the availability of the fibrinogen binding sites during ADP-induced aggregation, nor the necessity to define the conditions under which binding is studied.

In this article, we present further data on the nature of the ADP-induced binding of fibrinogen to rabbit platelets and on its relationship to platelet aggregation and deaggregation. We have investigated the following points: (1) the conditions under which the fibrinogen binding sites on the platelet surface become available; (2) the conditions under which the fibrinogen binding sites become unavailable; (3) the effect of the association and dissociation reactions of fibrinogen with platelets on the platelet receptor for fibrinogen, as determined during a second ADP stimulation; and (4) the effect of the association and dissociation reactions on the fibrinogen that has been bound to platelets.

MATERIALS AND METHODS

Human fibrinogen (grade L) from AB Kabi, Stockholm, Sweden, was adsorbed with Al(OH)3 to remove contaminating procoagulants. In some experiments, the fibrinogen was treated with diisopropyl fluorophosphate as described previously, but since this treatment did not affect the results, it was omitted in later experiments. Human thrombin (lot H-I), a gift from Dr. D.L. Aronson, Bureau of Biologics, USDA, Bethesda, Md., was dissolved in water at 100 U/ml and stored at -20°C. Apyrase, prepared by the method of Molnar and Lorand, was dissolved in 0.15 M NaCl solution and stored at -20°C. At a concentration of 9 μl/ml, it caused 91% conversion of 9 μM ADP to AMP and adenosine in 2 minutes.

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min at 37°C. ADP, diisopropyl fluorophosphate, creatine phosphate, creatine phosphokinase, gelatin (type 1, approximately 300 Bloom), and adenosine deaminase (type 1) were from Sigma Chemical Co., St. Louis, Mo. Bovine albumin (Pentex Fraction V) was from Miles Laboratories Inc., Elkhart, Ind. Al(OH)₃ was C₇Gel from Calbiochem, San Diego, Calif. Trasylol was from Boehringer Ingelheim (Canada) Ltd., Dorval, P.Q. Na¹²⁵I (carrier free, NEZ 033L) and Na¹⁰³CrO₄ (specific activity 200–500 Ci/g Cr, NEZ 030) were from New England Nuclear, Boston, Mass. All other reagents were analytical grade.

**Preparation of Platelet Suspensions**

Suspensions of washed rabbit platelets were prepared essentially as described by Ardlie et al.¹⁰¹¹ The final suspending medium was Tyrode solution containing 3.5 mg/ml of albumin (Tyrode-albumin). Final platelet counts in typical experiments were in the range of 5.7 – 8 x 10⁹/µl. Apyrase was added to some suspensions at 0.25 – 1.0 µl/ml to degrade any ADP released and stabilize the platelets. Rapid deaggregation of platelets, within 2 min of the addition of ADP, was accomplished by adding to the platelet suspension either apyrase at 9 – 10 µl/ml or creatine phosphate and creatine phosphokinase (CP/CPK) at 1.6 mM and 0.6 U/ml, respectively.¹² Suspensions without apyrase or CP/CPK were kept at room temperature, and aliquots were transferred to aggregometer cuvettes and incubated at 37°C for 3 min just before use. Suspensions containing apyrase were incubated at 37°C for the duration of the experiment. Since adenosine acts as an inhibitor of platelet aggregation, adenosine deaminase was added to some of the suspensions to destroy any adenosine produced by the degradation of ADP.¹⁵ Aggregation of platelets was studied by measuring light transmission through a stirred suspension in an aggregation module (Payton Associates, Scarborough, Ontario, Canada). The baseline was adjusted so that the initial decrease in light transmission due to shape change upon addition of the aggregating agent could be observed, and the sensitivity was adjusted so that the change in light transmission when platelets were stimulated with thrombin (1–2 U/ml) was approximately 80% of full scale. In aggregation studies, platelets were stirred at 1100 rpm, but in some of the binding studies, in order to minimize aggregation, the platelets were stirred at a slower rate (85 rpm), just sufficient to mix the reactants. All substances added to the platelet suspensions were dissolved in 0.15 M NaCl solution, and concentrations are expressed as final concentrations after all additions.

**Determination of ¹²⁵I-Fibrinogen Bound to Platelets**

The amount of ¹²⁵I-fibrinogen bound to platelets was determined as described by Mustard et al.,¹⁴ by rapid centrifugation for 1 min in an Eppendorf centrifuge and measurement of the radioactivity of the platelet pellet in a well-type γ-counter. Since it had been shown that rabbit and human fibrinogens were equally effective in binding to rabbit platelets, and since human fibrinogen is more readily available, it was used throughout this study. Trivalent ¹⁰³Cr was used as a space marker to measure trapped fluid in the pellet.¹³ Unless otherwise stated, all reagents were added before the ADP, and samples were centrifuged approximately 0.2 min after the addition of ADP. When this was not the case, the appropriate experimental details are given in the footnotes of the tables. After the contents of an aggregometer cuvette were transferred to a centrifuge tube the cuvette was washed with 1 ml of 0.15 M NaCl solution, the radioactivity in the washing fluid was determined, and the appropriate correction was made in calculating the amount of ¹²⁵I bound to the platelets.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was performed by a slight modification²⁰ of the method of Weber and Osborn.²¹ In cases where samples were to be reduced, β-mercaptoethanol was added to the urea-SDS incubation mixture at a concentration of 20 µl/ml.

**Characterization of ¹²⁵I-Fibrinogen**

Fibrinogen was labeled using the iodine monochloride method of McFarlane²² and conditions were selected so that the final degree of substitution was approximately 0.5 atoms of iodine per molecule of fibrinogen. The product was dialyzed against 0.15 M NaCl solution and stored in small aliquots at –20°C. The specific activity of the ¹²⁵I-fibrinogen was in the range 2.5–60 x 10⁶ cpm/µg. The protein was characterized by dilution with unlabeled fibrinogen followed by precipitation with trichloroacetic acid or clotting with thrombin and determination of the proportion of radioactivity incorporated into the precipitate or clot, respectively. The trichloroacetic acid precipitate contained 98%–99% of the radioactivity and 97%–98% of the protein, determined from the absorbance at 280 nm; the fibrin clot contained 92% of the radioactivity and 89%–93% of the protein. These values for precipitable and clottable protein are similar to those obtained for unlabeled fibrinogen.

A sample of ¹²⁵I-fibrinogen was added to unlabeled fibrinogen, reduced, and studied by SDS-PAGE. When the locations of the radioactive bands in the gels were compared with those of the bands stained with Coomassie Blue, it was found that the radioactivity was distributed in all three chains of the molecule and that 85%–100% was recovered in 3 peaks in the same positions as the stained bands, which corresponded to the Aα, Bβ, and γ chains of fibrinogen.²³

The equivalence of unlabeled fibrinogen and ¹²⁵I-fibrinogen in binding to platelets was shown by determining the percentage of ¹²⁵I-fibrinogen bound for an experiment in which the total volume and amount of fibrinogen added were kept constant, but the specific activity of the ¹²⁵I-fibrinogen was varied by using different proportions of unlabeled and labeled fibrinogens (Table 1). Since the percentage of the fibrinogen that bound to the platelets was unaffected by the proportion of unlabeled fibrinogen in the mixture, it was evident that the labeled and unlabeled fibrinogens were behav-

![Table 1. Test of the Similarity of Binding of ¹²⁵I-Fibrinogen and Unlabeled Fibrinogen to Washed Rabbit Platelets During ADP-Induced Shape Change](from www.bloodjournal.org by guest on September 23, 2017. For personal use only.)
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Table 2. Effect of the Removal of a Small Percentage of the Radioactivity From a Preparation of *125*I-Fibrinogen by Adsorption to Rabbit Platelets Exposed to ADP Upon Subsequent Binding of the *125*I-Fibrinogen to Fresh Platelets During ADP-Induced Shape Change

<table>
<thead>
<tr>
<th>Fibrogen Concentration in Platelet Suspension (µg/ml)</th>
<th><em>125</em>I-Fibrinogen Previously Exposed to Platelets During ADP Stimulation</th>
<th>Control <em>125</em>I-Fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>638</td>
<td>1.1</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>104</td>
<td>2.0</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>17</td>
<td>2.8</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>2.8</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Final concentration of ADP in each cycle, 1.8 µM; platelet count, 6.4 x 10⁵/µl.

One of three experiments that gave similar results.

Results

* Determination of *125*I-Fibrinogen Bound to Platelets

Figure 1 shows typical responses of platelets to ADP in suspensions stirred rapidly (1100 rpm) or slowly (85 rpm), and with apyrase omitted or added at 9 µl/ml, a concentration that caused the platelets to deaggregate within 2 min. Figure 1 A and C are typical aggregation curves, and Fig. 1 B and D show that when the platelets were stirred slowly they changed shape in response to ADP but they did not cause an appreciable increase in light transmission that would indicate aggregation. However, it could be seen by microscopic

Fig. 1. Typical light transmission curves observed when washed rabbit platelets (6.3 x 10⁵/µl) were stimulated with ADP (10 µM) in the presence of fibrinogen (76 µg/ml). Arrows indicate additions of fibrinogen (Fbg) and ADP. (A) Platelet suspension without apyrase, stirred at 1100 rpm; (B) Platelet suspension containing apyrase (9 µl/ml), stirred at 1100 rpm; (C) Platelet suspension containing apyrase (9 µl/ml), stirred at 85 rpm.
Fig. 2. Effect of time of centrifugation of platelet suspension on the binding of \textsuperscript{125}I-Fibrinogen to washed rabbit platelets stimulated with ADP. \textsuperscript{125}I-Fibrinogen was added before the ADP, and the time of centrifugation was measured from the time of addition of ADP. Final concentrations: ADP, 10 \mu M; \textsuperscript{125}I-Fibrinogen, 5 \mu g/ml; platelet count, \(8 \times 10^9/\mu l\) suspended in Tyrode-albumin without apyrase. These results are typical of several experiments with concentrations of \textsuperscript{125}I-Fibrinogen in the range of 6-150 \mu g/ml.

examination that the platelets formed small aggregates. Figure 2 shows the time course of binding of \textsuperscript{125}I-fibrinogen to platelets during ADP-induced aggregation in a rapidly stirred suspension without apyrase. It was shown previously that, in the presence of apyrase, fibrinogen bound to the platelets during shape change and aggregation and was rapidly lost during deaggregation.\textsuperscript{8} From Fig. 2 it can be seen that when there was no apyrase in the suspension, maximum binding occurred within 0.2 min after the ADP was added, and this was followed by slow dissociation of the fibrinogen from the platelets. Similar results were obtained when the platelet suspension was stirred slowly and large aggregates did not form, showing that extensive aggregation is not necessary for binding to occur. Figure 3 shows the effect of ADP concentration on the binding of \textsuperscript{125}I-fibrinogen (0.12 \mu M) to platelets, and it can be seen that binding was independent of ADP concentration in the range between 2 \mu M and 40 \mu M. When the experiment was repeated with concentrations of fibrinogen up to 13.5 \mu M, it was found that, at each fibrinogen concentration tested, the extent of binding was independent of ADP concentration in the same range of ADP concentrations. However, the amount of fibrinogen bound was dependent on the concentration of fibrinogen added to the platelet suspension. Since the binding was unaffected by ADP concentrations between 2 \mu M and 40 \mu M, concentrations of 9-10 \mu M were used in subsequent experiments. Figure 4 shows the effect of fibrinogen concentration on the amount of \textsuperscript{125}I-fibrinogen that binds to platelets stimulated with 9 \mu M ADP. It can be seen that the binding is dependent on fibrinogen concentration and that it is saturable. By comparing fibrinogen binding in Tyrode-albumin with that observed in Tyrode solution containing unlabeled fibrinogen at 18.6 mg/ml, the amount of specific binding of \textsuperscript{125}I-fibrinogen to platelets could be calculated (Fig. 5A). Although a state of equilibrium binding had not been established, thereby ruling out a calculation of binding affinities and the number of
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Table 3. Displacement of Bound 125I-Fibrinogen From Washed Rabbit Platelets by Unlabeled Fibrinogen in the Absence of Apyrase, as a Function of the Time of Addition of Fibrinogen and of Stirring Rate

<table>
<thead>
<tr>
<th>Stirring Rate</th>
<th>Before ADP</th>
<th>0.2 min</th>
<th>0.4–1.0 min</th>
<th>1.1–2.0 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1100 rpm (extensive aggregation)</td>
<td>75 ± 8 (8)</td>
<td>69 ± 8 (7)</td>
<td>38 ± 6 (5)</td>
<td>43 ± 8 (8)</td>
</tr>
<tr>
<td>85 rpm (limited aggregation)</td>
<td>80 ± 11 (3)</td>
<td>73 ± 12 (3)</td>
<td>71 ± 10 (3)</td>
<td>63 ± 10 (5)</td>
</tr>
</tbody>
</table>

Table 3 shows the effectiveness of unlabeled fibrinogen in displacing bound 125I-fibrinogen from platelets suspended without apyrase. When 125I-fibrinogen and a large excess of unlabeled fibrinogen were added together before the ADP, only 20% as much of the 125I-fibrinogen bound to the platelets as when no unlabeled fibrinogen was present. Unlabeled fibrinogen added after the ADP was less effective in displacing 125I-fibrinogen that had already bound, and the degree of effectiveness decreased progressively as the length of time before the addition of unlabeled fibrinogen was increased. However, this decrease was less pronounced when the platelets were stirred slowly and large aggregates did not form (Table 3).

Without apyrase in the suspension to degrade the added ADP, deaggregation was slow, and some 125I-fibrinogen bound to ADP-stimulated platelets whether it was added before or after the ADP (Table 4). However, less binding was observed when the 125I-

fibrinogen receptors per platelet, a Scatchard type of analysis24 (Fig. 5B) indicates that for the range of fibrinogen concentrations studied, 0.6 μg/ml to 1.1 mg/ml, there appears to be only one class of binding sites. This range includes the fibrinogen concentrations effective in enhancing ADP-induced aggregation as well as those used in typical binding studies.

Without apyrase in the suspension to degrade the added ADP, deaggregation was slow, and some 125I-fibrinogen bound to ADP-stimulated platelets whether it was added before or after the ADP (Table 4). However, less binding was observed when the 125I-

Final concentrations: ADP, 9.1 μM; 125I-fibrinogen, 6–31 μg/ml; unlabeled fibrinogen, 1.1–1.3 mg/ml; platelet count, 6.7 × 10^9/μl.

Similar results for the displacement of 125I-fibrinogen by unlabeled fibrinogen added before ADP were obtained with concentrations of 125I-fibrinogen as high as 107 μg/ml. 125I-fibrinogen was added before the ADP; amount bound in the absence of added unlabeled fibrinogen is defined as 100%, and displacement is expressed as a percentage of this value. Percentage of 125I-fibrinogen that bound to platelets to which no ADP had been added was subtracted from each value before calculations were made. Suspensions were centrifuged 2.5 min after the addition of ADP. Number of determinations is given in parentheses beneath each value; values are means ± SD.
fibrinogen was added after the ADP, and the amount bound was further diminished as the interval between the additions of ADP and fibrinogen was lengthened. The progressive decrease was more pronounced when the suspension was stirred rapidly and large aggregates formed than when extensive aggregation was avoided by slow stirring. It was also observed that fibrinogen added after the ADP enhanced ADP-induced aggregation, but to a lesser extent than when it was added before the ADP. In contrast, although the errors of the determinations are large, it is clear from Table 5 that when sufficient apyrase or CP/CPK was present in the platelet suspension to remove the ADP and cause the platelets to deaggregate within 2 min, the $^{125}$I-fibrinogen had to be added before or almost immediately after the ADP for detectable binding to occur. (Apparent discrepancies between Tables 4 and 5 in the values for platelet suspensions without apyrase or CP/CPK arise because the times of centrifugation were not the same.) When platelets were aggregated by ADP in the presence of a high concentration of apyrase, fibrinogen enhanced aggregation only when it was added before, or less than 1 min after, the ADP.

### ADP-Induced Aggregation of Platelets Previously Aggregated With ADP

We performed an experiment with platelets that had previously been aggregated with ADP to determine the effect of ADP-induced aggregation and deaggregation on the fibrinogen binding site.

ADP (9.1 μM) was added to a platelet suspension (7.3 × 10$^5$/μl) containing apyrase (1 μl/ml) at 37°C. The suspension was stirred for 10 min, during which period the platelets aggregated and deaggregated almost completely; the suspension was centrifuged for 8 min at 1200 g, the supernatant was aspirated, the platelets were washed twice with Tyrode-albumin and then resuspended in Tyrode-albumin containing apyrase at 37°C. The experiment was repeated with a platelet suspension containing unlabeled fibrinogen at a concentration of 2 mg/ml, and with a control suspension that contained 0.15 M NaCl in place of both the fibrinogen and the ADP. After 2 hr at 37°C, the 3 suspensions were tested for their ability to aggregate when stimulated with ADP, both with and without added fibrinogen, and for their ability to bind $^{125}$I-fibrinogen during ADP-induced shape change.

The results are summarized in Table 6. Unless the platelet suspensions were incubated at 37°C for 1.5–2.5 hr after the first aggregation, their response to the second stimulation was diminished with respect to

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**Table 4. Effect of Stirring Rate and Time of Addition of $^{125}$I-Fibrinogen on its Binding to Washed Rabbit Platelets Stimulated With ADP in the Absence of Apyrase**

| Stirring Rate | Time of Addition of $^{125}$I-Fibrinogen to Platelet Suspension (Minutes After Addition of ADP) |
|---------------|---------------------------------------------------------------------------------|---|
| 1100 rpm      | 0.2 min                           | 0.4–0.8 min | 1.0 min | 1.2–1.8 min | 2.0 min |
| (extensive aggregation) | 89 ± 25                         | 71 ± 21          | 52      | 23 ± 8     | 5 ± 5   |
| 85 rpm        | (9)                               | (4)            | (1)     | (5)        | (5)     |
| (limited aggregation) | 116 ± 24                       | 107 ± 30        | 80 ± 4  | 67 ± 17    | 50 ± 4  |
| (9)           | (3)                               | (7)            | (4)     | (7)        | (7)     |

Final concentrations: ADP, 9.1 μM; $^{125}$I-fibrinogen, 7–70 μg/ml; platelet count, 5.7 × 10$^5$/μl.

*Percentage of $^{125}$I-fibrinogen that bound to platelets to which no ADP had been added was subtracted from each value before calculations were made; values are means ± SD. Platelet suspensions were centrifuged for 2.5 min after the addition of ADP. The number of determinations is given in parentheses beneath each value; values are means ± SD.

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**Table 5. Effect of Apyrase or CP/CPK on the Binding of $^{125}$I-Fibrinogen to Washed Rabbit Platelets Exposed to ADP When the $^{125}$I-Fibrinogen Was Added After the ADP and Extensive Aggregation Was Prevented by Slow Stirring of the Suspension**

| Platelet Suspension | Time of Addition of $^{125}$I-Fibrinogen to Platelet Suspension (Minutes After ADP) |
|---------------------|---------------------------------------------------------------------------------|---|
| Suspension† without apyrase or CP/CPK | 0.2 min                           | 1.0 min | 2.0 min |
| Suspension‡ containing apyrase (9 μl/ml) | 101 ± 16                         | 48 ± 12 | 20 ± 10 |
| Suspension‡ containing CP/CPK (9 μl/ml) | 97 ± 16                          | −8 ± 26 | −21 ± 13 |
| Suspension‡ containing CP/CPK (9 μl/ml) | 81 ± 5                           | 12 ± 11 | 2 ± 4   |

Final concentrations: ADP: 10 μM; $^{125}$I-fibrinogen: 10–15 μg/ml; platelet count, 6–7 × 10$^5$/μl.

*Percentage of $^{125}$I-fibrinogen that bound to platelets to which no ADP had been added was subtracted from each value before calculations were made; values are means ± SD. Platelet suspensions were centrifuged for 2.5 min after the addition of $^{125}$I-fibrinogen.

†Six determinations.

‡Eight determinations.
both enhancement of aggregation and fibrinogen binding, although they had recovered their disc shape much sooner than this. It was also necessary to wash the platelets twice after the first aggregation. Otherwise traces of fibrinogen, contaminating the platelets that had previously been aggregated with fibrinogen, caused them to aggregate more strongly and bind a lower percentage of 125I-fibrinogen than the platelets that had not previously been exposed to fibrinogen. When these precautions were taken, no significant differences were found between control platelets and those that had already undergone aggregation.

Characterization of 125I-Fibrinogen That Had Been Bound to Platelets During ADP Stimulation

We examined the nature of the radioactivity lost from platelets upon deaggregation to try to establish whether it was in the form of intact fibrinogen or of a fragment of fibrinogen, such as might be produced by proteolytic cleavage. When Tyrode-albumin was used as the resuspending medium, the presence of the albumin caused the peak of radioactivity corresponding to the Aa chain to smear because of the similarity of their molecular sizes. The use of gelatin instead of albumin, as the resuspending medium, the presence of the proteolytic cleavage. When Tyrode-albumin was used as the resuspending medium, the presence of the albumin caused the peak of radioactivity corresponding to the Aa chain to smear because of the similarity of their molecular sizes. The use of gelatin instead of albumin, therefore, we conclude that essentially all of the 125I bound to the platelets had been 125I-fibrinogen, and we have not demonstrated any change in the 125I-fibrinogen during its interactions with platelets.

ADP (10 μM) was added to a slowly stirred platelet suspension (109/μl) containing 125I-fibrinogen (80 μg/ml) and apyrase (0.25 μl/ml). The suspension was centrifuged 0.5 min after the addition of the ADP, the supernatant was aspirated, and the platelet pellet was resuspended in one-fifth the volume of Tyrode solution containing gelatin (2.5 mg/ml) in place of albumin, apyrase (1.0 μl/ml), Trasylol (10 μl/ml), and unlabeled fibrinogen (500 μg/ml) as carrier. This suspension was incubated at 37°C for 30 min to achieve complete dissociation of the 125I-containing material from the platelets. It was then centrifuged, and the supernatant, containing the 125I that had originally been bound to the platelets and trapped with the pellet, was studied by SDS-PAGE after reduction with β-mercaptoethanol. The gel was stained with Coomassie Blue and then sliced in 1-mm slices, which were counted for radioactivity. The radioactivity pattern was compared with one obtained from a sample of untreated 125I-fibrinogen dissolved in the Tyrode-gelatin buffer. Parallel binding experiments were run to determine the proportion of bound 125I-fibrinogen that was displaced by a 50-fold excess of unlabeled fibrinogen and the proportion that was nonspecifically bound to the platelets or trapped in the platelet pellet.

Figure 6 shows the radioactivity profiles. Figure 6A represents 125I-fibrinogen dissolved in Tyrode solution containing gelatin, apyrase, and Trasylol, but not exposed to platelets. Figure 6B represents 125I-fibrinogen that had been associated with platelets during ADP stimulation and had dissociated upon removal of the ADP. A parallel binding experiment in the

<table>
<thead>
<tr>
<th>Treatment of Platelet Suspension</th>
<th>Aggregation Peak Height (% of Control)</th>
<th>Percent 125I-Fibrinogen Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Added Fibrinogen</td>
<td>With Added Fibrinogen</td>
</tr>
<tr>
<td>Platelets previously aggregated with ADP</td>
<td>58 ± 13.6</td>
<td>110</td>
</tr>
<tr>
<td>Platelets previously aggregated with fibrinogen and ADP</td>
<td>64 ± 9.6</td>
<td>101</td>
</tr>
<tr>
<td>Control platelets</td>
<td>49 ± 6.8</td>
<td>100</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>0.22 ± 0.03</td>
<td>1.30 ± 0.10</td>
</tr>
</tbody>
</table>

Final concentrations: ADP, 10 μM; unlabeled fibrinogen, 82 μg/ml (for peak heights); 125I-fibrinogen, 77 μg/ml (for binding). Platelet count, 7.2 x 109/μl. Peak heights are expressed as a percentage of the peak height observed when control platelets were aggregated with ADP in the presence of added fibrinogen. Suspensions were centrifuged 0.2 min after the addition of ADP for the measurement of 25I-fibrinogen bound.

One of two experiments that gave similar results.

* Three determinations.
† One determination.
‡ Four determinations.
presence of a 50-fold excess of unlabeled fibrinogen indicated that approximately 75% of the $^{125}$I-fibrinogen isolated had been specifically bound to the platelets, while 25% had been nonspecifically bound or trapped in the fluid with the platelet pellet. The positions of the peaks of radioactivity in both figures coincide with the positions of the $\alpha_2$, $\beta_2$, and $\gamma$ chains of fibrinogen established by staining with Coomassie Blue. The ratios of the radioactivities associated with the three chains for each of the $^{125}$I-fibrinogen samples analyzed showed no significant differences.

**DISCUSSION**

It had been shown that when fibrinogen is present in a suspension of human or rabbit platelets during stimulation with ADP under physiologic conditions of pH, temperature, and cation concentration, it binds to the platelets immediately upon addition of the ADP, and when $^{125}$I-fibrinogen is used to measure the binding, the radioactive material is lost from the platelets as they deaggregate. Added fibrinogen is not required for the aggregation of washed rabbit platelets by ADP, but does enhance the aggregation, indicating that although some fibrinogen may be present on the surface of these platelets, it is insufficient to support maximum aggregation. Unlabeled fibrinogen, if present on the platelets, would affect the stoichiometry and kinetics of fibrinogen binding, but it does not mask the observations of reversible association of $^{125}$I-fibrinogen during aggregation and deaggregation.

We have confirmed that fibrinogen binds to rabbit platelets during ADP-induced shape change and that the binding reaches a maximum within 0.2 min after the addition of ADP, but the fibrinogen dissociates from the platelets gradually after that. The rate of dissociation is increased when the stimulus is removed, as with apyrase or CP/CPK, but even in the continuing presence of ADP, platelets apparently undergo further change and lose their ability to bind fibrinogen. Deaggregation and reversal of fibrinogen binding can be prevented by lowering the temperature or calcium ion concentration, but since the binding of fibrinogen appears to be important in ADP-induced platelet aggregation in vivo, it is preferable to study factors affecting its association and dissociation in a system that reflects physiologic conditions as closely as possible. Under the conditions used, the release reaction does not take place and there is no secretion of ADP or fibrinogen, both of which would complicate the interpretation of the results.

Binding of fibrinogen is dependent on platelet shape change, and we postulate that when ADP binds to platelets and causes them to change shape, fibrinogen binding sites are revealed. It is unlikely that ADP is involved as a cofactor in fibrinogen binding, since the amount of fibrinogen that binds is independent of ADP concentration over a wide range (2–40 $\mu$M) and this relationship holds for concentrations of fibrinogen from 0.12 $\mu$M to 13.5 $\mu$M. Thus, there appears not to be a stoichiometric relationship between ADP and fibrinogen. On the other hand, the amount of fibrinogen that binds to platelets is dependent on fibrinogen concentration and shows saturation at high concentrations of fibrinogen. Approximately 80% of the $^{125}$I-fibrinogen can be displaced by an excess of unlabeled fibrinogen, showing that most of the binding is specific, with approximately 20% apparently nonspecific. This value of 20% is in the same range as the amount of $^{125}$I-fibrinogen usually associated with disc-shaped rabbit platelets before the addition of an ADP stimulus. In order to study the refractoriness that develops as the platelets lose their ability to bind fibrinogen, the length of time after stimulation that the binding sites remain available was studied by measuring the binding of $^{125}$I-fibrinogen added after ADP. The amount able to bind decreases the later the fibrinogen is
added, and the rate of this decrease is affected by two factors: (1) the state of aggregation of the platelets, since if the platelets form large aggregates, less fibrinogen is bound (possibly because the fibrinogen is unable to penetrate the core of the aggregate so that the binding sites become less accessible); and (2) the rate of removal of the ADP stimulus, since if the ADP is removed rapidly with apyrase or CP/CPK, the platelets lose their ability to bind fibrinogen more rapidly. The same effects operate in the experiments in which bound $^{125}\text{I}$-fibrinogen is displaced with unlabelled fibrinogen, suggesting that the fibrinogen is unable to penetrate readily into the core of large aggregates and so less displacement is observed than when extensive aggregation is prevented. Thus, it appears that platelets in their normal disc shape do not have fibrinogen binding sites available; upon ADP stimulation, fibrinogen binding sites are exposed and fibrinogen binds to the platelets; within a short period, the binding sites become unavailable again and the fibrinogen-binding capacity is lost.

To examine whether the reduced ability of platelets to bind fibrinogen added after stimulation with ADP might be due to a permanent blocking of the fibrinogen binding sites on the platelets, we studied the long-term effect of reversible ADP-induced aggregation on the amount of fibrinogen bound during a second stimulation of the platelets with ADP. Platelets did not regain the ability to bind fibrinogen upon a second ADP stimulation until after a lengthy recovery period, but previous aggregation had no significant permanent effect on the sensitivity of the platelets to ADP with respect to extent of aggregation or of fibrinogen binding; provided that sufficient time had been allowed for complete recovery of the platelets and all traces of ADP and fibrinogen had been removed from the platelet suspending fluid before the second stimulation with ADP and fibrinogen. This was observed even when the first aggregation was carried out in the presence of fibrinogen at a concentration of 2 mg/ml, an almost saturating concentration. Thus, it can be concluded that although platelets lose their ability to bind fibrinogen within a short period after exposure to ADP, the receptors for fibrinogen are not altered irreversibly by the binding of fibrinogen during aggregation, and the recovery of platelets from the refractory state observed by other investigators$^{25-27}$ is accompanied by the recovery of their ability to bind fibrinogen upon ADP stimulation.

Because of the possibility that fibrinogen might be altered during its association with platelets, we examined the nature of the radioactive material that had been associated with platelets during stimulation with ADP in the presence of $^{125}\text{I}$-fibrinogen and then lost from them when they were removed from the ADP-containing solution. SDS-PAGE of this material showed that it was apparently unchanged $^{125}\text{I}$-fibrinogen, since the peaks of radioactivity on the gels corresponded to the positions of the Aα, Bβ, and γ chains of fibrinogen, and there were no differences in the ratios of the radioactivities in the three chains when compared with control $^{125}\text{I}$-fibrinogen. The possibility of cleavage of a small peptide is not ruled out by this experiment, but it is unlikely that such a peptide remained attached to the platelets, since the binding sites on the platelets were not permanently blocked as a result of aggregation followed by deaggregation. It should be emphasized that the fibrinogen analyzed in this experiment had been bound to platelets and then dissociated, under physiologic conditions of pH, temperature, and cation concentrations, upon removal of the ADP stimulus. The fibrinogen had not been extracted from the platelets by a chemical procedure. This experiment confirms that it was fibrinogen, rather than a contaminant of the fibrinogen preparation, that was binding to the platelets and enhancing ADP-induced aggregation. Although SDS-PAGE is not a suitable technique for detecting contaminants present at very low levels, 75% of the radioactivity in the sample studied by this technique represented material that had been specifically bound to the platelets, and if it had been a protein other than fibrinogen, it would have been easily detectable in the gels.

The results of this study are consistent with the concept that when rabbit platelets are in their normal disc shape few binding sites for fibrinogen are available on the platelet membrane, but they become available and fibrinogen binds when the platelets change shape in response to stimulation with ADP. Within a short time after stimulation, the fibrinogen binding sites become unavailable again, fibrinogen is lost, and the platelets deaggregate. Fibrinogen binding sites may become permanently available as a result of the action of proteolytic enzymes on platelets.$^{28,29}$ Neither the fibrinogen nor its receptors appear to be permanently altered as a result of the binding and dissociation, and the platelets slowly recover their ability to aggregate and bind fibrinogen upon a second ADP stimulation.

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REFERENCES

2. McLean JR, Maxwell RE, Hertler D: Fibrinogen and adeno-
Reversibility of the association of fibrinogen with rabbit platelets exposed to ADP

EJ Harfenist, MA Packham and JF Mustard