Acceleration of Fibrin Polymerization by Calcium Ions

By Markley H. Boyer, John R. Shainoff, and Oscar D. Ratnoff

Calcium ions are known to accelerate the formation of a visible clot upon the addition of thrombin to plasma or fibrinogen. The experiments described demonstrate that this effect of calcium ions is not upon the rate of release of fibrinopeptides from fibrinogen, but rather upon the aggregation of soluble fibrin monomers or polymers, independent of the action of fibrin-stabilizing factor.

The final steps of the clotting process, in which fibrinogen (Factor I) is transformed to fibrin through the action of thrombin, is customarily described as taking place in three stages. Initially, thrombin, a proteolytic enzyme of high substrate specificity, cleaves four arginyl-glycine bonds in each fibrinogen molecule, so that two pairs of peptide fragments are released. During the second stage (aggregation), the cleaved fibrinogen (now called fibrin monomer) coagulates to form a visible fibrin clot; in systems containing purified reagents this clot is soluble in 5 M urea or monochloroacetic acid. In the third step (polymerization), probably coincident with the second, a plasma transamidase, fibrin-stabilizing factor (Factor XIII), converts the urea-soluble fibrin to an insoluble "cross-linked" form. This final reaction takes place only if calcium ions are present.

The need for calcium as an adjunct to the formation of insoluble fibrin is well established, but the role of this ion in the first two steps of the fibrinogen-to-fibrin transformation is unclear. Lorand and Konishi, using purified bovine fibrin, have reported accelerated aggregation in the presence of low concentrations of calcium. Elias and Iyer showed that calcium ions were capable of counteracting the effect of certain inhibitors of fibrin monomer aggregation. Problems in separating the three stages of clot formation have made interpretation of other reported observations difficult.

The experiments to be described were designed to study the formation of a clot in the presence or absence of calcium ions and to localize the effect of these ions to the first or second steps of fibrin production. The technique used to dissect the two stages was based on studies of fibrin formation in patients with dysfibrinogenemia. Our data indicate that calcium ions do not enhance...
the release of fibrinopeptides from fibrinogen and indeed may be slightly inhibitory. In contrast, they accelerate the aggregation of soluble fibrin monomers or polymers. This clot-promoting effect of calcium ions is not dependent on the presence of functional fibrin-stabilizing factor.

MATERIALS AND METHODS

Purified human fibrinogen was prepared in three ways. Fraction 1-2, a partially purified fibrinogen fraction of plasma containing fibrin-stabilizing factor, was used in most experiments on fibrin aggregation. It was separated from Cohn fraction I by the method of Blombäck. This fraction, which was 85–90% coagulable, was dissolved in barbital-saline buffer at a concentration of 9.3 mg/ml and stored at −20°C in polyethylene vials. For all experimental procedures, it was brought to pH 5.3 by addition of an equal part of Michaelis buffer. Fibrinogen depleted of fibrin-stabilizing factor was prepared by the method of Mosesson and was used to test the speed of fibrin aggregation in the absence of this enzyme. Clots formed from this fibrinogen dissolved rapidly in 1% monochloroacetic acid. The preparation was 90–95% coagulable and was stored at a concentration of 2.1 mg/ml at −20°C in barbital-saline buffer. To study the release of fibrinopeptides, Fraction 1-2 was further purified to remove plasminogen and cryoprecipitable protein, dialyzed at a concentration of 16 mg/ml against barbital-saline buffer, and then divided into two portions, one of which was treated to remove calcium ions and the other diluted directly to a concentration of 3.6 mg/ml.

Partially purified bovine thrombin was prepared from Topical Thrombin (Parke, Davis and Co., Detroit, Mich.) by the method of Prentice and his collaborators and was stored in barbital-saline buffer at −20°C in polyethylene containers until used. Thrombin prepared in this way contains 2000–3000 NIH units/mg of protein. Before use, the thrombic activity of the preparation was measured by comparing its clot-promoting effect to that of freshly prepared crude Topical Thrombin.

Removal of calcium from fibrinogen solutions and buffers was effected by treating with Chelex 100 (Calbiochem, Los Angeles, Calif.), a chelating resin with imino-diacetate exchange groups and specificity similar to ethylenediaminetetraacetic acid (EDTA). The extent of removal of calcium by the resin was not measured but could be assumed to be essentially complete, except for nonexchangeable forms incapable of chelation. The resin was equilibrated with the barbital-saline buffer before use by suspending 20 ml in 100 ml of buffer, adjusting pH, and washing with 2 liters of buffer. The fibrinogen solution (6 ml) was applied to a 1 × 8 cm column of the resin and flushed (6 ml/hr) through with 4 ml of buffer. All but about 10% of the protein was recovered with no discernable loss of coagulability (95–96%) by thrombin. Thrombin was decalcified by adding 30–40 mg of spin-dry resin to 0.5 ml of the enzyme solution (240 U/ml) containing 25% glycerol, stirring intermittently for 30 min, and retrieving the solution in a dropper fitted with a glass-fiber filter at the tip. No substantial loss of clotting activity resulted from the treatment. Glassware used with the decalcified solutions was rinsed with 1 mM EDTA and, subsequently, resin-treated buffer and water.

Crude hirudin grade III (Sigma Chemical Co., St. Louis, Mo.) was dissolved at appropriate concentrations in Michaelis buffer (pH 5.3).

Acetate-barbital buffer solutions (Michaelis buffers) were prepared over a range of pH values. These buffers contain no calcium salts and do not bind calcium ions. They have a constant ionic strength equivalent to that of blood at all pH values. In experiments to test the influence of calcium ions, four parts of Michaelis buffer were mixed with one part of 0.05 M calcium chloride solution, and the effect of this mixture compared to an iso-ionic mixture of four parts of Michaelis buffer and one part of 0.15 M sodium chloride.

Trisodium EDTA was prepared by adjusting the pH of solutions of disodium EDTA to pH 7.0. Barbital-saline buffer was composed of 0.025 M sodium barbitol and 0.125 M NaCl at pH 7.5–7.6.

The effect of calcium ions on the aggregation of soluble fibrin monomers and polymers was tested by taking advantage of Mommaerts' observation that fibrin monomers and
perhaps dissolved polymers can form at pH 5.2 but aggregate only when brought to neutrality. One tenth milliliter of Fraction I-2, at pH 5.3, was incubated at 37°C with 0.6 ml of Michaelis buffer (pH 5.3) for 120 sec in polystyrene tubes (12 × 75 mm). At the start, 0.01 ml of bovine thrombin (23 NIH units/ml) in Michaelis buffer (pH 5.3) was added to the fibrinogen. At intervals thereafter, 0.2-ml aliquots of the mixture were added to polystyrene tubes (10 × 75 mm) containing 0.035 ml hirudin (500 U/ml, pH 5.1). Finally, the pH of the mixture was brought to pH 7.4 by the addition of 0.3 ml Michaelis buffer (pH 9.45) containing one-fifth part of either 0.05 M calcium chloride or 0.15 M sodium chloride.

In all experiments the concentration of hirudin was sufficient to neutralize the added thrombin. No clot formed when Fraction I-2, Michaelis buffer (pH 5.3), and hirudin were incubated together for 1000 sec. The addition of thrombin to this mixture failed to initiate clot formation even when the pH of the solution was raised to pH 7.4 with Michaelis buffer (pH 9.45) in the presence of calcium ions.

To test the effect of calcium ions on the proteolytic stage of the reaction, rates of release of fibrinopeptides from both decalcified and untreated fibrinogen were measured in presence or absence of added calcium ions or EDTA. The measurements were performed by incubating 1 ml portions of decalcified or untreated fibrinogen solution (3.6 mg/ml) with 0.036 NIH units of thrombin for intervals of time up to 80 min, tenfold the clotting time observed with untreated fibrinogen. The reaction was terminated by rapidly compressing and removing the clot and adding 0.2 ml of 30% trichloroacetic acid (TCA) to the extruded fluid. Since less than 3% of reaction period was spent in compressing the clots, and since prior measurements had shown loss of fibrinopeptides to be negligible, no correction was made for this operation. The protein precipitated by TCA was removed by centrifugation, and the supernatant fluid was recovered in a dropper fitted with a glass fiber filter-tip. A portion (0.5 ml) of the fluid was then analyzed for total arginine content by the Sakaguchi reaction and another portion (0.4 ml) was processed for electrophoretic determination of the fibrinopeptides. The concentration of fibrinogen in Fraction I-2 and in fibrin-stabilizing factor-depleted fibrinogen was determined by the method of Ogston et al.

RESULTS

Effect of Calcium Ions on Release of Fibrinopeptides

At a concentration of thrombin (0.036 NIH units/ml) such that coagulation proceeded slowly, the release of fibrinopeptides from fibrinogen at pH 7.6
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was not accelelerated by calcium ions at a final concentration of 0.0025 M and indeed may have been slightly impeded (Fig. 1). Calcium ions did not accelerate the release of fibrinopeptides from native fibrinogen or fibrinogen treated with Chelex to reduce its concentration of bivalent cations. The addition of EDTA at a concentration of 0.00125 M did not alter the rate of release of fibrinopeptides.

**Effect of Calcium Ions on Aggregation of Dissolved Fibrin Monomers and Polymers**

In studies of fibrinopeptide release, the speed of visible clot formation was not a simple function of the amount of peptide produced but was dramatically increased by the presence of calcium ions (Table 1). The addition of EDTA impeded clot formation until almost all fibrinopeptide A had been released. These data indicate that monomer aggregation is strongly influenced by the presence of calcium ions. In direct tests where the monomer aggregation step was separated from the proteolytic stage by inactivation of thrombin by hirudin, calcium ions at a final concentration of 0.003 M accelerated visible clot formation (Table 2). This effect was not dependent on the presence of fibrin-stabilizing factor.

**DISCUSSION**

Although the dramatic effect of calcium ions in accelerating the formation of a visible clot has been recognized for many years, the relative importance of these ions at the various stages of fibrinogen to fibrin transformation is incompletely understood. Lorand and Konishi have shown that calcium accelerates monomer aggregation in a purified bovine fibrin system, but no data are available on the role of these ions in the first proteolytic step.

In the study reported here the problem was reexamined using the approach originally suggested by Mommaerts, that is, to use techniques separating the clotting of fibrinogen into a proteolytic and a polymerization stage. The results of the experiments indicate that calcium ions do not accelerate the release of fibrinopeptides from fibrinogen. In contrast, at a concentration comparable to that found in circulating plasma, calcium accelerates the aggregation of fibrin monomers or soluble polymers to form a visible clot.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Delay Until Visible Clotting (min)</th>
<th>Fibrinopeptide A Released at Time of Visible Clotting (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen + calcium ions</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>Fibrinogen alone</td>
<td>8</td>
<td>61</td>
</tr>
<tr>
<td>Fibrinogen + EDTA</td>
<td>16</td>
<td>90</td>
</tr>
</tbody>
</table>

*Thrombin was added to each mixture to induce clotting.
†Final concentration 0.0025 M.
‡Final concentration 0.00125 M.
Table 2. Effect of Calcium Ions on Aggregation of Dissolved Fibrin Monomers

<table>
<thead>
<tr>
<th>Incubation Time* (sec)</th>
<th>Visible Clot Formation† (sec) Calcium ions</th>
<th>Sodium ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0‡</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>15</td>
<td>&gt;320</td>
<td>&gt;320</td>
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<tr>
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<td>&gt;320</td>
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</tr>
<tr>
<td>180</td>
<td>4</td>
<td>49</td>
</tr>
<tr>
<td>240</td>
<td>Instantaneous</td>
<td>14</td>
</tr>
</tbody>
</table>

*Period of incubation of mixture of fibrinogen and thrombin at pH 5.3.
†Delay until visible clotting after neutralization of thrombin (in aliquots of first mixture) by addition of hirudin, adjustment of pH to 7.4, and addition of either calcium chloride or sodium chloride solutions.
‡In this experiment, hirudin was added to initial mixture before addition to thrombin.

The biochemical role of calcium in the aggregation of fibrin was not elucidated in the present study. In earlier experiments, it was noted that repeated washing of a calcium-fibrin clot removed essentially constant amounts of calcium each time. These studies suggested that calcium and fibrin clot were bound by adsorptive forces rather than by a chemical bond. Evidence to support this conclusion is still needed.

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REFERENCES

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