Multiple active forms of thrombin: Binding to platelets and effects on platelet function

(thrombin/modified thrombins/platelet aggregation and release/hemostasis)

S. FAZAL MOHAMMED*, C. WHITWORTH†, H. Y. K. CHUANC*, R. L. LUNDBLAD†*, AND R. G. MASON*

* Department of Pathology, Memorial Hospital, Pawtucket, Rhode Island 02860, and Brown University, Providence, Rhode Island 02912; † Departments of Pathology and Biochemistry and ‡ The Dental Research Center, University of North Carolina, Chapel Hill, N.C. 27514

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ABSTRACT The effect of various forms of thrombin on certain platelet functions has been investigated. Partially purified bovine thrombin, which is a mixture of multiple active forms of thrombin, was chromatographed to yield molecular species termed α, β, and γ-thrombin, each of which has varying degrees of fibrinogen clotting and esterase activities. A direct correlation was observed between the ability of the different forms of thrombin to clot fibrinogen and to influence platelet function. In general, thrombin with high fibrinogen clotting activity was also a potent inducer of platelet aggregation and the release reaction, while those species with low clotting activity were poor inducers of aggregation and release. On the other hand, thrombin with both fibrinogen clotting activity and esterase activity nearly completely inactivated by treatment with N-α-tosyl-L-lysine chloromethyl ketone had no aggregation or release inductive activity. Studies performed with partially purified thrombin or individual species of thrombin iodinated with 125I indicated that despite differences in fibrinogen clotting activities, α-thrombin and its degraded forms were capable of binding to the platelet surface. The possibility exists that thrombin binding to the platelet surface may be a means of strategically anchoring and concentrating large amounts of enzymatically active thrombin within the white thrombus at a site of vascular injury.

The plasma membrane is the primary site of action of many agents that affect platelet function. Biologically important agents that are known to influence platelet function include thrombin, adenosine diphosphate (ADP), collagen, epinephrine, and serotonin (1–14). Thrombin, a proteolytic enzyme pivotal in the blood coagulation reaction and a potent inducer of platelet aggregation and release, also interacts with the platelet plasma membrane and liberates a small glycoprotein (5–8). Recently, Tollefsen et al. (9) and Ganguly (10) have reported that thrombin or thrombin inhibited by diisopropylphosphofluoridate (Dip-F) or phenylmethylsulfonyl fluoride (PheSO2-F) is capable of tightly binding to the platelet surface. It has become apparent that thrombin can be isolated in several different molecular forms which differ significantly in fibrinogen–clotting and esterase activity (11–18). Since the effect of these different species of thrombin on platelet functions has not been determined, the present studies were initiated to compare the platelet aggregating, release inducing, and membrane binding properties of these forms of thrombin.

Studies performed with partially purified thrombin and with α-, β-, or γ-thrombin or with thrombin inhibited by N-α-p-tosyl-L-lysine chloromethyl ketone (Tos-LysCH2Cl) indicate that all the species of thrombin studied were capable of binding to the platelet surface, irrespective of their fibrinogen clotting or esterase activity. A direct correlation was observed between the ability of these species of thrombin to clot fibrinogen and to induce platelet aggregation and the release reaction.

MATERIALS AND METHODS

Blood was obtained by the two-syringe technique from normal healthy donors who denied receiving medication of any type for at least 1 week preceding venipuncture. The afibrinogenemic and thrombasthenic patients had not been transfused during the preceding 3 months. Eight parts of blood were drawn into one part of 0.108 M sodium citrate and mixed immediately. Platelet-rich plasma (PRP) was obtained by centrifugation of anticoagulated blood at 300 × g for 12 min at 23 °C. Platelets were then separated from plasma either by gel filtration (19) or by a method reported by us earlier (20). Washed platelets were suspended finally in Mg++-free Tyrode’s solution (MPTS) prior to their exposure to the various thrombins.

Purification of Thrombins. Partially purified bovine thrombin was obtained by stepwise chromatography of Parke-Davis topical thrombin on sulfopropyl Sephadex C-50 as previously described (21). This material is henceforth referred to as partially purified thrombin and had a specific activity of 1200–1800 NIH units/mg of protein. This material could be resolved further into α-, β-, and γ-thrombin by rechromatography on sulfopropyl Sephadex C-50 using gradient elution. The partially purified sample was dialyzed against 0.05 M sodium phosphate buffer at pH 6.5 and applied to a 2.5 × 28 cm column of sulfopropyl Sephadex C-50 previously equilibrated with the same solvent. The various forms of thrombin were resolved with a gradient of 0.15–0.45 M sodium phosphate at pH 6.5. A complete description of this procedure and the chemical characterization of these proteins appears elsewhere (22). Assays for fibrinogen-clotting activity and esterase activity with N-α-tosyl-L-arginine methyl ester were performed as previously described (21). Fibrinogen-clotting activity is expressed in NIH units with NIH Standard B3.1. Protein concentration was determined by the ninhydrin reaction (23) after alkaline hydrolysis (24) using crystalline bovine serum albumin (Pentex; Miles Laboratories, Elkhart, Ind.) as a standard. α-Thrombin was inactivated by Tos-LysCH2Cl as described by Glover and Shaw (13).

In view of differences in the fibrinogen clotting activity per mg of protein (NIH units) in various thrombin preparations and for the sake of clarity and comparison, results will be expressed in terms of nM concentrations of the various thrombins. A value of 39,000 daltons was used for α-thrombin while a value of 28,000 daltons was used for both β- and γ-thrombins (14).

Platelet Aggregation. Platelet aggregation was studied by the use of a Payton dual channel aggregometer at 37 °C. The rations was examined by adding 0.05 ml of a thrombin solution in saline (0.154 M NaCl) to 0.45 ml of a platelet suspension containing 400,000 platelets per ml. Percent aggregation was

Abbreviations: Dip-F, diisopropylphosphofluoridate; MPTS, magnesium free Tyrode’s solution; PheSO2-F, phenylmethylsulfonyl fluoride; PRP, platelet rich plasma; Tos-LysCH2Cl, N-α-p-tosyl-L-lysine chloromethyl ketone.
calculated by determining the weight of the paper under the resultant aggregometer curve (25), a procedure used in earlier studies.

Release Reaction. Isolated platelets were labeled with 5-hydroxy [side chain-2-14C]tryptamine creatinine sulfate ([14C]serotonin; Amersham-Searle, Chicago, Ill.) following the method described by Holmsen et al. (26). Subsequently, 0.45 ml of the washed platelet suspension containing 400,000 platelets per µl was stirred with 0.05 ml of a thrombin preparation for 4 min at 37°C. Platelets were then centrifuged for 1 min and an aliquot of the supernatant removed for counting of [14C]serotonin in a Nuclear Chicago three-channel scintillation counter. The primary and secondary scintillators used in toluene were 2,5-diphenyloxazole and 1,4-bis[2(5-phenyloxazoyl)]benzene, respectively.

Iodination of Thrombins. Purified thrombins were iodinated with 125I (Na125I, Amersham-Searle, Chicago, Ill.) according to the method of Hunter (27) as used by Tollefsen et al. (9). The amount of 125I bound to thrombin varied from 45 to 95% of the total 125I added to the reaction mixture. After iodination, the thrombins, dissolved in 0.75 M NaCl, were recharacterized for their protein content and fibrinogen clotting and esterase activities. Iodination under these conditions did not affect the catalytic activity of the various thrombins. Iodinated thrombins were stored at −20°C and were used within 1 week after labeling.

Binding of Thrombins to Platelets. Varying concentrations of one of the 125I-labeled thrombins (in saline) were mixed with a 2 ml solution of isolated platelets containing 350,000 platelets per µl in a silicone-coated test tube (20) and allowed to stand at 23°C for 15 min. After this incubation, 2.7 mM ethylenediaminetetraacetic acid, disodium salt (EDTA) was added and the reaction mixture was centrifuged at 820 × g for 2 min. The resultant supernatant was discarded and the sedimented platelets were resuspended in 2 ml of MPTS and incubated again for 10 min at 37°C. An additional 2.7 mM EDTA was added immediately prior to the next centrifugation at 820 × g for 2 min. This supernatant was discarded also and the sedimented platelets were resuspended in 1.6 ml of MPTS. An aliquot was removed from this control tube to determine the final platelet count. This platelet count, performed in duplicate, was used to determine the amount of thrombin bound to the surfaces of individual platelets. Washing of platelets up to 10 times did not change significantly the amount of labeled thrombin bound to their surfaces. After the first washing procedure, platelet suspensions were transferred to fresh, unused, silicone-coated tubes to eliminate the possibility of non-specific binding of free 125I-labeled thrombin to container walls. Washed erythrocytes were exposed to the 125I-labeled thrombin, reashed under the same conditions as described above, and examined for bound 125I-labeled thrombin. Less than 1% of the amount of 125I bound to platelets was found associated with erythrocytes when comparison was made on the basis of cell counts.

RESULTS

Effects of unmodified and modified thrombin on platelet functions

(a) Platelet Aggregation. A direct correlation was observed between the fibrinogen clotting activity and the ability of a particular thrombin preparation to induce platelet aggregation. Partially purified thrombin and α-thrombin were potent platelet aggregating agents (Fig. 1). At approximately 5 nM concentration, nearly a maximal degree of platelet aggregation was observed in the presence of these thrombins. β-Thrombin was less effective, whereas γ-thrombin was a poor aggregating agent at lower concentrations. Inhibition of α-thrombin by Tos-LysCH2Cl resulted in nearly complete loss of platelet aggregating activity.

(b) Release Reaction. Examination of [14C]serotonin released by platelets under the influence of a range of concentrations of the various thrombins revealed a pattern similar to that obtained in platelet aggregation studies. These release inducing activities of the different forms of thrombin were directly proportional to their fibrinogen clotting activities. At 10 nM concentration, both partially purified thrombin and α-thrombin induced nearly a maximal degree of release (Table 1). β-Thrombin was less effective, whereas γ-thrombin was a poor inducer of release. No release of [14C]serotonin was observed with concentrations of Tos-LysCH2Cl α-thrombin up to 50 nM.

Binding of various thrombins to the platelet surface

The various forms of thrombin were capable of binding to the platelet surface irrespective of their fibrinogen clotting or esterase activities. Overall, the binding ability of thrombins with high fibrinogen clotting activity was poor, the only exception being γ-thrombin which was found to have a binding pattern similar to that of α-thrombin or partially purified thrombin. Determination of the number of molecules of each of the various types of thrombin bound to the surface of platelets indicated that the binding efficiency of thrombin and partially purified thrombin was less than that of β- or Tos-LysCH2Cl α-thrombin (Fig. 2). Exposure of platelet suspensions to a maximum 400 nM concentration of α-thrombin or a 200 nM concentration of Tos-LysCH2Cl inactivated α-thrombin showed no indication of saturation of the binding sites under these experimental conditions (Fig. 3). It was also observed that the thrombin binding efficiency of platelets was better at lower than at higher thrombin concentrations. Platelets obtained from at fibrinogenemic and thrombasthenic patients were investigated also for their ability to bind thrombin. Preliminary studies on the binding of α- and Tos-LysCH2Cl α-thrombin to these platelets indicated a pattern similar to that observed in studies with normal platelets.
Table 1. Effects of various forms of thrombin on the release of platelet [14C]serotonin

<table>
<thead>
<tr>
<th>Thrombin (nM)</th>
<th>% of [14C]serotonin released by</th>
<th>Tos-LysCH2Cl-α-thrombin†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thrombin*</td>
<td>α-thrombin</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>43</td>
<td>41</td>
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<td>70</td>
</tr>
<tr>
<td>50.0</td>
<td>73</td>
<td>71</td>
</tr>
</tbody>
</table>

* Partially purified unmodified bovine thrombin.
† α-Thrombin inactivated by Tos-LysCH2Cl.

DISCUSSION

The ability of β- or γ-thrombin to cause weak platelet aggregation and to induce the release reaction at molar concentrations higher than that of α-thrombin is probably due to the residual fibrinogen-clotting activity of these proteins. If we consider that β- and γ-thrombin possess approximately 5–10% of the fibrinogen-clotting activity of α-thrombin, and if one assumes that there is a direct correlation between the ability to aggregate platelets and the ability to clot fibrinogen, then one would expect, irrespective of binding capability, that it would require some 20-fold more β- or γ-thrombin than α-thrombin to elicit the same physiological response. That is, in fact, what is observed in the current experiments. It is also possible that the residual fibrinogen clotting activity present in the preparations of β- and γ-thrombin is due to contamination by small amount of α-thrombin. This is considered unlikely, but does not affect the argument regarding the relationship between the physiological action of thrombin on platelets and the ability to clot fibrinogen.

Binding affinities of the various forms of thrombin for the platelet surface indicated generally a reverse pattern to that obtained in studies of platelet aggregation and release. The preparations of thrombin with high fibrinogen clotting activity appeared to bind to the platelet surface less efficiently than those with little or no fibrinogen clotting activity; the behavior of γ-thrombin being an unexplained exception of this rule. Since partially purified thrombin and α-thrombin are capable of inducing rapid aggregation, it is highly likely that in the presence of these forms of thrombin the platelet surface undergoes rapid secondary changes that are eventually manifested as aggregation and the release reaction. Apparently, similar secondary changes are not induced by β-, γ-, or Tos-LysCH2Cl-α-thrombins. Thus, it is possible that the decreased binding of partially purified thrombin or α-thrombin that was ultimately quantitated may be due to the secondary changes induced by these molecules on the platelet surface. Of interest also is the fact that Tollefsen and coworkers (9) have reported a similar binding affinity for partially purified thrombin and for thrombin inhibited by Dip-F. The only conclusion drawn by binding studies presented here is that various thrombins with varying degrees of fibrinogen clotting and esterase activities are capable of binding to platelet surfaces. It is realized that more detailed and critical studies will have to be carried out before the differences in the binding affinities of various thrombins could be considered meaningful.

Our failure to observe saturation of binding sites on the platelet plasma membrane raises the question of whether new binding sites may be uncovered as exposed sites are saturated with bound thrombin. Ganguly (10) has reported similar observations with washed platelets exposed to partially purified thrombin, although he observed a saturation when platelets were in plasma. Platelets in plasma were not used in the present study due to the inherent complexity of this medium. Failure to demonstrate binding saturation is unsettling and deserves further investigation.

Internalization of labeled thrombin by platelets is another possible explanation for failure to demonstrate saturation of binding sites. While this possibility cannot be ruled out completely, it appears unlikely in light of the work of others (9) who demonstrated binding of thrombin to platelet plasma mem-

![Fig. 2](image-url) Binding of various thrombins to platelets. See Fig. 1 for notations.

![Fig. 3](image-url) Concentration dependent binding of α-thrombin and Tos-LysCH2Cl-α-thrombin to platelets. Note that saturation of binding sites is not observed.
branes by autoradiography. These workers (9) were unable to demonstrate cytoplasmic localization of thrombin in platelets.

Observations reported here suggest that enzymatically active sites on the thrombin molecule apparently are not involved in the binding of the molecule to the platelet surface. The observed binding abilities of β-, γ-, and Tos-LysCH₂Cl-α-thrombin support this assumption. If the active site serine or histidine residues of the different thrombins were involved in this binding, blockage of these sites by Tos-LysCH₂Cl should have inhibited greatly the binding ability of thrombin. The present study does suggest the interesting possibility that a thrombin molecule may bind to the platelet surface in a manner that leaves the enzymatically active sites free to clot fibrinogen in and around the cell. Such an attachment would produce a means of anchoring and concentrating enzymatically active thrombin at the site of blood vessel injury, since platelets would be attracted avidly to exposed subendothelial components. If this is true, it may in fact be an important step in hemostasis. Whether binding of the thrombin molecule to the platelet surface is necessary for thrombin to act upon the cell, however, remains unknown.

In summary, this study shows for the first time that various forms of thrombin react differently with platelets. The ability of the various forms of thrombin to induce aggregation of platelets and the release reaction correlated well with their ability to clot fibrinogen. However, the abilities of the various forms of thrombin to bind to platelets displayed a pattern generally the reverse of that found in studies of platelet aggregation or release. Thrombin appears to bind to platelets in such a manner that the enzymatically active sites of the molecule are unimpaired, thus providing an effective means of anchoring this crucial enzyme at sites of vascular injury. Saturation of thrombin binding to platelets would be desirable for overall efficiency, if platelet aggregation was the only reaction desired. On the other hand, lack of saturation of the binding of thrombin to platelets would enhance the efficiency of this method of concentrating a key enzyme at precisely the point where it can function best in achievement of hemostasis by inducing fibrin formation to stabilize the already formed platelet aggregate.

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