The Binding of Thrombin to the Surface of Human Platelets*

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SUMMARY

We previously postulated that thrombin might initiate platelet aggregation and the release reaction by acting at the platelet surface. We have now demonstrated surface binding of thrombin using highly purified bovine thrombin labeled with 125I. At least two classes of binding sites have been demonstrated. When studies are done at relatively high thrombin concentrations, platelets bind 50,000 thrombin molecules per platelet at saturation with an apparent dissociation constant of 2.9 units per ml (30 nM thrombin). Binding studies carried out at lower thrombin concentrations disclose that approximately 500 thrombin molecules bind per platelet with an apparent dissociation constant of 0.02 unit per ml (0.21 nM thrombin). The other properties of these two types of thrombin-binding sites were identical. Diisopropyl fluorophosphate-treated thrombin (DIP-thrombin) binds to platelets with equal affinity and competes for the same sites as native thrombin, even though DIP-thrombin does not cause platelet aggregation or the release reaction.

Experiments using high resolution electron microscopic autoradiography established that 125I-thrombin binds to the cell surface. The mean position of 400 grains was 490 ± 90 A (S.E.M.) external to the platelet plasma membrane, suggesting that bound thrombin is located in the "glycoprotein coat" of the platelet surface. Similar localization was found for 125I-labeled phytohemagglutinin, which is known to bind to a platelet surface oligosaccharide. Addition of excess unlabeled thrombin rapidly displaced >95% of 125I-thrombin previously bound to platelets, further suggesting that thrombin does not enter the cell.

Although DIP-thrombin competitively inhibits binding of native thrombin, DIP-thrombin increases the extent of [3H]serotonin release when platelets are exposed to suboptimal concentrations of native thrombin. These findings suggest that thrombin induces the release reaction by a complicated mechanism involving both binding and proteolytic activities.

Platelets play an important role in the processes of hemostasis and thrombosis. In response to blood vessel injury and in certain pathological conditions, platelets aggregate and release numerous constituents, including adenine nucleotides, serotonin, and specific proteins (1, 2). Thrombin and a variety of other agents (e.g. collagen, ADP, and antigen-antibody complexes) induce aggregation or the release reaction or both in preparations of washed human platelets. We have suggested that thrombin interacts with the platelet surface to initiate these events. As evidence to support this hypothesis we have reported that adenylate cyclase activity is reduced in the particulate fraction of thrombin-treated platelets (3), and that the erythroagglutinating phytohemagglutinin from Phaseolus vulgaris, which binds to a receptor oligosaccharide on the platelet surface, mimics the effects of thrombin by producing adenylate cyclase inhibition and the release reaction (4). In addition, thrombin causes the release of a glycoprotein (mol wt 190,000) termed "thrombin-sensitive protein" from the particulate fraction of platelets (5, 6). It is possible, however, that these effects are secondary to the action of thrombin at a site other than the platelet surface.

We now describe quantitative binding studies and autoradiography using 125I-labeled thrombin which indicate that thrombin binds reversibly to high affinity sites on the platelet surface. We also report the unexpected finding that DFP-treated thrombin, which does not induce platelet aggregation or the release reaction but which binds normally to platelets, potentiates the effect of suboptimal concentrations of both native thrombin and E-PHA.1

MATERIALS AND METHODS

Isotopes were purchased from the following sources: carrier-free sodium[125I]iodide, Mallinckrodt Nuclear, St. Louis, Mo.; [3H]serotonin binoxalate (92.1 Ci per mmole), New England Nuclear, Boston, Mass. Bovine serum albumin (crystallized and lyophilized) and diisopropyl fluorophosphate were obtained from Sigma Chemical Co., St. Louis, Mo. Millipore filters (RAWP 025-00, 1.2-μm pore diameter) were purchased from Millipore Corp., Bedford, Mass. E-PHA and lentil PHA were purified and iodinated as previously described (4).

Preparation of Platelets—Platelets were isolated from 60 to 70 ml of human blood as follows. Whole blood was placed in 50-ml plastic centrifuge tubes containing Na2EDTA (5 mM final concentration) and centrifuged for 3 min at 1400 × g. The supernatant platelet-rich plasma was centrifuged at 2250 × g for 15 min and the resulting pellet was resuspended in 40 ml of isotonic phosphate-buffered saline (pH 6.5) containing 0.113 M NaCl.

1 The abbreviations used are: E-PHA, erythroagglutinating phytohemagglutinin from Phaseolus vulgaris; lentil PHA, lentil phytohemagglutinin; DFP, diisopropyl fluorophosphate; DIP-thrombin, diisopropyl phosphoryl-thrombin.

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36,600 (13). Purified from topical thrombin (Parke, Davis and Co., Detroit, pH 6.5, with 5 mg per ml and incubated with 5% radioactive iodide covalently bound to thrombin was G-25-80 equilibrated with 0.75 M NaCl-0.05 M sodium phosphate, pH 6.5, without loss of specific clotting activity. The amount of reaction was started by addition of 0.05 ml of chloramine-T (4 mg per ml) with rapid mixing.

Addition of 0.1 ml of sodium metabisulfite (2.4 mg per ml) after 1 min.

The clotting activity of 2630 units per mg of protein. Clotting activity was determined by the method of fibrinogen clotting activity of 2000 to 2500 units per mg of protein. Was the generous gift of Dr. Earl W. Davie and Mr. Mark E. Legaz, Bovine thrombin further purified by affinity chromatography (9) was stored at -20° and used within 10 days, since we observed 25 to 50% of the total present in the reaction. Iodinated thrombin showed a single peak of radioactivity which coincided with the protein band.

![Image](https://via.placeholder.com/150)

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**RESULTS**

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**Binding of Thrombin to Platelets**—In preliminary experiments we found that a correction was necessary for nonspecific (i.e. nonsaturable) binding of thrombin to platelets and for adsorption of free thrombin to Millipore filters. In subsequent experiments, duplicate incubations were performed at several concentrations of 125I-thrombin with and without the addition of a 20-fold or greater excess of unlabeled thrombin. 125I-Thrombin bound to platelets in the presence of excess thrombin was proportional to the total amount of thrombin added and was considered to be...
bound nonspecifically. In the absence of platelets, $^{125}$I-thrombin adsorbed to filters was proportional to the total added and was undiminished by excess unlabeled thrombin. We therefore corrected each value for $^{125}$I-thrombin bound by subtracting the value obtained in a duplicate incubation containing excess unlabeled thrombin. The magnitude of the correction for nonspecific binding varied as shown in Fig. 1 from 7.5% to 30% of the total thrombin bound. In the range of thrombin concentrations used in these experiments, equilibrium of binding occurred within 10 to 15 min, and in most experiments 30-min incubations were performed. Fig. 1 shows that thrombin binds minimally to erythrocytes, indicating that thrombin binding is specific with regard to cell type.

To determine the average number of thrombin receptor sites per platelet as well as their affinity, binding data were plotted (Figs. 2 and 3) according to the method of Steck and Wallach (18). When plotted in this manner, the number of molecules bound at saturation can be calculated from the intercept on the ordinate, and the apparent dissociation constant ($K_{	ext{diss}}$) can be calculated from the intercept on the abscissa. From a number of similar experiments (e.g. Fig. 2), using different thrombin and platelet preparations, we determined that platelets bind 40,000 to 60,000 thrombin molecules per cell with a dissociation constant of 1.3 to 3.9 units per ml (13 to 40,000 thrombin molecules per cell with a dissociation constant of 0.02 unit per ml). At higher thrombin concentrations the curve is distinctly nonlinear, and the points approach the line (dashed) extrapolated from the binding data of Fig. 2. These results suggest that platelets may possess at least two populations of thrombin-binding sites. The properties of the high affinity sites were similar to those of the other thrombin sites in that native unlabeled thrombin competes for these sites.

Thrombin treated with diisopropyl fluorophosphatadose does not produce platelet aggregation or the release reaction (20). Using iodinated thrombin that was subsequently treated with DFP, we have found that platelets possess an equivalent number of binding sites having similar affinity for DFP-thrombin as for native thrombin. Further studies demonstrated that unlabeled DIP-thrombin competitively inhibits binding of native $^{125}$I-thrombin (Fig. 2) with a calculated $K_i$ (3.5 units per ml) nearly equal to the $K_{	ext{diss}}$ (2.9 units per ml) for native thrombin determined in the same experiment. Similar competition for the high affinity thrombin sites was observed (data not shown). These results suggest that native thrombin and DIP-thrombin compete for the same platelet receptor sites with nearly equal affinity.

**Localization of Bound Thrombin—**We have employed electron

![Fig. 1. Binding of $^{125}$I-thrombin to platelets.](image)

Each incubation included 0.5 X $10^8$ platelets and the specified amount of thrombin in 0.5 ml of Tris-buffered saline (pH 7.5) containing 5 mg per ml bovine serum albumin. Incubations were initiated by addition of platelets. After 30 min at room temperature, the incubations were terminated by Millipore filtration as described under "Materials and Methods." $\bullet$ — $\bullet$, total $^{125}$I-thrombin bound; $\square$ — $\square$, nonspecific binding of $^{125}$I-thrombin in the presence of 200 units of unlabeled thrombin; $\bigcirc$ — $\bigcirc$, net $^{125}$I-thrombin bound calculated by subtracting nonspecific binding; $\bigtriangleup$ — $\bigtriangleup$, total $^{125}$I-thrombin bound to 0.25 X $10^8$ erythrocytes.

![Fig. 2. Binding of $^{125}$I-thrombin to platelets and competitive inhibition by DIP-thrombin.](image)

Incubations were conducted as in Fig. 1 with 0.2 X 4.25 units of $^{125}$I-thrombin and 0.5 X $10^8$ platelets in a total volume of 0.5 ml. Data are plotted according to the method of Steck and Wallach (18). The intercept on the ordinate equals 1/thrombin bound at saturation per 0.5 X $10^8$ platelets. The intercept on the abscissa equals $-1$/thrombin free per 0.5 ml ($K_{	ext{diss}}$) at which level half-maximal binding occurs. $\bullet$ — $\bullet$, $^{125}$I-thrombin; $\bigcirc$ — $\bigcirc$, $^{125}$I-thrombin + 0.95 unit of unlabeled DIP-thrombin.

![Fig. 3. Binding of $^{125}$I-thrombin to platelets.](image)

This experiment is analogous to that described in Fig. 2 except that lower levels of $^{125}$I-thrombin (0.00125 to 0.600 unit/0.5 ml) were used. $\cdots$ — $\cdots$, extrapolation of the data presented in Fig. 2 for $^{125}$I-thrombin binding in the absence of DIP-thrombin.
microscopic autoradiography to localize $^{125}$I-thrombin bound to platelets. A typical autoradiograph is shown in Fig. 4. About 100 cells were examined and a total of 400 grains were counted. For each grain the smallest possible circle was drawn which would circumscribe the grain, and the perpendicular distance from the center of the circle to the nearest surface membrane was measured. For the purpose of these measurements, membranes of the platelet canalicular system were considered as surface membrane, since these membranes are continuous with the platelet surface (7). Thus the lumen of a channel was considered external to the platelet. Approximately 15% of the grains were judged to be in association with channels. The distribution of grains is shown in Fig. 5. The mean position for $^{125}$I-thrombin was 490 ± 90 Å outside the platelet plasma membrane, suggesting that thrombin is localized in the “glycoprotein coat” of the platelet surface. A similar localization was obtained using $^{125}$I-lentil PHA (mean = 240 ± 50 Å S.E.M. outside), which has been shown to bind to a platelet surface oligosaccharide (4). As further support for the surface localization of bound thrombin, we noted that $^{125}$I-thrombin could be rapidly displaced from platelets by excess unlabeled DIP-thrombin as shown in Fig. 6. Thrombin was removed from both “low” and “high” affinity sites in these experiments.

**Effect of DIP-Thrombin on Release of Serotonin from Platelets—**
The role that thrombin binding might play in induction of the platelet release reaction was investigated using DIP-thrombin, which does not induce release, but which binds to the same sites as active thrombin. Thus one might expect DIP-thrombin to inhibit the release reaction induced by thrombin. The dependence of the extent of [14C]serotonin release on native thrombin concentration (in the absence of DIP-thrombin) is presented in Fig. 7. At each thrombin level tested, release occurred rapidly during the first 1 to 2 min following thrombin addition and very slowly, if at all, thereafter. The maximum amount of serotonin

![Fig. 4. Autoradiograph of $^{125}$I-thrombin-treated platelets. Platelets were incubated with $^{125}$I-thrombin (2.0 units per ml, ~5 µCi per unit) for 10 min in phosphate-buffered saline (pH 6.5). Platelets were then fixed with glutaraldehyde and washed to remove unbound thrombin. Autoradiography was performed as described under "Materials and Methods." × 17,300](image1)

![Fig. 5. Distribution of autoradiographic grains in relation to the platelet plasma membrane. Autoradiography was performed as described under "Materials and Methods" and in the legend to Fig. 4. The perpendicular distance was measured from the center of each grain to the plasma membrane. Top, 2.0 units per ml of $^{125}$I-thrombin incubated with platelets for 10 min prior to fixation; bottom, 50 µg per ml of $^{125}$I-lentil PHA, 10 min prior to fixation.](image2)

![Fig. 6. Time course of binding of $^{125}$I-thrombin to platelets and displacement by excess unlabeled thrombin. Each point represents a separate incubation conducted as in Fig. 1 (0.5-ml final volume) with addition of platelets at 0 min. Unlabeled DIP-thrombin was added at 10 min as indicated by the arrows. All values were corrected for nonspecific binding. Top, 1.0 unit per ml of $^{125}$I-thrombin, 66 units per ml of unlabeled DIP-thrombin. Bottom, 0.06 unit per ml of $^{125}$I-thrombin, 33 units per ml of DIP-thrombin.](image3)
FIG. 7. Thrombin-induced release of \(^{14}\)C-serotonin from platelets. Platelets were loaded with \(^{14}\)C-serotonin as described under "Materials and Methods." Ten-minute incubations were conducted as in Fig. 1 using unlabeled thrombin. Release is expressed as the percentage of serotonin retained as compared to control incubations without thrombin.

FIG. 8. Effect of DIP-thrombin on \(^{125}\)I-thrombin binding and on \(^{14}\)C-serotonin release. Two-minute incubations were performed at 0.01 unit per ml (left panels) and 0.1 unit per ml (right panels) of thrombin (total volume 0.5 ml). DIP-thrombin (unlabeled) was varied from 3.3 \(\times\) 10\(^{-2}\) to 3.3 \(\times\) 10\(^{-4}\) units per ml. Binding and release experiments were otherwise conducted as in Figs. 1 and 7, respectively. \(\bullet--\bullet\), DIP-thrombin alone; \(\square--\square\), 0.01 unit per ml of unlabeled thrombin; \(\triangle--\triangle\), 0.1 unit per ml of unlabeled thrombin; \(\square--\square\), 0.01 unit per ml of \(^{125}\)I-thrombin; \(\bigtriangleup--\bigtriangleup\), 0.1 unit per ml of \(^{125}\)I-thrombin.

that could be released by thrombin was 85%, and this extent of release was observed at thrombin concentrations as low as 0.1 unit per ml at a platelet concentration of 10\(^6\) cells per ml. About 50% of \(^{14}\)C-serotonin was released at 0.02 unit per ml of thrombin.

At concentrations up to 33 units per ml, DIP-thrombin had very little effect on \(^{14}\)C-serotonin release induced by 0.1 unit per ml of native thrombin, even though binding of native thrombin was reduced by over 90% (Fig. 8). Furthermore, at 0.01 unit per ml of native thrombin, a level at which only partial release is observed, DIP-thrombin (0.033 to 3.3 units per ml) slightly increased the amount of \(^{14}\)C-serotonin released, while decreasing the amount of native thrombin bond by 60 to 90%.

These experiments indicate that DIP-thrombin has an effect on platelets other than merely competing for binding with native thrombin. We also studied the effect of DIP-thrombin on the release reaction induced by E-PHA to further elaborate this point. DIP-thrombin enhanced E-PHA-induced release of serotonin to a small but reproducible degree when suboptimal concentrations of E-PHA were used (Fig. 9). These results further support the hypothesis that DIP-thrombin in some way enhances the efficiency of both thrombin and E-PHA in inducing the release reaction.

DISCUSSION

Our data support the hypothesis that thrombin acts at the platelet surface to initiate the release reaction. Thrombin binds to the platelet surface as shown by autoradiography and by the fact that \(^{125}\)I-thrombin can be displaced from platelets by excess unlabeled (DFP-treated) thrombin. Human platelets have two types of thrombin-binding sites; one population of approximately 50,000 sites per platelet with an apparent dissociation constant of about 2.9 units per ml and another smaller population of sites (~800 per platelet) with a 100-fold greater affinity for thrombin (~0.02 unit per ml). Greater than 90% of \(^{125}\)I-thrombin bound to either low affinity or high affinity sites can be displaced by unlabeled DFP-treated thrombin (Fig. 4), suggesting that the binding observed does not represent thrombin taken up into the cells. The high affinity population of binding sites may be more important in thrombin-induced aggregation and release, since the \(K_{dis}\) for these sites is nearly equal to the thrombin concentration at which half-maximal serotonin release is observed after 2 min. On the other hand, higher concentrations of thrombin result in more rapid release, and others have reported that inhibition of adenylyl cyclase (3) and release of calcium (21, 22) and nucleotides (21, 23) require greater concentrations of thrombin (0.1 to 0.5 unit per ml), and thus the larger population of sites of relatively lower affinity may also be important physiologically.

The fact that DIP-thrombin binds to platelets similarly to native thrombin without causing serotonin release suggests that thrombin has a separate catalytic site for release distinct from the site of binding to the cell surface. Similar conclusions have been reached in studies of the reaction between fibrinogen and thrombin in which separate proteolytic and fibrinogen-binding sites have been defined (24).

We have observed that DIP-thrombin does not prevent thrombin-induced release of serotonin at concentrations which greatly inhibit binding of native thrombin (Fig. 8). For example, at 33 units per ml of DIP-thrombin plus 0.1 unit per ml of native thrombin, native thrombin binding is reduced by 90% to 0.2 milliunit per 0.5 \(\times\) 10\(^6\) platelets, while serotonin release (~70% of control) is only slightly affected. By comparison, at 0.01
unit per ml of native thrombin in the absence of DIP-thrombin, 0.8 milliunit was bound but only about 10% of serotonin was released. At 0.33 unit per ml of DIP-thrombin plus 0.01 unit per ml of native thrombin, we observed a slight but reproducible increase in serotonin release. Similarly, DIP-thrombin potentiates release of serotonin induced by suboptimal (25) concentrations of E-PHA (Fig. 9). These observations cannot be explained at the present time, although they suggest that binding of DIP-thrombin to the platelet surface potentiates the effect of native thrombin.

The effects of DIP-thrombin are not explained by kinetic differences in binding or release, since time course studies of rates of thrombin binding and of serotonin release in the presence and absence of DIP-thrombin show no difference. In fact, the kinetics of serotonin release which we obtain is essentially identical to those observed for calcium and nucleotide release (22, 23) with an apparent first order rate constant of 0.015 to 0.03 s⁻¹.

Dewiler and Feinman (22, 23) have recently reported studies of the kinetics of calcium and ATP release induced by thrombin using human platelets. These authors conclude that platelets contain approximately 40,000 thrombin receptors per cell and that the only kinetic models which fit their data indicate cooperativity between thrombin sites. They further postulate that there are fewer thrombin sites on platelets at low thrombin concentration than at higher thrombin concentrations. Our results using direct binding studies largely support these results. Thus we observe two types of thrombin-binding sites: a few sites of very high affinity and a larger number of lower affinity sites. Our data do not distinguish whether these are physically distinct sites or represent a single class of sites which undergo a negative cooperative interaction as thrombin concentration increases. Furthermore, the experiments using DIP-thrombin suggest that the efficiency of active thrombin in inducing release may be enhanced as larger numbers of sites are occupied by either thrombin or DIP-thrombin.

The physiological advantage of this system might be to prevent triggering of the platelet release reaction by low concentrations of thrombin in vivo. Thus, lower concentrations of thrombin would bind to the high affinity, potentially less efficient sites on platelets and prevent release by a few platelets. As a higher thrombin concentration was generated, greater numbers of sites would be saturated on all of the platelets, thus triggering the release reaction. This mechanism might serve as a damper on platelet aggregation and release. An alternative explanation for two types of thrombin receptors would be that they occur on different cells and that a subpopulation of the platelets has high thrombin affinity.

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