Binding of the Products of Prothrombin Activation to Human Platelets

DOUGLAS M. TOLLEFSEN, CRAIG M. JACKSON, and PHILIP W. MAJERUS

From the Divisions of Hematology and Oncology, Departments of Internal Medicine and Biochemistry, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT Binding of prothrombin and activation intermediates 1 and 2 to human platelets was tested with ^{125}I-labeled protein preparations. None of these precursors of thrombin bound to platelets under conditions in which high affinity binding of thrombin was observed, nor did they cause platelet aggregation or serotonin release. The molecular conformation required for binding to platelets as well as for induction of platelet aggregation and release is present, therefore, only after the final step in prothrombin activation.

INTRODUCTION

We recently reported that thrombin binds specifically to about 50,000 receptor sites on the surface of intact human platelets (1). The kinetics of thrombin binding are complex in that the affinity of platelets for thrombin decreases as more thrombin is bound. This could be explained either by a negative cooperative interaction among receptors of a single kind or by multiple kinds of receptors with different affinities for thrombin. At this time the nature of the thrombin receptor(s) is unknown.

Native thrombin and thrombin inactivated with diisopropyl fluorophosphate (DIP-thrombin)[1] bind indistinguishably to platelet receptor sites (1), although only the native, proteolytically active thrombin can initiate platelet aggregation and the release reaction (2), i.e., the rapid secretion of serotonin, calcium, adenine nucleotides, and certain other substances (3). We observed that DIP-thrombin potentiates serotonin release induced by submaximal levels of native thrombin, suggesting that thrombin binding per se might play a role in induction of the release reaction (1).

To study the specificity of the binding of thrombin to platelets, we now have tested binding of the thrombin precursors, prothrombin and activation intermediates 1 and 2 (nomenclature of Owen, Esmon, and Jackson (4); see Fig. 1). These experiments indicate that the molecular conformation required for binding to platelets is present only after the final step in prothrombin activation.

METHODS

Platelets were isolated from human blood, washed, and suspended in isotonic Tris-saline (pH 7.5) containing glucose (1 mg/ml), as previously described (1).

Bovine prothrombin, intermediate 1, and intermediate 2 were prepared as described previously (4, 5). Purified prothrombin was cleaved by thrombin to yield intermediate 1 and fragment 1, which were separated by chromatography on QAE-Sephadex Q-50 (4). Intermediate 2 was prepared by incomplete activation of prothrombin with factor X_a (without factor V and phospholipid) and was isolated by QAE-Sephadex chromatography (4). Bovine thrombin was purified by the method of Glover and Shaw (6). The concentrations of these proteins were estimated by measurement of ultraviolet absorption with the following extinction coefficients: prothrombin, $E^{1\%}_{280} = 15.5$; intermediate 1, $E^{1\%}_{280} = 19.2$; intermediate 2 and thrombin, $E^{1\%}_{280} = 21.4$ (4).

The erythroagglutinating phytohemagglutinin of *Phaseolus vulgaris* (E-phytohemagglutinin) was prepared as described previously (7).

Samples of proteins (5–10 μg) were prepared for gel electrophoresis by boiling for 2 min in 0.1 M sodium phosphate (pH 7.5), containing 5% sodium dodecyl sulfate and 0.1 M β-mercaptoethanol. Electrophoresis was carried out with 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate (pH 7.5). The gels were stained for protein with Coomassie blue. Molecular weights of the proteins were determined by plotting the logarithm of molecular weight versus electrophoretic mobility, with the following molecular weight standards: serum albumin (mol wt 65,000), alkaline phosphatase (mol wt 43,000), aldolase (mol wt 40,000), pepsin (mol wt 35,000),

Mr. Tollefsen is the recipient of U. S. Public Health Service Medical Scientist Training Program Award GM 02016.

Received for publication 3 February 1975 and in revised form 4 April 1975.

[1] *Abbreviations used in this paper:* DIP-, diisopropyl phosphoryl-; E-phytohemagglutinin, erythroagglutinating hemagglutinin of *Phaseolus vulgaris*.

myoglobin (mol wt 17,200), and fibrinogen α, β, and γ chains (mol wt 63,500, 56,000, and 47,000, respectively).

Proteins were labeled with ^{125}I by a modified Chloramine-T procedure (1), and binding studies with platelets were performed with the Millipore filtration assay described previously (1), with the following modification: incubations were terminated by adding 5 ml of isotonic Tris-saline (pH 7.5) and the platelet suspensions were immediately filtered under vacuum. This procedure took less than 5 s. No further washing of the filtered platelets was performed. A small amount of binding to the filters was observed in the absence of platelets. This "nonspecific" binding was proportional to added protein over the ranges studied and amounted to 0.7%, 1.0%, and 8.0% of the prothrombin, intermediate 1, and intermediate 2 added, respectively, in the experiments shown in Figs. 3 and 4. Each point in Figs. 3 and 4 represents the difference between incubations in the presence and in the absence of platelets.

Prothrombin, intermediate 1, and intermediate 2 were converted to thrombin (8) with venom from the Australian taipan snake (*Oxyuranus scutellatus scutellatus*) obtained from Sigma Chemical Co. (St. Louis, Mo.). Reaction mixtures contained 18–48 μg of the thrombin precursor (unlabeled or labeled with ^{125}I) and 2.5 μg of venom in 0.5 ml of isotonic Tris-saline (pH 7.5). Incubations were carried out for 4 h at room temperature, and the development of thrombin activity was followed by the fibrinogen clotting assay of Seegers and Smith (9) with purified bovine fibrinogen (10).

Release of [^{14}C]serotonin from platelets was measured as previously described (1).

RESULTS

The structural relationships among the proteins used in this study are diagramed in Fig. 1. Prothrombin (mol wt 73,000) is a single polypeptide chain precursor of thrombin (mol wt 37,000). Activated factor X (X_a) catalyzes the proteolytic cleavage of prothrombin to

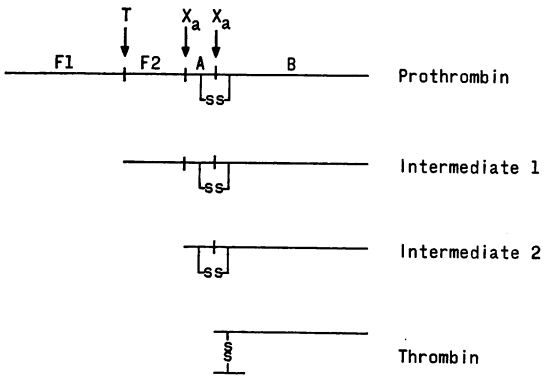

FIGURE 1 Structural relationships among prothrombin, intermediate 1, intermediate 2, and thrombin. Polypeptide chains are represented by horizontal lines proportional in length to the molecular weights of the chains. Vertical lines indicate sites of the proteolytic cleavage catalyzed by thrombin (T) and activated factor X (X_a). F1, fragment 1; F2, fragment 2; A and B, A and B chains of thrombin, respectively. Data from ref. 4.

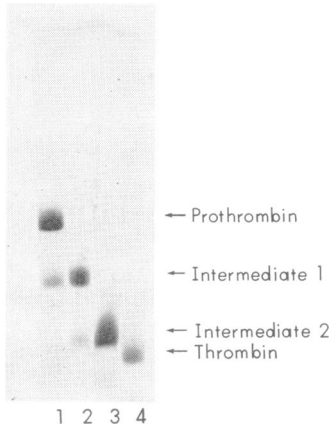

FIGURE 2 Sodium dodecyl sulfate gel electrophoresis of protein preparations used in these studies. 1, prothrombin; 2, intermediate 1; 3, intermediate 2; 4, thrombin. Gels containing standard proteins (see Methods) were run simultaneously to confirm the identities of the protein bands (shown by the arrows at right) according to their known molecular weights (4). The A chain of thrombin is not detected in this gel system.

yield two polypeptide chains, designated intermediate 2 (mol wt 37,000) and fragment 1·2 (mol wt 36,000) (5). X_a further cleaves intermediate 2 to yield the A and B chains of thrombin (mol wt 6,000 and 31,000, respectively), which are linked by a disulfide bridge. Thrombin cleaves prothrombin at one site, yielding intermediate 1 (mol wt 49,000) and fragment 1 (mol wt 24,000) (4). The appearance of these intermediates and fragments during prothrombin activation in vitro has been elucidated previously (4, 5) and is discussed briefly below.

We prepared prothrombin, intermediates 1 and 2, and thrombin by previously published methods (4–6). To determine the purity of these preparations, we carried out polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol (Fig. 2). Intermediate 2 and thrombin were homogeneous by this criterion. The prothrombin and intermediate 1 preparations each contained a single minor component co-migrating with intermediates 1 and 2, respectively. The fibrinogen clotting activities of these preparations were as follows: prothrombin, < 4 U/mg; intermediate 1, < 7 U/mg; intermediate 2, 1.0 U/mg; and thrombin, 2,300 U/mg.

The thrombin precursors were labeled with ^{125}I, and these derivatives were tested for binding to platelets (Fig. 3). Nonspecific binding to the Millipore filters, determined as described in the Methods section, was subtracted to give the data presented. Prothrombin, intermediate 1, and intermediate 2 (closed symbols) did not bind or bound only slightly, although the experiments shown in Fig. 3 were done over a range of

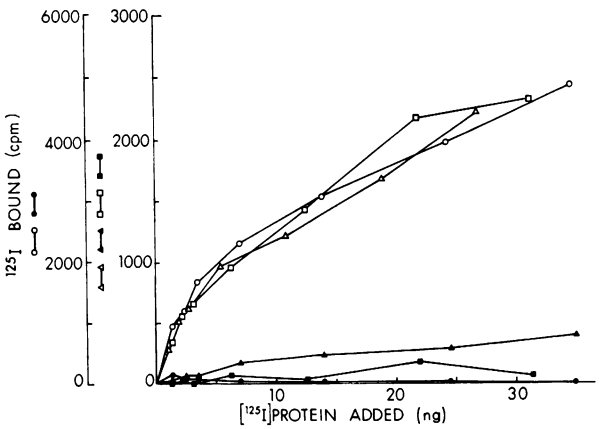

FIGURE 3 Binding of ^{125}I-labeled proteins to intact platelets. Each incubation included 0.5×10^8 platelets and the amount of protein specified on the abscissa in 0.5 ml of Tris-buffered saline (pH 7.5) containing 1 mg/ml glucose. Incubations were initiated by addition of platelets. After 30 min at room temperature the incubations were terminated by Millipore filtration as described in Methods. Added ^{125}I-proteins were: ●—●, prothrombin (2,000 cpm/ng); ■—■, intermediate 1 (1,190 cpm/ng); ▲—▲, intermediate 2 (331 cpm/ng). In control experiments the iodinated proteins were activated with taipan venom and retested for binding. Venom-activated proteins were: ○—○, prothrombin; □—□, intermediate 1; △—△, intermediate 2.

protein concentrations in which we previously observed high-affinity binding of thrombin (1). A direct comparison can be made with the binding of thrombin derived from each of these precursors (open symbols) as discussed below.

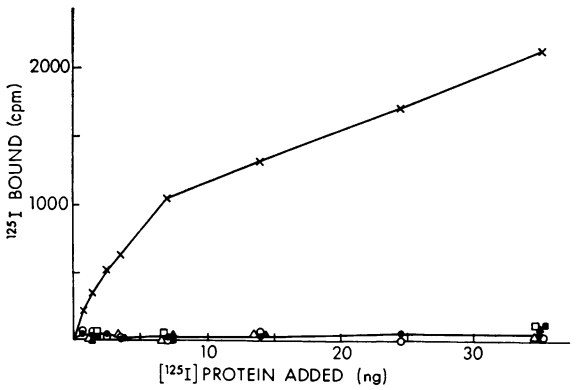

FIGURE 4 Binding of ^{125}I-prothrombin to platelets in the presence and absence of calcium. Incubations were performed as in Fig. 3. ^{125}I-Prothrombin was present along with the following additions: ○—○, none; ●—●, 2 mM $CaCl_2$; △—△, 0.1 U/ml thrombin; ▲—▲, 0.1 U/ml thrombin + 2 mM $CaCl_2$; □—□, 100 μg/ml E-phytohemagglutinin; ■—■, 100 μg/ml E-phytohemagglutinin + 2 mM $CaCl_2$. The ^{125}I-prothrombin was also activated with taipan venom and tested for binding in the absence of calcium (×—×).

The following experiments were done to establish that the preparation and iodination of the thrombin precursors did not denature these proteins and thereby prevent their binding to platelets. (*a*) The thrombin precursors were converted to thrombin with taipan venom, which contains an enzyme capable of hydrolyzing the same peptide bonds as factor X_a (8). The percentages of theoretical clotting activity obtained after venom activation were: prothrombin, 94%; intermediate 1, 96%; and intermediate 2, 46%.[2] This indicates that most of the protein in these preparations was in a native conformation. (*b*) The iodinated precursors were activated with taipan venom to approximately the same extent as the unlabeled proteins, and after activation the labeled preparations bound to platelets (Fig. 3, open symbols). The bound material in each case was displaced (> 80%) by 30 μg/ml of unlabeled thrombin (data not shown). In these control experiments, the taipan venom was not removed before binding was tested, but we showed that the venom had no effect by itself on platelets (i.e., it did not induce aggregation or serotonin release) and that thrombin activity and the ability to bind to platelets appeared simultaneously during incubation with the venom.

The binding experiments were repeated at higher protein concentrations (up to 42–92 μg/ml), and no binding was observed with any of the precursors unless they were first converted to thrombin.

Since prothrombin and fragment 1 have been shown to bind to phospholipid vesicles in a process requiring divalent cations (12), we also studied the binding of these two proteins to platelets in the presence of 2 mM calcium chloride. Prothrombin binding could not be detected in a range from 1.4 ng/ml to 84 μg/ml in the presence of calcium ions, although the venom-activated protein did bind. This experiment is shown in Fig. 4 in the range 1.4–70 ng/ml. Similarly, fragment 1 did not bind either in the presence or absence of calcium ions (data not shown).

Platelets undergoing the release reaction in response to 0.1 U/ml of thrombin (1) or to 100 μg/ml of E-phytohemagglutinin (7) also did not bind detectable prothrombin either in the presence or absence of calcium (Fig. 4).

In additional experiments we observed that the thrombin precursors did not cause release of [^{14}C]serotonin from platelets at concentrations equivalent to 10 U/ml of thrombin (100–1,000-fold greater than normally required for release; ref. 1). The thrombin precursors at these concentrations also had no effect on

[2] Intermediate 2 could be activated fully by [X_a, V_a, phospholipid, Ca^{++}] in the presence of fragment 1·2 (11).

the kinetics of thrombin-induced serotonin release or on ^{125}I-thrombin binding to platelets (tested at 0.02–0.1 U/ml thrombin). These observations are consistent with the lack of detectable binding of the thrombin precursors.

DISCUSSION

Our experiments demonstrate a high degree of specificity in the binding of thrombin to platelets. Neither prothrombin nor intermediates in the conversion of prothrombin to thrombin bind (Fig. 3). It is particularly interesting that intermediate 2 does not bind, since hydrolysis of a single peptide bond (4) converts intermediate 2 to thrombin (see Fig. 1) and confers upon the protein its enzymatic as well as its platelet-binding properties. These properties apparently represent independent conformational changes in the protein, since the proteolytic active site of thrombin can be blocked with diisopropyl fluorophosphate while DIP-thrombin retains the ability to bind to platelets (1). The finding that intermediates 1 and 2 also do not affect thrombin-induced serotonin release further implies that the accumulation of these intermediates during prothrombin activation does not modulate platelet function mediated by thrombin.

It is well established that phospholipid dispersions enhance the rate of prothrombin activation in vitro. Prothrombin and factor X_a both bind to phospholipid vesicles in a process requiring divalent cations, while thrombin does not bind. Gitel, Owen, Esmon, and Jackson have demonstrated that the fragment 1 region of the prothrombin molecule is required for binding to phospholipid vesicles (12). It is generally assumed that platelets provide a catalytic surface similar to that of phospholipid vesicles for prothrombin activation in vivo. The procoagulant activity of platelets (platelet factor 3) appears in response to a variety of platelet-aggregating agents (3). Although the molecular basis of platelet factor 3 is not understood, one might predict by analogy to phospholipid vesicles that activated platelets would bind prothrombin in the presence of divalent cations.

In contrast to the above prediction, control platelet preparations or platelets that had undergone the release reaction in response to 0.1 U/ml of thrombin (1) or to 100 μg/ml of E-phytohemagglutinin (7) did not bind detectable prothrombin in the presence (or absence) of calcium ions (Figs. 3 and 4). We do not conclude from this that prothrombin binding to platelets or activation on the platelet surface does not occur, since further experiments are required to determine whether prothrombin binding can be detected under other conditions or by other techniques. Specifically, we would not have detected binding if the dissociation rate were very rapid, such that a large fraction of the bound prothrombin dissociated within the time (< 5 s) taken for dilution and filtration of the platelet suspension. If prothrombin binding does take place, our data suggest that it is qualitatively very different from thrombin binding. A further possibility is that our washing procedure removes some factor necessary for prothrombin binding. Alternatively, platelets might bind prothrombin very tightly, so that the binding sites of our washed platelets would already be saturated with prothrombin.

The receptor(s) for thrombin binding has not been identified. Using monovalent anti-fibrinogen antibody fragments (Fab), we have tested the hypothesis that platelet surface fibrinogen molecules bind thrombin. The Fab fragments bound to the platelet surface but had only a marginal inhibitory effect on the binding of thrombin, although they markedly inhibited platelet aggregation (13). We concluded that platelet fibrinogen is required for thrombin-induced aggregation of washed platelets but probably does not constitute a major population of thrombin receptor sites.

Recent experiments in this laboratory have demonstrated a strong correlation between thrombin binding and the extent of [^{14}C]serotonin release from platelets.[3] At a given total thrombin concentration, changes in the anionic composition of the buffer in which platelets were suspended resulted in large differences in the amount of thrombin bound, and the extent of serotonin release was directly proportional to the thrombin bound. The various buffers used did not appear to have a general effect on the platelet surface, since lectin-induced release (7) was not affected. Thrombin binding thus appears to be a highly specific process that is directly involved in induction of the platelet release reaction.

[3] Shuman, M., and P. W. Majerus. Manuscript in preparation.

ACKNOWLEDGMENTS

This research was supported by Grants HL 14147 and HL 16634 from the National Institutes of Health and by Grant PRA 33 from the American Cancer Society.

REFERENCES

1. Tollefsen, D. M., J. R. Feagler, and P. W. Majerus. 1974. The binding of thrombin to the surface of human platelets. *J. Biol. Chem.* **249**: 2646–2651.
2. Davey, M. G., and E. F. Lüscher. 1967. Actions of thrombin and other coagulant and proteolytic enzymes on blood platelets. *Nature (Lond.)*. **216**: 857–858.
3. Holmsen, H., H. J. Day, and H. Stormorken. 1969. The blood platelet release reaction. *Scand. J. Haematol. Suppl.* **8**: 1–26.

4. Owen, W. G., C. T. Esmon, and C. M. Jackson. 1974. The conversion of prothrombin to thrombin. I. Characterization of the reaction products formed during the activation of bovine prothrombin. *J. Biol. Chem.* **249**: 594–605.
5. Esmon, C. T., W. G. Owen, and C. M. Jackson. 1974. The conversion of prothrombin to thrombin. II. Differentiation between thrombin- and factor X_a-catalyzed proteolyses. *J. Biol. Chem.* **249**: 606–611.
6. Glover, G., and E. Shaw. 1971. The purification of thrombin and isolation of a peptide containing the active center histidine. *J. Biol. Chem.* **246**: 4594–4601.
7. Tollefsen, D. M., J. R. Feagler, and P. W. Majerus. 1974. Induction of the platelet release reaction by phytohemagglutinin. *J. Clin. Invest.* **53**: 211–218.
8. Owen, W. G., and C. M. Jackson. 1973. Activation of prothrombin with *Oxyuranus scutellatus scutellatus* (Taipan snake) venom. *Thromb. Res.* **3**: 705–714.
9. Seegers, W. H., and H. P. Smith. 1942. Factors which influence the activity of purified thrombin. *Am. J. Physiol.* **137**: 348–354.
10. Straughn, W., III, and R. H. Wagner. 1966. A simple method for preparing fibrinogen. *Thromb. Diath. Haemorrh.* **16**: 198–206.
11. Esmon, C. T., W. G. Owen, and C. M. Jackson. 1974. The conversion of prothrombin to thrombin. IV. The function of the fragment 2 region during activation in the presence of Factor V. *J. Biol. Chem.* **249**: 7791–7797.
12. Gitel, S. N., W. G. Owen, C. T. Esmon, and C. M. Jackson. 1973. A polypeptide region of bovine prothrombin specific for binding to phospholipids. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1344–1348.
13. Tollefsen, D. M., and P. W. Majerus. 1975. Inhibition of human platelet aggregation by monovalent anti-fibrinogen antibody fragments. *J. Clin. Invest.* **55**: 1259–1268.