When thrombin is complexed to the endothelial cell surface receptor thrombomodulin, it loses its procoagulant activities in that it no longer clots fibrinogen or activates factor V. Studies were initiated to determine if complex formation also blocks thrombin's other major procoagulant function, the activation of platelets. When bound to thrombomodulin, thrombin no longer induces platelets to either aggregate or release \( ^{14} \)C serotonin. Binding studies using \( ^{125} \)I-labeled thrombin or diisopropyl phosphorothrombin indicate that the complex does not bind to the platelet. When thrombomodulin is added after thrombin has bound to the platelets, the thrombin rapidly redistributes onto the thrombomodulin. These data suggest that in addition to its other anticoagulant effects, thrombomodulin may also act to inhibit and/or reverse platelet activation by thrombin.

Regulation of the blood-clotting process requires control of both plasma and cellular events. Thrombin appears to be a focal point in the control of this complex process. Depending on the exact conditions and environment, thrombin may either facilitate (1) or inhibit (2-4) the clotting process. Thrombin is a potent stimulator of platelet aggregation and release. At present, the exact mechanism by which thrombin activates platelets remains obscure (5). Many studies indicate that platelet activation involves a receptor-thrombin interaction and that binding to high affinity sites (\( \sim 1 \) nM) correlates with serotonin release and aggregation (6-9). Evidence also exists that binding alone is not sufficient for activation. Thrombin which has been blocked in the catalytic site is able to bind to platelets equivalently to native thrombin, but can neither induce release and aggregation (6, 10, 11) nor inhibit activation by native thrombin (6, 12). Destruction of a platelet surface protein by chymotrypsin not involved in thrombin binding greatly diminishes thrombin's ability to activate the platelets (13). The observation that thrombin covalently immobilized on Sepharose 2B can activate platelets (14) supports the hypothesis that proteolysis of a membrane protein(s) is also necessary.}

Recently, a role of the vascular endothelium in the regulation of both thrombin formation and thrombin clearance has begun to emerge. The endothelial cell surface binds thrombin with relatively high affinity (\( K_d \sim 1 \) nM) (15-18). Both the activation of protein C by thrombin (19-22) and the inhibition of thrombin by antithrombin III (17, 23) are greatly accelerated on the endothelial cell surface. Furthermore, the binding of thrombin to the endothelial cell surface possibly triggers intracellular prostacyclin formation (3, 4).

Of these putative endothelial receptors for thrombin, only the receptor for protein C activation, thrombomodulin, has been isolated (24). Thrombomodulin is a membrane protein (\( M_r \sim 74,000 \)) which has only been detected on the surface of endothelial cells (24). Thrombomodulin forms a high affinity (\( K_d \sim 0.5 \) nM) 1:1 molar complex with thrombin (24). While Ca\(^{2+} \) is required for the activation of protein C by this complex, the high affinity interaction between thrombin and thrombomodulin does not require Ca\(^{2+} \) (25).

Focal point in the control of this complex process. Depending on the exact conditions and environment, thrombin may either facilitate (1) or inhibit (2-4) the clotting process. Thrombin is a potent stimulator of platelet aggregation and release. At present, the exact mechanism by which thrombin activates platelets remains obscure (5). Many studies indicate that platelet activation involves a receptor-thrombin interaction and that binding to high affinity sites (\( \sim 1 \) nM) correlates with serotonin release and aggregation (6-9). Evidence also exists that binding alone is not sufficient for activation. Thrombin which has been blocked in the catalytic site is able to bind to platelets equivalently to native thrombin, but can neither induce release and aggregation (6, 10, 11) nor inhibit activation by native thrombin (6, 12). Destruction of a platelet surface protein by chymotrypsin not involved in thrombin binding greatly diminishes thrombin's ability to activate the platelets (13). The observation that thrombin covalently immobilized on Sepharose 2B can activate platelets (14) supports the hypothesis that proteolysis of a membrane protein(s) is also necessary.

**Experimental Procedures**

**Materials**—Bovine thrombin (26) and rabbit lung thrombomodulin (24) were purified by published methods. DIP-thrombin\(^{1} \) was prepared by reacting thrombin (0.5 mg/ml) with 2 mM diisopropyl fluorophosphate in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4, for 10 min. Excess diisopropyl fluorophosphate was removed by dialysis against the same buffer. Molecular weights and extinction coefficients \( (E_1^1 %) \) used were 37,000 and 21 for thrombin and 74,000 and 8.8 for thrombomodulin. Carrier-free Na\(^{125} \)I was purchased from New England Nuclear, \(^{14} \)C serotonin from Amersham Corp., and bovine serum albumin from Sigma.

**Platelet Preparations**—The preparation of PRP and gel-filtered platelets as well as the measurement of \(^{14} \)C serotonin secretion was carried out as previously described (27). The Tangen buffer used in these studies contained 0.145 M NaCl, 5 mM KCl, 50 \( \mu \)M CaCl\(_2\), 100 \( \mu \)M MgCl\(_2\), 5.5 mM glucose, 15 \( \mu \)M HEPES, and 3.5 mg/ml of bovine serum albumin.

**Measurement of Aggregation**—Aggregometry was carried out in a Payton dual channel aggregometer thermostated at 37 °C with continuous stirring at 1000 rpm.

**Labeling of Thrombin**—Thrombin was iodinated with Na\(^{125} \)I using Enzymobeads (Bio-Rad). Reactions were performed at 4 °C and contained 20 \( \mu \)g of thrombin, 500 \( \mu \)g of Enzymobeads in 0.15 M NaCl, 0.02 Tris-HCl, pH 7.4, in a total volume of 150 \( \mu \)l. The reaction was started by the addition of glucose (0.3% final...
concentration). After 20 min, the reaction was stopped by pelleting the beads. The labeled protein was dialyzed overnight versus four changes of buffer and bovine serum albumin was added to 5 mg/ml. The resultant product was able to form a complex with antithrombin III (>95%) as judged by autoradiography of Na dodecyl sulfate gels. The specific activity of the $^{125}$I-thrombin was 5-10 pCi/µg. The labeled thrombin was used within 1 week.

The specific activity of the $^{125}$I-thrombin was 5-10 pCi/µg. The labeled protein was dialyzed overnight changes of buffer and bovine serum albumin was added to 5 mg/ml. The labeled protein was then added to the beads. The labeled protein was dialyzed overnight changes of buffer and bovine serum albumin was added to 5 mg/ml.

Binding Studies—Platelets (2.5-5 × 10⁸/ml) were incubated at 37 °C with $^{125}$I-thrombin (0.025–11.3 nM) or $^{125}$I-DIP-thrombin in siliconized aggregometer cells. Unless noted, the samples were stirred for 30 s and then left unstirred for 3 min. At this time, 0.1-ml samples were layered on Apiezon A:n-butyl phthalate (1:9) oil (0.35 ml in 0.4-ml centrifuge tubes) and centrifuged for 2 min at 10,000 rpm in a Beckman Microfuge II. Aliquots of supernatant were removed and the bottom of the tubes were cut for determining the free and bound thrombin, respectively.

Binding mixtures containing thrombomodulin were prepared in two ways. In most cases, thrombomodulin was mixed with the labeled thrombin or DIP-thrombin at a molar ratio of 20:1 before addition to the platelets. Higher concentrations of thrombomodulin were obtained by the direct addition of thrombomodulin to the platelets to yield a final concentration of 110 nM. Labeled protein was then added to the mixture.

RESULTS

Effect of Thrombomodulin on Thrombin-induced Platelet Aggregation—When thrombomodulin is added to PRP at 45 nM, no effect on the platelets is observed (Fig. 1, tracing a). When thrombin is then added to a level sufficient to cause rapid aggregation (tracing b) in the absence of thrombomodulin (2.25 nM), the platelets initially react by undergoing shape change (tracing a, arrow 2). However, they quickly return to discoid shape as indicated in the aggregometer tracing. A similar effect is seen if high concentrations of preformed thrombomodulin-thrombin complex are added to the PRP (data not shown). When 10 µM ADP is added to PRP treated with thrombomodulin and thrombin (tracing a, arrow 3), normal aggregation occurs, indicating that the platelets have not become refractory to all aggregating agents. When thrombin is added at concentrations in excess of the thrombomodulin, rapid platelet aggregation occurs.

Thrombomodulin is also able to reverse platelet aggregation induced by thrombin, as shown in Fig. 2. In this experiment, aggregation was initiated by the addition of 2.25 nM thrombin (arrow 1, all tracings). When 45 nM thrombomodulin was added (arrow 2, all tracings) 2.5 s (tracings a), 10 s (tracing b), or 15 s (tracing c) after the thrombin stimulus, aggregation could be reversed. ADP was added to the 15-s interval sample to indicate aggregation was not complete (tracing c, arrow 3). Thrombomodulin does not reverse aggregation or block clot formation if added later than 15 s after the thrombin stimulus (tracing d). Similar tracings are obtained when lower doses of thrombin are used as the initial stimulus (data not shown) or if hirudin is added shortly after the thrombin (28).

Results similar to those obtained in Fig. 1 are obtained when gel-filtered platelets are substituted for PRP. To establish that thrombin had not been irreversibly altered by interaction with thrombomodulin, DIP-thrombin was employed to displace active thrombin from the preformed complex. When a molar excess of DIP-thrombin (56 nM) was added to the preformed thrombomodulin-thrombin complex (35 nM thrombomodulin, 2.3 nM thrombin) in the presence of platelets, the platelets aggregated within 2 min (data not shown). Presumably, the aggregation reflects the release of thrombin from thrombomodulin due to competition by DIP-thrombin (20, 21, 25). The activation observed is not due to active thrombin since this level of DIP-thrombin did not aggregate the platelets in the presence or absence of thrombomodulin.

In addition to inhibiting aggregation, thrombomodulin also inhibits the thrombin-initiated platelet release reaction (Fig. 3). $[^{14}C]$Serotonin release induced by 5.6 nM thrombin was completely blocked by the simultaneous addition of 50 nM thrombomodulin (Fig. 3, I). Aggregometer tracings indicated

**Fig. 1.** Effect of thrombomodulin on thrombin-induced platelet aggregation in PRP. Aggregation in PRP was measured as described under "Experimental Procedures." At the times indicated by the arrows, thrombin or thrombomodulin was added to the following concentrations: tracing a: arrow 1, thrombomodulin, 45 nM; arrow 2, thrombin, 2.25 nM; arrow 3, 10 µM ADP; tracing b: arrow 1, thrombin, 2.25 nM. The star indicates clot formation.

**Fig. 2.** Reversal of thrombin-induced platelet aggregation by thrombomodulin. PRP was equilibrated in the aggregometer cuvette, and thrombin was added to 2.25 nM at the time indicated by arrow 1. Thrombomodulin was added to 45 nM at the times indicated by arrow 2. Addition of 10 µM ADP is indicated by arrow 3. The star indicates clot formation. The time intervals between thrombin and thrombomodulin were: 2.5 s (tracing a), 10 s (tracing b), 15 s (tracing c), and 20 s (tracing d). See Fig. 1 for no thrombomodulin control.
that shape change of the platelets had occurred in this sample (data not shown). Lower levels of thrombomodulin were only partially effective in blocking $[^{14}C]$serotonin release.

The effect of thrombomodulin on thrombin stimulation of platelets can be explained in either of two ways. First, the thrombomodulin-thrombin complex may bind to the platelets but lead to an inactive complex on the platelet surface. Alternatively, when bound to thrombomodulin, thrombin may be unable to bind to the platelet receptor required for activation.

To distinguish these possibilities, direct binding studies using iodinated thrombin and DIP-thrombin were performed.

**Thrombin Binding to Platelets in the Presence of Thrombomodulin**—The binding of thrombin to platelets was studied as a function of the thrombin concentration as depicted in Fig. 4. As it has previously been shown that thrombin and DIP-thrombin bind to platelets equivalently (6, 10, 11), both forms of thrombin were used. Since platelets are not activated in the presence of thrombomodulin, it was felt the use of DIP-thrombin would avoid binding differences due to variable activation states of the platelets in the presence of differing levels of thrombin and the presence or absence of thrombomodulin. In the absence of thrombomodulin, binding could be observed to both high (Fig. 4A) and low (Fig. 4B) affinity sites, as has been previously reported (5, 6, 9, 29). When the labeled thrombin or DIP-thrombin is first complexed to thrombomodulin at a molar ratio of 20:1 (thrombomodulin:thrombin) at relatively high concentrations ($\geq 1 \text{nM}$ thrombin), binding of the labeled protein is decreased to less than 10% of that seen in the absence of thrombomodulin. At lower concentrations of thrombin, where only high affinity platelet sites are observed, higher concentrations of thrombomodulin were necessary to achieve comparable decreases in binding. This is most likely a reflection of the intrinsic dissociation constant of thrombomodulin for thrombin and its ability to compete successfully with the high affinity platelet site.

Evaluation of nonspecific binding was attempted by the inclusion of $2 \mu$M unlabeled thrombin or DIP-thrombin in the reactions (Fig. 4, ---). Although this is not a totally appropriate control for thrombomodulin-containing mixtures (see "Discussion"), it can be seen that the presence of $110 \text{nM}$ or less thrombomodulin reduces thrombin binding to platelets well below the nonspecific level.

**Dissociation of DIP-thrombin from Platelets by Thrombomodulin**—Addition of thrombomodulin to a platelet suspension preincubated with $^{125}I$-DIP-thrombin caused a rapid dissociation of the label from the platelets (Fig. 5). The preincubation of $^{125}I$-thrombin or $^{125}I$-DIP-thrombin to platelets in the presence and absence of thrombomodulin. Labeled thrombin or DIP-thrombin at the concentrations indicated was added to platelets (2.5-5 x $10^8$/ml) in the presence or absence of thrombomodulin in a total volume of 0.4 ml at 37°C. Thrombomodulin-thrombin complexes were formed as described under "Experimental Procedures." After 3.5 min, triplicate 0.1-ml aliquots were removed and centrifuged to determine the amount bound and free as described under "Experimental Procedures." Nanomoles of thrombin bound ($T_B$) per 10$^9$ platelets uncorrected for nonspecific binding are plotted versus the total concentration of labeled protein added ($[T]_{\text{tot}}$). Solid symbols represent binding when native thrombin was the labeled ligand; open symbols represent radiolabeled DIP-thrombin. $\bullet$ and $\square$, no thrombomodulin present; $\bullet$ and $\square$, thrombomodulin-thrombin complex, 20:1 molar ratio; $\triangle$, thrombomodulin, final concentration 110 nM, mixed with platelets before addition of labeled protein. Dashed lines represent binding in the presence of $2 \mu$M unlabeled thrombin or DIP-thrombin whether or not thrombomodulin was present. $A$, thrombin concentrations varied between 0.01 and 0.4 nM; $B$, thrombin concentrations varied between 0.01 and 10 nM.
Thrombin-Platelet Interaction and Thrombomodulin

FIG. 5. Dissociation of $^{125}$I-DIP-thrombin from platelets upon the addition of thrombomodulin. Gel-filtered platelets (3 $\times$ 10$^8$/ml) were preincubated with $^{125}$I-DIP-thrombin (0.23 nM) plus unlabeled DIP-thrombin or thrombin (0.11 nM). At time 0, samples were removed to determine maximum binding and thrombomodulin was added to 110 nM. Aliquots were withdrawn at the times indicated and centrifuged to determine the counts remaining bound. These values were converted to per cent of the counts bound at zero time. ○, platelets preincubated 30 s before the addition of thrombomodulin; □, platelets preincubated 3.5 min prior to the addition of thrombomodulin. Dashed line represents the addition of unlabeled DIP-thrombin (110 nM) 30 s after the addition of the preincubation mixture.

In the presence of thrombomodulin, $^{125}$I-DIP-thrombin rapidly dissociated from the platelet. The time course of dissociation was equivalent in both reaction mixtures (○). Again, thrombomodulin was able to dissociate more of the bound $^{125}$I-DIP-thrombin from the platelets than an equivalent (110 nM) concentration of unlabeled DIP-thrombin (--;--). After rapid dissociation of label from the platelets upon addition of the unlabeled DIP-thrombin, a slow reuptake was consistently observed. When the period of preincubation was increased from 30 s to 3/4 min (□), the rate of dissociation appeared slower and biphasic, but the thrombomodulin was still capable of removing the prebound DIP-thrombin.

When the platelets were preincubated for 30 s with 5.6 nM $^{125}$I-thrombin (sufficient thrombin for extensive activation), the rate and extent of dissociation upon thrombomodulin addition were intermediate to those represented in Fig. 5 (data not shown).

DISCUSSION

Previous studies have demonstrated that complex formation between thrombin and thrombomodulin blocks thrombin’s ability to perform its procoagulant functions, factor V activation and fibrinogen clotting (25), while promoting a major anticoagulant function, the activation of protein C to form the anticoagulant activated protein C (22, 24). The studies presented here indicate that thrombomodulin blocks an additional procoagulant property of thrombin, the activation of platelets. When complexed to thrombomodulin, not only is thrombin incapable of activating platelets, it is also incapable of binding to the platelets. In the presence of thrombomodulin, platelet-bound thrombin rapidly redistributes onto thrombomodulin and platelet activation is circumvented. Unlike the thrombin inhibitor hirudin, which reduces binding to the levels observed in the presence of excess unlabeled thrombin (29, 30), thrombomodulin decreases binding well below this level.

The inhibition of thrombin’s ability to activate platelets does not result from an irreversible alteration in the specificity of thrombin. DIP-thrombin, which binds to thrombomodulin, displaces thrombin from the complex, and the free thrombin rapidly activates the platelets, consistent with previous observations (25). These experiments also demonstrate that the presence of thrombomodulin does not render the platelets refractory to activation. This conclusion is supported further by the observation that platelets aggregate rapidly in response to ADP in the presence of the thrombomodulin-thrombin complex.

Although complex formation between thrombin and thrombomodulin inhibits the ability of thrombin to activate platelets, the mechanism of this inhibition is uncertain. Clearly, the complex no longer binds to the platelet surface. Studies of Wallace and Bensusan (14) in which platelets were passed over columns of immobilized thrombin at 4 °C and then warmed to 37 °C indicated that proteolysis but not binding was required for activation, as secretion and aggregation could occur after the removal of thrombin. Studies by Holmsen et al. (28) are in agreement with these studies, although the continued presence of thrombin was required for other platelet responses to occur. Suggestive evidence has also been presented that cleavage of glycoprotein V (31) is involved in platelet activation. Further studies are required to determine if the thrombomodulin-thrombin complex is able to carry out any of these functions which do not require binding per se.

From the binding data presented, it is tempting to calculate the relative affinities of thrombin for thrombomodulin and for the platelet surface. However, such estimates of the binding affinities were not attempted because of the complexity of the system. While thrombin interaction with platelets can be modeled either as two classes of binding sites (9, 11, 32–34) or a single class of sites exhibiting negative cooperativity (35), accurate measurement of the binding affinity requires accurate assessment of nonspecific binding (5). It is apparent from our studies that the nonsaturable or “nonspecific” binding measured in the presence of excess unlabeled thrombin is significantly greater than that measured in the presence of thrombomodulin. In addition, this is not a totally appropriate control for the binding in the presence of thrombomodulin. Unlabeled thrombin or DIP-thrombin will compete with the label for thrombomodulin (25) in addition to competing for the platelet sites. High concentrations of unlabeled thrombomodulin-thrombin complex are also inappropriate, as this would change the equilibrium for the formation of the complex, as well as change the relative concentrations of the competing receptor species, thrombomodulin and the platelet.

Thus, accurate assessment of the binding of thrombin to the platelet requires determination of which, if either, of these measurements represents true nonspecific binding.

The physiological relevance of the observation that thrombomodulin blocks thrombin activation of platelets is not certain. It is likely that thrombomodulin on the endothelial cell surface also exhibits this property. Based on estimates of the endothelial cell surface to blood volume ratio in the microcirculation (36) and the yield of thrombomodulin from rabbit lung (24), it can be estimated that thrombomodulin is present in the microcirculation at $\approx 100$ nM. In comparison, assuming $\approx 1000$ high affinity binding sites/platelet and $\approx 2.5 \times 10^6$ platelets/ml, these sites would be present at $\leq 1$ nM. Thus, a physiological role for thrombomodulin in the inhibition and/or reversal of thrombin-mediated platelet activation may exist. Whether other thrombin-binding molecules on the endothelial cell surface also block thrombin activation of the platelet remains to be determined.

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