Evidence for a Single Class of Thrombin-Binding Sites on Human Platelets†

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ABSTRACT: We previously demonstrated that human platelets bind a small number of thrombin molecules with high affinity and a larger number with relatively lower affinity (Tollefsen, D. M., Feagler, J. R., and Majerus, P. W. (1974), *J. Biol. Chem. 249*, 2646). In the present report, equilibrium binding studies using [^{125}I]DIP-thrombin (diisopropylphosphoryl-thrombin) over the range of 0.0002–10 U/ml yield a Hill coefficient of 0.775. Measurements of the rate of dissociation of [^{125}I]DIP-thrombin bound to platelets at relatively high affinity (0.04 U/ml added) indicate a much faster dissociation in the presence of 14 U/ml unlabeled DIP-thrombin ($T_{1/2}$ = 1.0 min) than in its absence ($T_{1/2}$ = 140 min). [^{125}I]DIP-thrombin bound at low affinity (1.0 U/ml added) dissociates from platelets with a $T_{1/2}$ = 1.7 min in the absence of unlabeled DIP-thrombin. These results suggest a negative cooperative interaction among receptor sites for thrombin; i.e., as thrombin binds to unoccupied sites, high-affinity receptors are apparently converted to low-affinity receptors. In an attempt to detect whether there is heterogeneity of thrombin receptors, [^{125}I]DIP-thrombin was covalently cross-linked to intact platelets using 1 mM glutaraldehyde. A single complex (apparent molecular weight, 200 000) containing [^{125}I]DIP-thrombin was formed throughout a range of thrombin concentrations in which both high- and low-affinity binding was observed. Since incorporation of [^{125}I]DIP-thrombin into this complex did not occur in the absence of platelets and was inhibited by unlabeled thrombin, the complex may represent thrombin cross-linked to its receptor. We conclude that a single class of receptor sites can account for both high- and low-affinity binding of thrombin to platelets, although interaction between nonidentical sites cannot be excluded.

Platelets are small, anucleate cells in blood that are responsible for initiating hemostasis after vascular injury. At a site of damage to a blood vessel, platelets adhere to collagen or other substances beneath the vascular endothelium, aggregate with one another to form a platelet plug, and apparently increase the rate of activation of certain coagulation factors (Marcus, 1969). Before or during aggregation, platelets secrete a variety of substances, including Ca^{2+}, adenine nucleotides, serotonin, and hydrolytic enzymes (Holmsen et al., 1969). Thrombin at low concentrations (<1 nM)[1] is a stimulus for platelet aggregation and secretion in vitro, although its role in the initial stages of hemostasis in vivo remains hypothetical.

Numerous studies have defined other effects of thrombin on platelets, including effects on cAMP (cyclic adenosine 3′,5′-monophosphate) metabolism (Salzman and Weisenberger, 1972), protein phosphorylation (Lyons et al., 1975), lipid synthesis (Lewis and Majerus, 1969), prostaglandin synthesis (Hamberg and Samuelsson, 1974), and carbohydrate metabolism (Marcus, 1969). We have postulated that thrombin initiates some or all of these events by interacting with a component(s) of the platelet surface. Thrombin binds reversibly to approximately 50 000 receptor sites on the surface of intact human platelets (Tollefsen et al., 1974). This binding appears to be highly specific since closely related proteins, including prothrombin and the intermediates in prothrombin activation, do not bind to platelets under conditions in which binding of thrombin is observed (Tollefsen et al., 1975). Moreover, human and bovine thrombin bind to platelets nearly identically (Shuman et al., 1976), even though there are important structural differences between these two proteins detected by partial amino acid sequence studies (Walz and Seegers, 1974; Thompson et al., 1974) and by immunological techniques (Shuman et al., 1976). Studies in which the affinity of platelets for thrombin was altered by buffers containing various anions have established a close correlation between the number of thrombin molecules bound to platelets and the extent of serotonin secretion, suggesting that thrombin binding is an important step in the mechanism of secretion by platelets (Shuman and Majerus, 1975).

In equilibrium studies we have found that the binding of thrombin to platelets is kinetically complex (Tollefsen et al., 1974). Thus, the affinity of platelets for thrombin decreases as more thrombin is bound, with apparent dissociation constants ranging from 0.2 to 30 nM. This type of binding could result (a) from heterogeneous receptor sites on platelets with different affinities for thrombin or (b) from a negative cooperative interaction among a homogeneous population of receptor sites. In this report we present evidence for a single population of receptor sites that exhibit negative cooperativity in binding thrombin. These experiments also reconcile the apparent discrepancy between our previous demonstration of reversible, equilibrium thrombin binding and the very slow turnover of enzyme observed by Detwiler and Feinman (1973) when they studied platelet secretion at submaximal thrombin concentrations.

Materials and Methods

Platelets were isolated from human blood and washed to remove plasma as previously described (Tollefsen et al., 1974). Erythrocyte ghosts were prepared by the method of Fairbanks et al. (1971).

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[1] Note: 1 nM thrombin ≃0.1 U/ml.

Materials were obtained from the following sources: carrier-free [^{125}I]iodide from Mallinckrodt Nuclear, St. Louis, Mo.; diisopropyl fluorophosphate from Sigma Chemical Co., St. Louis, Mo.; 8% ultrapure glutaraldehyde from Biodynamics Research Corp., Rockville, Md.; and Metricel GA-3 (1.2 μm) filters from Gelman Instrument Co., Ann Arbor, Mich.

Preparation of Thrombin. Bovine thrombin was purified from topical thrombin (Parke, Davis and Co., Detroit, Mich.) by the method of Glover and Shaw (1971) and assayed as previously described (Tollefsen et al., 1974). Thrombin was treated with diisopropyl fluorophosphate to form DIP-thrombin[2] and labeled with ^{125}I using reported procedures (Tollefsen et al., 1974). The labeled thrombin contained approximately 0.5 I atom/molecule of thrombin when the specific activity of thrombin was 10 000 cpm/mU. Labeled thrombin binds to platelets similarly to unlabeled thrombin as described previously (Tollefsen et al., 1974; Shuman et al., 1976). Before use the thrombin solution was chromatographed on a column of Sephadex G-25 equilibrated with either Tris- or phosphate-buffered saline (see below), depending on the experiment.

Measurement of Association and Dissociation Rates. These experiments were performed with washed platelets suspended in isotonic Tris-buffered saline (0.0154 M Tris-HCl, 0.14 M NaCl, and 0.0055 M glucose; pH 7.5). To determine the association rate, the amount of thrombin bound to platelets after various times was measured in incubations containing 5×10^7 platelets in a final volume of 0.5 ml. Each incubation was initiated by the addition of [^{125}I]DIP-thrombin (either 1.0 or 0.04 U/ml final concentration) and was terminated by filtration (using Metricel GA-3 filters) as described previously (Tollefsen et al., 1974). The data were corrected for nonspecific binding by subtracting the amount of labeled thrombin bound in the presence of an excess of unlabeled thrombin. When high-affinity binding is measured, nonspecific binding represents less than 5% of total binding; when low-affinity binding is measured, the nonspecific binding ranges from 10 to 20%.

For measurements of the dissociation rate of thrombin from platelets, platelets were incubated at a concentration of 1×10^8/ml with [^{125}I]DIP-thrombin (either 1.0 or 0.04 U/ml) in a total volume of 10 ml. After a 30-min incubation, the platelet suspension was centrifuged at 2250g for 15 min at room temperature, and the supernatant containing unbound thrombin was removed. The platelets were quickly resuspended in the original volume of isotonic Tris-buffered saline. A timer was started at the beginning of the resuspension, which took about 20 s to complete. At various times, aliquots of 0.5 ml were removed, diluted to 5 ml with buffer, and filtered to determine the amount of thrombin bound. The dilution and filtration step took less than 5 s.

To compare the dissociation rates in the presence and absence of unlabeled thrombin, the platelets resuspended after centrifugation were divided into two portions, to one of which was added unlabeled thrombin (final concentration 14 U/ml). Aliquots were removed from each portion and filtered as described above.

Glutaraldehyde Cross-Linking Experiments. Since buffers containing Tris cannot be used with glutaraldehyde, washed platelets were suspended in isotonic phosphate-buffered saline (0.113 M NaCl, 0.0043 M K_2HPO_4, 0.043 M Na_2HPO_4, 0.0244 M NaH_2PO_4, and 0.0055 M glucose; pH 6.5). Platelets (1×10^8/ml) were incubated with [^{125}I]DIP-thrombin (0.02–1.5 U/ml) in 0.5 ml of buffer for 30 min at room temperature to allow binding to occur. Then 0.8 M (8%) glutaraldehyde was added to a final concentration of 1 mM, and the mixture was allowed to remain at room temperature for an additional 30 min. The platelet suspension was then boiled for 5 min with $\frac{2}{5}$ volume of a solution containing 8% sodium dodecyl sulfate, 37% sucrose, and 0.01% bromophenol blue. Electrophoresis was carried out with 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate (pH 7.5). Following electrophoresis, the gels were frozen and sliced using a razor blade gel slicer into approximately forty 2-mm gel slices. The molecular weight of the thrombin–platelet complex formed by glutaraldehyde was estimated by plotting the migration distance in this gel system vs. the logarithm of molecular weight compared with proteins of known molecular weight obtained from commercial sources except as noted below. The standard proteins were: platelet myosin (mol wt 230 000), gift from Dr. R. M. Lyons, University of Texas at San Antonio; β-galactosidase (mol wt 130 000); fibrinogen α, β, and γ chains (mol wt 73 000, 60 000, and 52 000, respectively); bovine serum albumin (mol wt 68 000); *E. coli* alkaline phosphatase (mol wt 43 000), gift from Dr. M. J. Schlesinger, Washington University, St. Louis; aldolase (mol wt 40 000); and pepsin (mol wt 35 000). The standard plot was linear throughout this range of molecular weights.

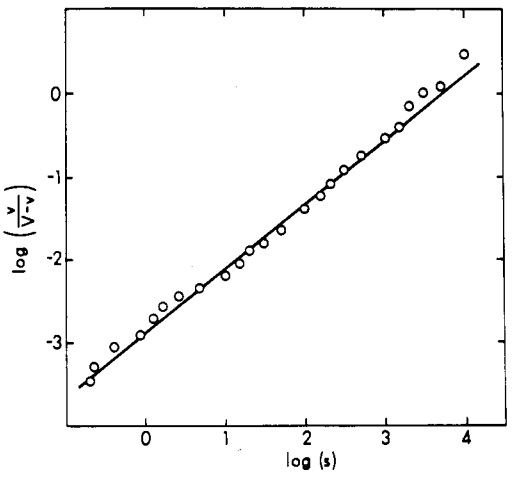

FIGURE 1: Hill plot of thrombin binding to platelets. Incubations were conducted in 0.5 ml of isotonic Tris-buffered saline (pH 7.5) with 0.0002–10 U/ml of [^{125}I]thrombin and a platelet concentration of 1×10^8/ml. Incubations were terminated by filtration after 30 min, and the amounts of bound and free thrombin were determined. The maximum number of thrombin molecules bound at a saturating thrombin concentration was calculated by the method of Steck and Wallach (1965), using data from the six highest thrombin concentrations to construct the double reciprocal plot. s = free thrombin (mU/ml). v = thrombin bound (mU/10^8 platelets). V = maximum amount of thrombin bound at saturation (mU/10^8 platelets).

Results

Kinetics of Thrombin Binding to Platelets. The experiments reported here were all performed using DIP-thrombin. We previously have shown that native and DIP-thrombin bind to platelets identically (Tollefsen et al., 1974). When equilibrium binding data from experiments using thrombin over the range from 0.0002 to 10 units/ml are plotted according to the Hill equation (Hill, 1913) as shown in Figure 1, the slope of the line yields a Hill coefficient of 0.775. A value of this coefficient less than 1.0 suggests (Levitzki and Koshland, 1969), but does not prove, a negative cooperative interaction among a single pop-

[2] Abbreviations used: DIP-thrombin, diisopropylphosphorylthrombin; cAMP, cyclic adenosine 3′,5′-monophosphate.

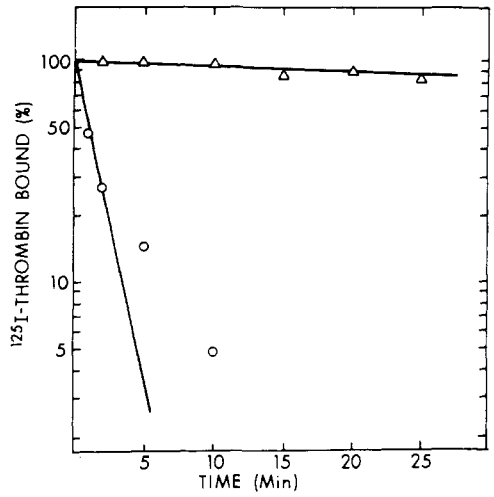

FIGURE 2: Dissociation of thrombin from high-affinity binding sites. Platelets were incubated at a concentration of 1×10^8/ml with 0.04 U/ml of [^{125}I]DIP-thrombin. After 30 min the platelet suspension was centrifuged (see Materials and Methods), and the supernatant containing unbound thrombin was removed. The platelets were quickly resuspended in the original volume of buffer and divided into two portions, to one of which was added 14 U/ml of unlabeled thrombin. At various times after the addition of the unlabeled thrombin, aliquots were removed and filtered to determine the thrombin bound. Data are plotted as the percentage of the thrombin originally bound (4.0 mU/10^8 platelets). (○) Tube containing excess unlabeled thrombin; (△) tube without additional thrombin.

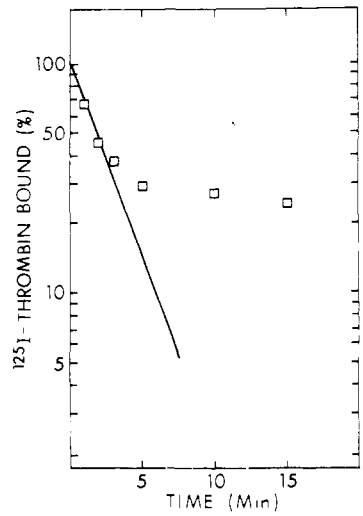

FIGURE 3: Dissociation of thrombin from low-affinity binding sites. This experiment was carried out in the same manner as in Figure 2, except that 1.0 U/ml of [^{125}I]DIP-thrombin was used. No unlabeled thrombin was added after resuspension of the platelets. The amount of thrombin originally bound (100%) was 30 mU/10^8 platelets.

ulation of platelet receptor sites for thrombin. From equilibrium binding measurements alone it is not possible to distinguish negative cooperativity from heterogeneous receptor sites with different affinities for thrombin. To distinguish between these possibilities, we have employed a procedure recently devised by DeMeyts et al. (1973) to test the negative cooperativity hypothesis of insulin binding to human lymphocytes. These investigators studied the dissociation of [^{125}I]insulin from its receptor both in the presence and in the absence of excess unlabeled hormone. Conditions were used in which rebinding of the dissociated hormone was minimized. They reasoned that if the receptor sites were heterogeneous and independent of each other, the dissociation rate should be the same in both cases. Alternatively, if the presence of unlabeled

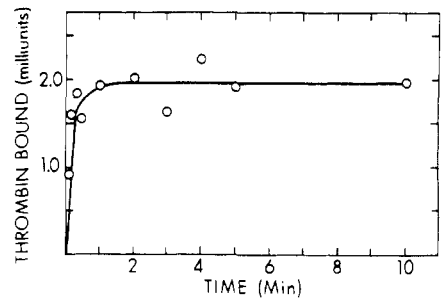

FIGURE 4: Association of thrombin with platelets. Each time point represents a separate incubation containing 5×10^7 platelets in 0.5 ml of buffer with 0.04 U/ml of [^{125}I]DIP-thrombin. At the indicated times the incubations were terminated by filtration. The data are corrected for nonspecific binding (see Materials and Methods).

hormone increased the dissociation rate of the labeled hormone, a negative cooperative interaction must be occurring.

We exposed platelets to a relatively low level of [^{125}I]DIP-thrombin (0.04 unit/ml) at which high-affinity binding is observed, and after equilibrium had been reached the platelets were sedimented and resuspended in the original volume of buffer in the absence of thrombin or in the presence of 14 units/ml of unlabeled thrombin. The amount of [^{125}I]DIP-thrombin that remained bound to the platelets was measured at various times after resuspension (Figure 2). The concentration of thrombin remaining in this experiment after resuspension was about 0.004 unit/ml. This level of thrombin would reach a new equilibrium of binding, as determined in previous experiments (Tollefsen et al., 1974), such that the amount of thrombin bound would be about $\frac{1}{5}$ the amount bound at 0.04 unit/ml. Thus, reassociation of labeled thrombin is negligible, at least at early time points.

As shown in Figure 2, the dissociation rate was much greater in the presence than in the absence of excess unlabeled thrombin. This observation suggests that as the unlabeled thrombin binds to the platelets the association is weakened between platelet receptor sites and the [^{125}I]DIP-thrombin originally bound. This could be explained by a negative cooperative interaction among receptor sites for thrombin. If there were no site–site interaction, the dissociation rate of thrombin from high-affinity sites would be independent of the binding of additional thrombin molecules to unoccupied sites.

The dissociation rate of low-affinity thrombin binding (1.0 unit/ml of [^{125}I]DIP-thrombin added) was also determined (Figure 3). As one would predict, the initial rate of dissociation was similar to that obtained in the previous experiment in the presence of excess unlabeled thrombin. Control experiments established that the dissociated radioactivity was contained in thrombin. Thus, the supernatants containing the dissociated thrombin under conditions of Figures 2 and 3 were analyzed both by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by quantitative precipitation by rabbit antibovine-thrombin antibody. Over 90% of the radioactivity remained as thrombin as judged by both analyses.

Association rates were also determined both at 0.04 unit/ml and at 1.0 unit/ml using the same preparation of [^{125}I]DIP-thrombin. These data are shown in Figures 4 and 5. Rate constants for thrombin binding were determined from the data above (see Table I). First-order rate constants for dissociation (k_2) were determined directly from the initial slopes of the semilogarithmic plots in Figures 2 and 3. Rate constants for association (k_1) were calculated from the equation: initial association rate = k_1[T][R], where [T] is the initial free thrombin concentration and [R] is the initial free concentration

of platelet receptor sites for thrombin. In this calculation the number of receptor sites per platelet was assumed to be 50 000 for both low- and high-affinity binding. The apparent dissociation constants for thrombin binding at low and at high affinity were calculated from the equation: $K_{diss} = k_2/k_1$. The calculated dissociation constants are similar to the dissociation constants determined previously from equilibrium measurements (Tollefsen et al., 1974). These latter constants were 2.9 units/ml for low-affinity binding and 0.02 unit/ml for high-affinity binding. If the apparent number of high-affinity thrombin binding sites (500 per platelet) as determined in the previous equilibrium studies by the method of Steck and Wallach (1965) is used in the calculation of k_1 rather than the total number of sites observed at high thrombin concentrations (50 000 per platelet), the K_{diss} calculated for high-affinity binding is about two orders of magnitude less than the value actually observed. This provides a further suggestion that all 50 000 thrombin receptor sites are available for high-affinity binding.

Cross-Linking of Thrombin to Platelets with Glutaraldehyde. While the results of kinetic experiments similar to those described above have been interpreted by others (DeMeyts et al., 1973) to indicate cooperativity within a single class of binding sites, it is also possible that there is cooperativity between nonidentical sites. In an attempt to detect possible platelet receptor heterogeneity, we carried out experiments in which [^{125}I]DIP-thrombin was covalently cross-linked to intact platelets with glutaraldehyde prior to electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. The concentration of [^{125}I]DIP-thrombin was varied 75-fold over a range in which both high- and low-affinity equilibrium binding is observed. The results of these experiments are shown in Figure 6.

The bulk of the [^{125}I]DIP-thrombin in each case migrated as a peak of radioactivity with an apparent molecular weight of 36 000 (not shown). The size and position of this peak were apparently unaffected by the presence or absence of platelets or glutaraldehyde. In incubations containing 1 mM glutaraldehyde and platelets (*closed circles*), a new radioactive peak representing 1–5% of the total radioactivity was found which had an apparent molecular weight of 200 000. The only other detectable radioactivity was located at the origin of the gel.[3] Neither of these peaks appeared in the absence of glutaraldehyde (*open triangles*) or in the absence of platelets (not shown). When 14 U/ml of unlabeled thrombin was included in the incubation of [^{125}I]DIP-thrombin with platelets before the addition of glutaraldehyde, the 200 000 molecular weight peak was reduced by 70–85% (*open circles*). Since incorporation of [^{125}I]DIP-thrombin into the 200 000 molecular weight peak requires platelets and is inhibited by unlabeled thrombin, this peak may represent thrombin cross-linked to the receptor detected by equilibrium binding studies. We have not identified the highly cross-linked material at the origin of the gels. Since there was no consistent effect of 14 U/ml of unlabeled thrombin on this material (Figure 6), it probably does not contain thrombin receptor. It may contain some aggregated, cross-linked thrombin since ½–⅔ of the radioactivity at the origin is seen in gels where no platelets are included in the incubation mixture. Additional control experiments were carried out using erythrocyte ghosts (3×10^8/gel) in place of platelets under conditions of Figure 6. No cross-linked material was seen within the gels. This is consistent with our previous observation that erythrocytes do not bind thrombin (Tollefsen et al., 1974).

[3] When these experiments were carried out using native [^{125}I]thrombin, an additional radioactive peak at a molecular weight of 90 000 was seen. This reflected a thrombin-antithrombin III complex rather than a thrombin-receptor complex. This was established by the observations that this complex could not be dissociated by excess unlabeled thrombin, did not sediment with the platelets, and was not observed when DIP-thrombin was used. The formation of the complex did not require glutaraldehyde and the complex was precipitated by bovine antibodies to human antithrombin III.

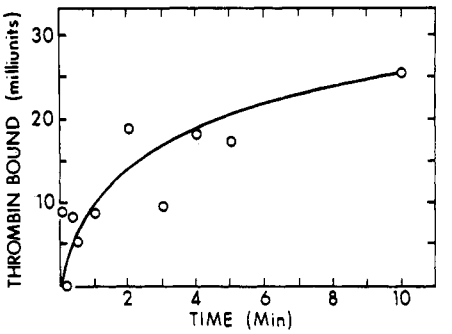

FIGURE 5: Association of thrombin with platelets. This experiment was carried out as in Figure 4, except that 1.0 U/ml of [^{125}I]DIP-thrombin was added.

TABLE I: Rate Constants for Thrombin Binding to Human Platelets.

	"High Affinity"[d]	"Low Affinity "[d]
k_1[a]	4.0×10^7 M^{-1} min^{-1}	4.2×10^6 M^{-1} min^{-1}
k_2[b]	4.8×10^{-3} min^{-1}	4.0×10^{-1} min^{-1}
K_{diss}[c]	1.2×10^{-10} M (= 0.011 U/ml)	9.5×10^{-8} M (= 8.6 U/ml)

[a] k_1 is the association rate constant calculated from data in Figures 4 and 5 according to the equation: initial association rate = k_1[T][R], where [T] is the initial free thrombin concentration and [R] is the initial free concentration of platelet receptor sites for thrombin (50 000 sites/platelet, see text). [b] k_2 is the first-order dissociation rate constant determined from the initial slopes of Figures 2 and 3. [c] K_{diss} is the apparent dissociation constant calculated from the equation $K_{diss} = k_2/k_1$. [d] "High affinity" and "low affinity" refer to experiments performed using 0.04 and 1.0 U/ml of thrombin, respectively.

The amounts of [^{125}I]DIP-thrombin incorporated into the 200 000 molecular weight peak were calculated from the specific radioactivity of the thrombin and the number of platelets applied to each gel. At total thrombin concentrations of 0.02, 0.1, 0.5, and 1.5 U/ml, these values were 1.7, 3.0, 10, and 15 mU/10^8 platelets, respectively. For comparison, equilibrium binding data from the experiment shown in Figure 1 indicate 2.3, 7.6, 32, and 66 mU bound/10^8 platelets at total thrombin concentrations identical with the above. This suggests a glutaraldehyde crosslinking efficiency ranging from 75% at the lowest thrombin concentration to 23% at the highest.

In other experiments, glutaraldehyde concentrations were varied under cross-linking conditions otherwise identical with those of Figure 6. At 0.5 mM glutaraldehyde, there was essentially no cross-linking of [^{125}I]DIP-thrombin to platelets detected in sodium dodecyl sulfate gels. At 1.5 mM and higher glutaraldehyde concentrations, there was a progressive decrease in ^{125}I cpm of the 200 000 molecular weight peak accompanied by an increase in radioactivity of material that did not enter the gel.

Since the sodium dodecyl sulfate gel migration of the com-

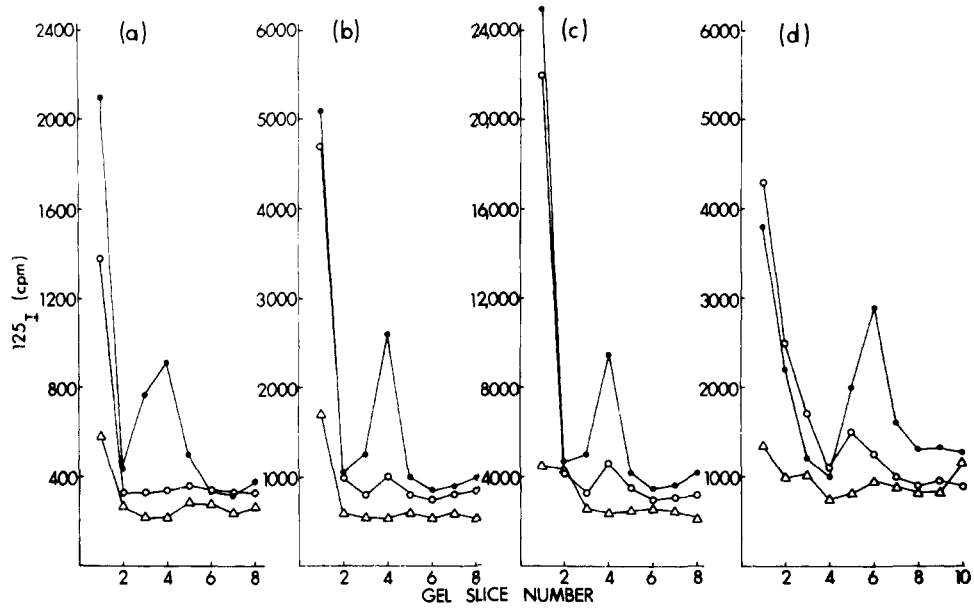

FIGURE 6: Cross-linking of thrombin to intact platelets with glutaraldehyde. Incubations with and without 1 mM glutaraldehyde were performed as described in Materials and Methods. Each gel contains 0.07×10^8 platelets incubated with the following: (△) [^{125}I]DIP-thrombin, no glutaraldehyde; (●) [^{125}I]DIP-thrombin, then 1 mM glutaraldehyde; (○) [^{125}I]DIP-thrombin + unlabeled thrombin (14 U/ml), then 1 mM glutaraldehyde. The concentration of [^{125}I]DIP-thrombin in each panel is as follows: (a) 0.02 U/ml; (b) 0.1 U/ml; (c) 0.5 U/ml; (d) 1.5 U/ml. Only the first 8 slices of 40 are shown for gel a–c. Gels a, b, and c were run using a single sample of [^{125}I]DIP-thrombin (specific radioactivity 10 000 cpm/mU). Gel d was from another experiment (specific radioactivity 2500 cpm/mU). The cross-linked peak in d migrated further than those in a–c due to longer electrophoresis time. The molecular weights of the cross-linked peaks are all 200 000 based on comparisons with those of known proteins (see Materials and Methods).

plex formed with 1 mM glutaraldehyde was identical at concentrations of [^{125}I]DIP-thrombin at which both high- and low-affinity binding is observed (see legend, Figure 6), these experiments are consistent with the presence of a single class of receptor sites that can bind thrombin at different affinities depending on the thrombin concentration. It is also possible that there are heterogeneous interacting receptors, all of similar molecular weight, which cannot be distinguished by our methods.

Discussion

The identity of the thrombin receptor on platelets remains unknown. Phillips and Agin (1974) have presented evidence that thrombin can hydrolyze a platelet surface glycoprotein (molecular weight 120 000); however, the relation of this hydrolysis to platelet secretion is not clear, due in part to the high thrombin concentrations employed (100- to 1000-fold greater than required for maximum secretion). Furthermore, preliminary experiments indicate that this glycoprotein when labeled by the lactoperoxidase iodination technique is not incorporated into the thrombin–receptor complex formed by glutaraldehyde cross-linking (Tollefsen and Majerus, unpublished observations).

The molecular weight of the thrombin–receptor complex was estimated in sodium dodecyl sulfate gel electrophoresis by comparison with proteins of known molecular weight. The molecular weight of 200 000 is only a tentative estimate since the possible effect of glutaraldehyde on the binding of sodium dodecyl sulfate to this complex or on the electrophoretic mobility of the complex cannot be assessed. Further experiments using cleavable cross-linking reagents are required to determine the stoichiometry of this complex. That the complex contains the platelet receptor for thrombin and is unlikely to be a simple oligomer of thrombin is suggested by the fact that its formation is platelet dependent and is inhibited by increasing concentrations of unlabeled thrombin.

Our kinetic observations may account for one aspect of the platelet response to thrombin that has been previously unexplained. Detwiler and Feinman (1973) have shown that the yield of secretion of Ca^{2+} or ATP depends on thrombin dose at low thrombin concentrations. They suggested that this finding could be explained by a lack of turnover of thrombin and further postulated that there was a slow dissociation of the thrombin–receptor complex. It was difficult to reconcile this lack of turnover with the reversible binding of thrombin that we demonstrated (Tollefsen et al., 1974). The present studies show that, while equilibrium binding of thrombin does occur, the dissociation rate of thrombin from platelets is very slow at low thrombin concentrations. For example, at 0.04 U/ml of thrombin, the $T_{1/2}$ for dissociation is about 140 min. At this level of thrombin in Tris-buffered saline, the yield of [^{14}C]serotonin secretion from platelets is about 75% (Tollefsen et al., 1974).

We have presented evidence in this report suggesting that thrombin binds to a single class of platelet receptor sites that undergo a negative cooperative interaction, although we cannot exclude interaction among receptor sites which are not identical. In addition to thrombin, several hormones exhibit dissociation rates in the presence of unlabeled ligand that suggest negative cooperativity. These include insulin binding to human lymphocytes (DeMeyts et al., 1973), nerve growth factor binding to sympathetic neurons (Frazier et al., 1974), and catecholamine binding to erythrocyte membranes (Limbird et al., 1975). In contrast, the binding of human growth hormone to human lymphocytes does not demonstrate negative cooperativity when tested in the same manner (DeMeyts et al., 1973). Thus, the thrombin–platelet interaction closely resembles the interaction observed between certain hormones and their target tissues.

Acknowledgment

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