Identification of the Thrombin Receptor on Human Platelets by Chemical Crosslinking

Junki Takamatsu, McDonald K. Horne III, and Harvey R. Gralnick

Hematology Service, Clinical Pathology Department, Clinical Center, National Institutes of Health, Building 10, Room 2C390, Bethesda, Maryland 20892

Abstract

To identify the molecular site of thrombin binding to the platelet membrane, we covalently linked 125I-thrombin to platelets by using the bifunctional chemical cross-linking agents disuccinimidyldiester and dithiobis(succinimidyl propionate). The proteins cross-linked to 125I-thrombin by this method were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and followed by autoradiography. Two radiolabeled thrombin complexes were identified, a major species of $M_t \sim 200,000$ and a minor one of $M_t \sim 400,000$. Hirudin prevented the formation of both complexes. The radioactivity of the $\sim 200,000-M_t$ complex was always 7-10-fold greater than the radioactivity of the $\sim 400,000-M_t$ complex regardless of the thrombin concentration to which the platelets were exposed (0.1-29 nM). Although 125I-thrombin complexes generated with thrombasthenic platelets (lacking glycoprotein Ib/IIa) were indistinguishable from normal, no complexes appeared when Bernard-Soulier platelets (lacking glycoprotein Ib [GPIb]) were used. Complex formation was blocked by rabbit antiguycoprotein antigen, but not by the monoclonal antibody 6D1, which is directed against the site on GPIb where von Willebrand factor (vWF) binds in the presence of ristocetin. Although cross-linking studies suggested that vWF might partially inhibit thrombin binding to platelets, this was not confirmed by equilibrium binding studies in the presence of vWF and ristocetin. The data suggest, therefore, that at all thrombin concentrations binding occurs at the same membrane site, despite evidence from equilibrium studies for high and low affinity classes of receptors, and that the $\sim 400,000-M_t$ complex is simply a dimer of the $\sim 200,000-M_t$ species. We conclude that the membrane site to which thrombin binds is the glycoprotein portion of platelet GPIb at a site remote from the point of ristocetin-dependent vWF binding.

Introduction

Alpha thrombin is an important and potent activator of platelet function (1). Although the mechanisms of its action are not completely understood, thrombin appears to bind to specific sites on the platelet surface. Tollefsen et al. (2) defined two classes of binding sites by equilibrium binding studies. One class is characterized by a relatively low surface density ($\sim 500$ sites/cell) and by binding at thrombin concentrations corresponding to the threshold at which physiological responses occur ($K_d \sim 0.2$ nM). The other class is present in greater numbers ($\sim 50,000$ sites/cell) but has a lower affinity for thrombin ($K_d \sim 30$ nM) and uncertain physiologic significance.

Whether these classes of binding sites represent different membrane structures or negative cooperativity within a single structure has never been clarified, nor have any of the membrane structures involved been identified with certainty. Nevertheless, several reports have implicated the membrane glycoprotein Ib (GPIb)$^3$ as the thrombin receptor. Purified GPIb and its derivative glycalcin competitively inhibit thrombin binding to both classes of sites (3, 4). Yet, Fab fragments of anti-GPIb antisera are reported to have minimal effect on platelet aggregation induced by thrombin (5). Platelets from patients with Bernard-Soulier syndrome, which lack GPIb, bind decreased amounts of thrombin (6). However, these platelets are known to have other membrane abnormalities as well (7).

We have attempted to identify the platelet receptor for thrombin by chemically cross-linking 125I-thrombin to intact platelets with bifunctional cross-linking agents and analyzing the radioactive complexes with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We have used normal platelets, Bernard-Soulier platelets, and platelets from patients with Glanzmann's thrombasthenia, which lack the membrane glycoprotein IIb/IIIa (GPIb/IIIa) complex (8). In addition, we have studied thrombin binding to normal platelets in the presence of several antibodies specific for certain membrane components and in the presence of von Willebrand factor (vWF) and ristocetin. In some instances, the cross-linking studies have been supplemented with binding studies under equilibrium conditions. We have obtained evidence that the platelet has only one binding site for thrombin, and that this is located on GPIb at a point remote from where vWF binds in the presence of ristocetin.

Methods

Human blood was obtained from healthy donors, patients with Glanzmann's thrombasthenia, and patients with the Bernard-Soulier syndrome. All subjects were informed about the scope of the study. Venipuncture was performed using a 19-gauge needle. Blood was collected into 0.1 volumes of 0.13 M sodium citrate, 10 mM Na$_2$EDTA, in polypropylene tubes. Platelet-rich plasma (PRP) was prepared from normal and throm-

---

A portion of this work has been reported at the meeting of the American Society for Clinical Investigation, Washington, DC, May, 1985.

Address correspondence to Dr. Gralnick. Dr. Takamatsu's present address is The First Department of Internal Medicine, Nagoya University School of Medicine, Showaku Nagoya 466, Japan.

Received for publication 24 June 1985 and in revised form 30 September 1985.

The Journal of Clinical Investigation, Inc.
Volume 77, February 1986, 362–368

1. Abbreviations used in this paper: DFP, diisopropylfluorophosphate; DSP, dithiobis(succinimidyl propionate); DSS, disuccinimidyl suberate; DTT, dithiothreitol; GPIb, glycoprotein Ib; GPIb/IIIa, glycoprotein IIb/IIIa; NEM, N'-ethylmaleimide; FRP, platelet-rich plasma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; vWF, von Willebrand factor.
bathemic blood by centrifugation at 500 g for 10 min at 24°C. Platelets were isolated from this PRP on an arabinogalactan gradient as previously described (9). Bernard-Soulier Platelet PRP was produced by allowing the whole blood to sediment at room temperature for 3 h. Platelets were collected from the Bernard-Soulier PRP by washing away the plasma with saline-phosphate-EDTA buffer (0.15 M NaCl, 10 mM sodium phosphate, and 10 mM Na3EDTA, pH 7.4) containing 1% bovine serum albumin (BSA). All platelets were suspended in this buffer, and in some experiments 6 mM N-ethylmaleimide (NEM) and 1 mM leupeptin were included.

Highly purified alpha thrombin, ~3,000 U/mg, was provided by Dr. John Fenton, New York State Dept. of Health, Albany, NY. The thrombin was iodinated (Na125I) using agarose-bound lactoperoxidase as previously described (10). Iodination did not alter the fibrinogen clotting activity of the thrombin (11). 125I-thrombin was treated with disopropyl pyrophosphoramide (DFP) by the method of Tollefson et al. (2).

Equilibrium binding studies. Isolated platelets were suspended in Tyrode's buffer (0.14 M NaCl, 3 mM KCl, 12 mM NaHCO3, 0.4 mM NaH2PO4, 2 mM CaCl2, and 1 mM MgCl2, pH 7.35) with 5% BSA to give a final concentration of 4.8 × 109/ml normal platelets and 2.4 × 109/ml Bernard-Soulier platelets. Binding experiments were performed at room temperature by mixing 0.2 ml of the platelet suspensions with 0.2 ml of 125I-thrombin (final concentration, 0.004-8.0 U/ml) in polystyrene tubes. The total radioactivity (cpm) of each sample was measured in an automatic gamma counter (Tracor Analytic, model 1185). The samples were incubated for 30 min. We (unpublished data) and others (2) have previously shown that binding equilibrium is reached within 10-15 min. After incubation the tubes were centrifuged at 2,000 g for 8 min, and the supernatants aspirated without disturbing the platelet pellets. The radioactivity of the tubes was remeasured after aspiration to obtain bound 125I-thrombin. Unbound (supernatant) 125I-thrombin was calculated as total minus bound cpm. Non specifically bound thrombin was measured by including hirudin 20 U/ml in the thrombin-platelet mixtures. Radioactivity measurements were converted to units of thrombin concentration by determining the specific activity of the labeled thrombin (3.3 × 105 cpm/μl).

Equilibrium binding of 125I-thrombin in the presence of vWF and ristocetin or antilygococalcin antiserum. Platelets (2 × 109/ml) were suspended in saline-phosphate-EDTA buffer containing 1% BSA, 2-100 μg/ml vWF, and ristocetin (0, 1, or 2 mg/ml). 0.2 ml of the platelet suspension was mixed with 0.2 ml of 20 nM 125I-thrombin for 15 min before the cells were isolated by centrifugation. In other experiments, the platelet-thrombin mixtures contained 20% (vol/vol) rabbit anti glycopocalcin antiserum obtained from Dr. N. O. Solum (12), University of Oslo, Norway, or an equivalent volume of rabbit preimmune serum with or without hirudin. The concentrations of bound and free thrombin were determined as described above.

Cross-linking protocol. Platelets (109/ml) were incubated for 15 min at room temperature with 125I-thrombin (0.1-100 nM). In our initial studies, this incubation was in saline-phosphate-EDTA, but in other experiments the protease inhibitors leupeptin and NEM were also added as a precaution. However, the presence of NEM and leupeptin did not alter the results obtained with SDS-PAGE. In some experiments inhibitors of thrombin or other agents were added to the platelets before thrombin or a cross-linking agent was added. Hirudin (20 U/ml), purified human vWF, ristocetin, the murine monoclonal antibodies 6D1 and 10E5 (gifts from Dr. Barry Coller, State University of New York at Stony Brook, NY), and the rabbit polyclonal antiserum against glycopocalcin (from Dr. Solum) were tested. The vWF was purified as previously described (13). The monoclonal antibody 6D1 is directed against the site on GPIb which vWF binds in the presence of ristocetin (14). The antibody 10E5 reacts with the GPIb/IIa complex where fibrinogen binds (15).

After incubation, the platelets were rinsed once with saline-phosphate-EDTA and resuspended in the same buffer. The bifunctional cross-linking agents diisuccinimidyl suberate (DSS) (16) or diethyldithiobis(succinimidyl propionate) (DSP) (17) were freshly prepared. The formation of the radio labeled cross-linked complexes was followed for 20 s to 60 min by analysis with SDS-PAGE and autoradiography. Although complexes appeared by 40 s and their radioactive intensity stabilized by 80 s, the cross-linking reaction was typically allowed to proceed for 15 min at ice-bath temperature before quenching with 10 or more volumes of ice-cold 10 mM Tris HCl, 1 mM EDTA (pH 7.4 for DSS; pH 8.5 for DSP). This mixture was centrifuged at 2,000 g for 30 min at 25°C. The pellet was dissolved in 3.3% SDS with heating at 100°C for 5-10 min. The sample was then centrifuged at 20,000 g for 60 min at 25°C. The supernatant was mixed with sample buffer (0.0625 M Tris HCl, 1% SDS, pH 6.8) for electrophoresis.

SDS gel electrophoresis and autoradiography. Samples were heated in sample buffer with or without reducing agent (10 mM dithiothretol [DTT]) at 100°C for 5 min before loading onto a 1.5-mm thick slab gel. The Laemmli system of electrophoresis buffers was used (18). The separating gels were composed of 5% acrylamide, 10% acrylamide, or a composite of 0.5% agarose and 2.25% acrylamide. Stacking gels of 3% acrylamide were used with the 5 gels. After electrophoresis the gels were stained with Coomassie Blue, dried and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) at -70°C. On occasion gels were sliced at 2-mm intervals, and the radioactivity of the individual slices was determined.

Reagents. DSS and DSP were purchased from Pierce Chemical Co. (Rockford, IL). Latex agaro was purchased from Accurate Chemical and Scientific Corp. (Denver, CO), and acrylamide and bisacrylamide were purchased from Bio-Rad Laboratories (Richmond, CA). Na125I was from Amersham Corp. (Arlington Heights, IL). Ristocetin was purchased from Lundbeck (Copenhagen, Denmark). NEM was obtained from Aldrich Chemical Co. (Milwaukee, WI). Leupeptin, lactoperoxidase, DFP, and hirudin were from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in this study were of reagent grade.

Results

Addition of the cross-linking agent DSP to preincubated mixtures of 125I-thrombin and platelets resulted in the appearance of three bands of radioactivity on 5% SDS-PAGE (Fig. 1). Identical results were obtained when 125I-DFP-thrombin was substituted. A control study performed with 125I-thrombin and DSS but without platelets showed only an 125I band corresponding to monomeric thrombin in 5% gels. Another control that included 125I-thrombin

![Image](image-url)
and platelets but no cross-linking agent revealed only an \(^{125}\)I-band that also co-migrated with free thrombin (not shown). These bands seen in the controls corresponded to the most rapidly migrating band of radioactivity in the cross-linked samples. Furthermore, when the cross-linked samples (formed with the thio-cleavable agent DSP) were treated with a reducing agent (DTT), the two bands of higher Mr disappeared, while the band at Mr \(\sim 38,000\) remained. This confirmed that the two bands of higher Mr were in fact due to covalent cross-linking by DSP, and that the band at Mr \(\sim 38,000\) was not a cross-linked species (Fig. 2).

The same pattern of radioactive bands was present in unreduced samples cross-linked with the noncleavable agent DSS (Fig. 2). In contrast, under reducing conditions, the middle band with Mr \(\sim 200,000\) migrated slightly further to a position corresponding to Mr \(\sim 180,000\). With 10% SDS-PAGE, only two autoradiographic bands appeared, one at the origin (Mr \(> 180,000\)) and one at Mr \(\sim 38,000\), co-migrating with free \(^{125}\)I-thrombin (data not shown).

The intensity of the unreduced middle band on 5% SDS-PAGE was maximal with 0.5 mM and 1 mM DSP (Fig. 1). At higher concentrations of DSP, less of the cross-linked material could be dissolved in SDS. When 10 mM DSP was used, only 38% of the radiolabeled material remained in the supernatant of samples centrifuged at 20,000 \(g\) for 60 min, whereas with 1 mM DSP, 90% of the radioactivity remained in the supernatant, and in the absence of DSP 94% remained soluble. Therefore, 1 mM DSP was used in subsequent experiments.

With an initial thrombin concentration of 20 nM and a platelet concentration of \(10^9/\text{ml}\), \(\sim 2.5\%\) of the total thrombin in an individual sample became bound to the platelets. With 1 mM DSP, \(\sim 15\%\) of this platelet-bound \(^{125}\)I-thrombin migrated with the \(\sim 200,000\)-Mr complex, as quantitated by measuring the radioactivity of 2-mm gel slices.

A third band of radioactivity remained at the top of 5% gels (Fig. 1). In more porous slab gels, however, this band diminished and a faint band appeared with Mr \(\sim 400,000\) (Fig. 3). On occasion, such slabs were cut into 2-mm slices and the radioactivity of the slices quantitated. Regardless of the thrombin concentration used (0.1–20 nM), the ratio of cpm in the \(\sim 200,000\)-Mr band to the cpm in the \(\sim 400,000\)-Mr band remained \(7:10:1\). When platelets, unexposed to thrombin, were incubated with DSP and separated by SDS-PAGE, Coomassie Blue staining of the gels revealed a protein pattern similar to that of non-cross-linked platelets, but with a large amount of material remaining at the origin (Fig. 4).

When hirudin was added to the thrombin-platelet mixtures, subsequent cross-linking with DSP produced no Mr \(200,000\) \(^{125}\)I-thrombin complex. A faint band was present on 5% SDS-PAGE, corresponding to free thrombin nonspecifically associated with the platelets, and a small amount of radioactivity remained at the top of the gel (Fig. 1, lane H and Fig. 9, lane 3). Unlabeled thrombin competed with \(^{125}\)I-thrombin for binding to platelets, as shown in Fig. 5. At a molar ratio of labeled to unlabeled thrombin of 1:1, there was a slight diminution in the appearance of the \(\sim 200,000\)-Mr band, and at ratios of 1:100 and 1:500 the radioactive \(\sim 200,000\)-Mr band was not seen.

When platelets from a patient with the Bernard-Soulier syndrome (lacking GPIb) were substituted for normal platelets in the cross-linking studies, no radioactive bands appeared at Mr \(\sim 200,000\) or \(\sim 400,000\) in gels of 0.5% agarose and 2.25% acrylamide (Fig. 6). To confirm this apparent lack of thrombin binding to the Bernard-Soulier platelets, an equilibrium binding study was performed. A Scatchard plot of the data is shown inFig. 7. For comparison, a parallel study with normal platelets is also presented. The only binding demonstrable to the Bernard-Soulier platelets is represented by a horizontal line (bound/free = 2.3±0.45 ml/10\(^9\) cells, mean±SD), which approximates the asymptote approached by the hyperbolic plot of the data for the normal platelets. The intercept of this line also coincides with the constant bound/free ratio observed in the presence of hirudin (2.6±0.40 ml/10\(^9\) cells, mean±SD) with free thrombin concentrations of 0.01–6 U/ml. By these criteria, the thrombin associated with the Bernard-Soulier platelets represents only non-specific (unsaturable) binding.

SDS-PAGE of the platelets from a patient with Glanzmann's
thrombasthenia incubated with thrombin and cross-linking agents is shown in Fig. 8. Despite the lack of the GPIIb/IIIa complex in these platelets (8), the radioactive bands derived by thrombin cross-linking were indistinguishable from those seen with normal platelets.

In an attempt to identify the submolecular components of the membrane site(s) involved in thrombin binding, we studied the effect of two monoclonal and one polyclonal antibody against platelet glycoproteins. Neither the monoclonal antibody, 6D1, which is directed against the ristocetin-dependent vWf binding site on GPIb, nor the monoclonal antibody, 10E5, which is directed against the fibrinogen binding site on GPIIb/IIIa, altered the appearance of the crosslinked bands appearing on 5% gels. However, the polyclonal antibody against glycopcalicin completely inhibited the formation of the $^{125}$I-thrombin–platelet complexes (Fig. 9). Preimmune serum had no discernible effect. The inhibition by antilygocalicin was confirmed by measuring the equilibrium binding of 20 nM thrombin to platelets in the presence of the antibody and in the presence of preimmune serum with and without hirudin. Even though the concentration of antiserum was less in these experiments than in the cross-linking studies (20 vs. 50%, vol/vol), ~80% of the specific binding at equilibrium was inhibited by the antibody.

Incubating platelets with $^{125}$I-thrombin in the presence of vWf and ristocetin appeared to reduce the intensity of the ~200,000-Mr band by ~20% (Fig. 10), whereas vWf alone had no apparent effect. However, equilibrium binding studies with 20 nM thrombin in the presence of ristocetin (1 or 2 mg/ml) and vWf (2–100 μg/ml) failed to demonstrate any inhibition (Fig. 11).

Figure 5. Effect of unlabeled thrombin on the formation of the Mr ~200,000 complex of $^{125}$I-thrombin and platelet protein. $^{125}$I-thrombin (20 mM) was mixed with native thrombin in a ratio of unlabeled to labeled of 1:1 (lane 2), 10:1 (lane 3), 500:1 (lane 4), or 1,000:1 (lane 5) before being added to the platelets and cross-linked with 1 mM DSP. No unlabeled thrombin was added to the $^{125}$I-thrombin used in the sample in lane 1. The $^{125}$I-thrombin was effectively displaced from the thrombin–platelet complexes by unlabeled thrombin.

Figure 6. Analysis of the $^{125}$I-thrombin–platelet complexes of normal (left) and Bernard-Soulier (right) platelets in an SDS-gel of 0.5% agarose and 2.25% acrylamide. Normal platelets were incubated with 20 and 50 nM $^{125}$I-thrombin, while the Bernard-Soulier patient's platelets were incubated with 20 nM and 100 nM $^{125}$I-thrombin. Cross-linking was performed with 1 mM DSS. Molecular weight standards were the same as in Fig. 3. The arrows indicate radiolabeled species of Mr, ~400,000, ~200,000, and ~38,000 (dye front). In contrast to normal platelets, the platelets from the Bernard-Soulier patient show only a band that co-migrates with free thrombin.

Figure 7. Scatchard plot representing the binding of thrombin to normal and Bernard-Soulier platelets. The horizontal line through the Bernard-Soulier points approximates the asymptote of the hyperbolic curve depicting the data for the normal platelets. The vertical intercept of this line (bound/free ~ 2.3 ml/10^10 cells) agrees well with the bound/free ratio measured in the presence of hirudin (2.6±0.40 ml/10^10 cells, mean±SD). The binding experiments were performed at 25°C.

Discussion

We have studied the binding of alpha thrombin to the platelet surface by cross-linking radiolabeled thrombin to platelets with the bifunctional agents DSP and DSS and by measuring the

Figure 8. Autoradiograph of normal platelets and platelets from a patient with Glanzmann's thrombasthenia, exposed to (left to right) 10, 20, and 100 nM $^{125}$I-thrombin and cross-linked with 1 mM DSS. The samples were unreduced. The migration distance corresponding to Mr, ~200,000 is indicated by an arrow. $^{125}$I-thrombin cross-linked complexes of the thrombasthenic patient's platelets are indistinguishable from those formed with normal platelets. Molecular weight standards were the same as shown in Fig. 1.
association of thrombin with platelets under equilibrium conditions. As we and others have shown, in the presence of the leech peptide hirudin, the only thrombin binding that occurs is unsaturable, and is equivalent to the nonspecific binding calculated from the limiting value of bound/free on a Scatchard plot (Fig. 7) (19, 20). When hirudin was added to 125I-thrombin before mixing with platelets and exposure to a cross-linking agent, the only autoradiographic bands appearing after electrophoretic separation (SDS-PAGE) were very faint, at the top of the gels (to be discussed below) and at Mr ~ 38,000, corresponding to a small amount of free thrombin (Figs. 1 and 9). Therefore, the 125I bands we observed on SDS-PAGE of samples prepared without hirudin are interpreted to have resulted from the reaction of thrombin with a specific membrane binding site.

When normal platelets were cross-linked to 125I-thrombin in the absence of hirudin, a consistent finding was a species with Mr ~ 200,000, compatible in size with a bimolecular complex of one thrombin (Mr ~ 38,000) and one GPIb (Mr ~ 165,000). In 5% gels, 125I-bands were also detected at the dye front and at the origin (Mr > 350,000). The possibility of complexes of Mr lower than 200,000 was excluded by 10% SDS-PAGE, in which the only bands visualized were at the top of the gel and at Mr ~ 38,000 (uncross-linked thrombin).

In gels of 0.5% agarose and 2.25% acrylamide, a minor cross-linked species of Mr ~ 400,000 was also evident (Fig. 3). Although it was not always well separated from the band at Mr ~ 200,000, the absence of any radiolabeled material in the Mr range 200,000~350,000 in 5% gels indicated that the faint band in the more porous system represented a distinct molecular complex rather than a group of complexes of Mr 200,000~400,000. Since there are no recognized membrane proteins sufficiently large to produce a bimolecular unit of this size with thrombin, the Mr ~ 400,000 band must be either (a) a dimer of the ~200,000-Mr complex, (b) a heteromultimer of the ~200,000-Mr complex and ~5 additional 125I-thrombin molecules, or (c) multiple 125I-thrombin molecules linked to a membrane protein or group of proteins other than that in the ~200,000-Mr band. The latter two possibilities are unlikely both because they would require multiple cross-linking reactions within a single structure and because there is no evidence of the expected family of less cross-linked components of such a conglomerate. Therefore, the most reasonable explanation for the ~400,000-Mr complex is that it is a dimer of the ~200,000-Mr species. This implies that the thrombin-receptor units in the intact platelet membrane are in close proximity, since the bridging distance of DSP is only 12 A. This possibility has previously been raised by Ganguly and Gould (21), who suggested that thrombin stimulation may lead to clustering of the thrombin receptors.

In addition to the cross-linked complexes with Mr ~ 200,000 and ~400,000, most of the polyacrylamide gels retained a variable amount of material at the origin of the lanes, regardless of the porosity of the slab matrix. Although the ~400,000-Mr complex would not be expected to enter the 5% gels, the material remaining at the top of these slabs was frequently too concentrated to be explained by this complex alone. Furthermore, small amounts of material remained at the origin of the 0.5% agarose, 2.25% acrylamide slabs. Even hirudin, which prevented the formation of cross-linked complexes, did not always eliminate retention of radioactivity at the top of the electrophoretic lanes. Similar observations have been commonly made by others using cross-linking agents (16, 17, 22). The phenomenon is generally attributed to entrapment of radiolabeled protein in an extensively cross-linked matrix of high molecular weight and limited solubility in SDS. This explanation is consistent with our observation that only 38% of the radioactivity of a thrombin-platelet sample
remained in the supernatant after solubilization with SDS if cross-linking was accomplished with 10 mM DSP, whereas 90% remained in the supernatant if the DSP was only 1 mM. Also, when platelet proteins (unexposed to thrombin) were separated on SDS-PAGE and stained with Coomassie Blue, previous treatment of the sample with cross-linking agents was associated with marked retention of material at the electrophoretic origin, consistent with a highly cross-linked matrix of platelet protein unrelated to thrombin. Therefore, we feel that the radioactivity remaining at the origin of the electrophoretic separations of the 125I-thrombin–platelet samples includes nonspecifically trapped material and not covalently bound thrombin-platelet complexes of $M_r > 400,000$.

Our observations, therefore, favor a single binding site for thrombin to the platelet membrane, although equilibrium studies have previously demonstrated two classes of receptors and the curvilinear configuration of our own Scatchard analysis of normal thrombin binding is consistent with this interpretation (Fig. 7). The possibility that we have selectively lost a class of cross-linked complexes during our sample preparation for SDS-PAGE appears unlikely, since ~90% of the 125I-thrombin originally bound to the platelets was solubilized in SDS for electrophoresis. Furthermore, the results of the equilibrium studies are as compatible with a single binding site that manifests negative cooperativity as they are with the presence of two independent binding sites.

Most of our experiments were performed with a thrombin concentration (20 nM), which should have been sufficient to saturate the high affinity receptor class ($K_d 0.2$ nM) and to bind extensively to the low affinity class ($K_d 30$ nM). To evaluate whether the minor ~400,000-$M_r$ complex identified under these conditions might represent the low capacity, high affinity binding site, studies were repeated with thrombin concentrations as low as 0.1 nM, which should have highlighted binding to the high affinity receptor. However, the ~200,000-$M_r$ complex remained dominant, approximately sevenfold as intense as the ~400,000-$M_r$ complex, just as when the much higher thrombin concentrations were used. This is further evidence, therefore, that thrombin binds to the same site regardless of the concentration of thrombin in solution.

Although this conclusion agrees with the work of Tollefsen and Majerus (22), who found a single complex of $M_r \sim 200,000$ when crosslinking DFP-thrombin to platelets with glutaraldehyde, it is not supported by the results of Larsen and Simons (23), who used a photo-activatable thrombin analogue. When this analogue was blocked at its active site, it formed cross-linked complexes of $M_r \sim 400,000, \sim 200,000$, and ~46,000 with platelets at thrombin concentrations > 20 nM, whereas only the ~200,000-$M_r$ complex was apparent at thrombin concentrations ≤ 2 nM. When the active site of the analogue was not blocked, the $M_r$ of the 200,000 complex shifted to ~120,000. The differences between these observations and ours may be related to the fact that in the earlier studies thrombin binding occurred with the platelets suspended in a modified Tyrode’s buffer without protease inhibitors. This may have allowed proteolysis of the platelet membrane or the thrombin-receptor complexes. Proteolysis appeared to be prevented in our study by the presence of EDTA or EDTA with leupeptin and NEM. Coller has shown that GPIb is susceptible to proteolysis, which can be inhibited by these agents (24). Harmon and Jameson (25) have also recently published evidence for thrombin receptor heterogeneity, but their technique of radiation inactivation determines the size of functional units rather than the size of the specific protein to which thrombin binds.

Glanzmann’s platelets, which lack GPIb/IIa, formed cross-linked complexes with 125I-thrombin indistinguishable from those seen with normal platelets (Fig. 8). GPIb/IIa, therefore, appears not to contain the thrombin binding site. This conclusion is consistent with the lack of binding inhibition in the presence of the monoclonal antibody 10E5. Furthermore, it agrees with the observations of White, Workman, and Lundblad (26), who demonstrated normal thrombin binding to Glanzmann’s platelet under equilibrium conditions.

On the other hand, our studies with Bernard-Soulier platelets, which neither generated the cross-linked thrombin complexes nor demonstrated any specific thrombin binding at equilibrium, strongly suggest that thrombin binds to GPIb. Nevertheless, because Bernard-Soulier platelets are known to have membrane abnormalities in addition to their absence of GPIb, other lines of evidence are required to assign the binding site to this glycoprotein (7). Such evidence is provided by the marked inhibition of thrombin binding in the presence of the polyclonal antigelatinic platelet antiserum. By equilibrium binding methods, this antiserum eliminated ~80% of specific thrombin binding, and by the cross-linking technique the antiserum prevented formation of the ~200,000-$M_r$ complex. These studies, therefore, localize the binding site to a particular domain of GPIb, the glycoprotein portion of the molecule. This localization is also supported by the behavior of the ~200,000-$M_r$ complex under reducing conditions (Fig. 2). In the presence of DT TT the $M_r$ of the complex, formed with the noncleavable cross-linking agent, decreased by ~20,000, consistent with the load expected by reductive cleavage of the beta (nonglycolicin) portion of GPIb ($M_r 23,000$) and the A chain of thrombin ($M_r 4,600$) (27, 28). Furthermore, this behavior of the reduced complex implies that the cross-linking is to the B chain of the thrombin molecule.

In an attempt to localize the thrombin binding site even more precisely, the cross-linking studies were performed in the presence of the monoclonal antibody 6D1, which inhibits the ristocetin-dependent binding of vWF to GPIb (14). This antibody had no apparent effect on formation of the ~200,000-$M_r$ complex. Therefore, similar studies were performed to see whether vWF itself would inhibit thrombin binding in the presence of ristocetin. Although the cross-linking studies suggested that the vWF multimers, which are manyfold larger than the 6D1 antibody, might have a moderate inhibitory effect on thrombin binding (~20%), this impression was not confirmed by equilibrium binding methods. Kao et al. (29) found a similar lack of competition of vWF and thrombin for platelet binding. Therefore, it appears that thrombin binds at a site beyond the realm where steric hindrance from the very large vWF is operative.

In summary, our data are consistent with the conclusion that platelets bind thrombin specifically to a single species of membrane protein, GPIb, despite evidence from equilibrium studies that two functional classes of binding sites exist. Furthermore, this binding occurs between the B chain of the thrombin molecule and the glycoprotein portion of GPIb, at a point remote from where vWF binds in the presence of ristocetin.

References


