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Platelet Activation by Thrombin in the Absence of the High-Affinity Thrombin Receptor†

Joan T. Harmon and G. A. Jamieson*
Cell Biology Laboratory, American Red Cross, Rockville, Maryland 20855
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ABSTRACT: The receptor status of the moderate-affinity platelet binding site for α-thrombin has been established by treating platelets with *Serratia marcescens* protease under conditions causing cleavage of 95–97% glycoprotein Ib (2.5 μg for 30 min). High-affinity binding was lost under these conditions, but the platelets continued to show moderate-affinity binding (K_{d_1} = 16 ± 5 nM; 930 ± 300 sites/platelet) and low-affinity binding (K_{d_2} = 4.6 ± 3 μM; 170 000 ± 90 000 sites/platelet), in good agreement with the values previously obtained for moderate- and low-affinity binding in intact platelets [Harmon, J. T., & Jamieson, G. A. (1986) *J. Biol. Chem. 261*, 15928–15933]. Platelets treated with *Serratia* protease under these conditions were about 4-fold less sensitive to activation by α-thrombin, as measured by serotonin secretion. Crossover studies with analogues showed that binding of α-thrombin was competable by both D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone treated thrombin and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone treated thrombin, and both analogues were capable of inhibiting activation of *Serratia*-proteolyzed platelets by α-thrombin. These studies establish that the moderate-affinity platelet binding site for α-thrombin is a receptor, occupancy of which is required for platelet activation in the absence of the high-affinity receptor.

The definition of a receptor is that the observed binding of a ligand must be saturable, reversible, and specific, and must lead to a physiological event (Kahn, 1976). We have recently shown (Harmon & Jamieson, 1986a) that the activation of intact platelets by thrombin proceeds via a receptor-dependent mechanism as shown by the fact that D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone treated thrombin (PPACK-thrombin)[1] binds to the high-affinity binding site for α-thrombin (K_d = 0.3 nM) and competes with it in platelet activation, while TLCK-thrombin does not bind to the high-affinity site accessible to α-thrombin and hence cannot compete with it in platelet activation (Harmon & Jamieson, 1986a). These results have resolved long-standing questions regarding the correlation of equilibrium binding of thrombin with its ability to activate platelets [for a review, see Phillips (1985)].

Intact platelets also contain a moderate-affinity binding site (~1700 sites/platelet; K_d = 11 nM) (Harmon & Jamieson, 1986a), and in contradistinction to the high-affinity site, both PPACK-thrombin and TLCK-thrombin compete with α-thrombin in binding to the moderate-affinity site. However, the question of whether this moderate-affinity binding site is a classical receptor, capable of fully activating platelets in the absence of the high-affinity receptor, cannot be answered with intact platelets since these are fully activated by α-thrombin at concentrations well below those at which antagonism by TLCK-thrombin at the moderate-affinity binding site would be observed. The low-affinity binding of α-thrombin to platelets is not thought to be of physiological relevance since its dissociation constant (K_d ~ 3 μM) is far above thrombin concentrations that are likely to be encountered physiologically.

In the present studies, we have removed the high-affinity thrombin receptor by treating platelets with *Serratia marcescens* protease and have shown that moderate-affinity binding identifies a receptor fully capable of activating platelets

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* Address correspondence to this author at the Cell Biology Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855.

[1] Abbreviations: TLCK-thrombin, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone treated thrombin; PPACK-thrombin, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone treated thrombin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GPIb, glycoprotein Ib; Tris, tris(hydroxymethyl)aminomethane.

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in the absence of the high-affinity α-thrombin receptor.

Materials and Methods

Serratia marcescens Protease. The lyophilized purified protease was generously supplied by Dr. Herbert A. Cooper, The University of North Carolina at Chapel Hill, and Dr. Arnold Kreger, Wake Forest University (Lyerly & Kreger, 1979; Cooper et al., 1982). It was reconstituted in 50 mM Tris, pH 7.4, to a concentration of 0.5 mg/mL and stored at −70 °C.

Platelet Preparation. Whole blood anticoagulated with CPD-A1 was obtained within 3 h of venipuncture from the American Red Cross Washington Regional Blood Services, Washington, D.C. Platelet-rich plasma was prepared by low-speed centrifugation, and the platelets were separated from the plasma according to the citrate wash procedure (pH 6.5) previously described (Harmon & Jamieson, 1985). After the platelets were washed, they were suspended in modified Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.5 mM glucose, 0.4 mM Na_2HPO_4, and 10 mM HEPES, pH 7.4) to a concentration of 1×10^9 platelets/mL.

Thrombin and Thrombin Analogues. Thrombin was purified from Cohn fraction III (Fenton et al., 1977) and iodinated with Iodogen as previously described (Tandon et al., 1983). TLCK-thrombin and PPACK-thrombin were prepared from α-thrombin by established procedures (Workman et al., 1977; Glover & Shaw, 1971; Kettner & Shaw, 1979).

Serratia Protease Treatment. Since there is evidence to indicate that GPIb is the high-affinity thrombin receptor (see Discussion), platelets were treated with *Serratia marcescens* protease which cleaves GPIb with a high degree of specificity (Cooper et al., 1981, 1982; McGowan & Detwiler, 1985).

Aliquots of washed platelets in modified Tyrode's buffer (1×10^9/mL) were incubated in the presence of increasing amounts of *Serratia* protease for 30 min at room temperature. Following this incubation, the platelet suspension was diluted with an equal volume of citrate wash buffer (pH 6.5) and sedimented at 1400*g* for 10 min. The platelet pellet was resuspended in modified Tyrode's buffer to a concentration of 1×10^9 platelets/mL, and total binding was determined at low concentrations of ^{125}I-thrombin as described below. Control platelets were obtained from the same donor as *Serratia*-treated platelets and were treated identically with *Serratia*-treated platelets except that the *Serratia* protease in modified Tyrode's buffer was replaced by buffer only.

Thrombin Binding Assay and Data Analysis. The binding assay was conducted in modified Tyrode's buffer, pH 7.4, with 1% bovine serum albumin at room temperature using low concentrations of ^{125}I-α-thrombin (~0.1 nM) (Harmon & Jamieson, 1985). Experiments to establish conditions for steady-state binding showed that nonproteolyzed, control platelets achieved steady-state binding by 10 min, as previously described, while *Serratia*-proteolyzed platelets required 60–120 min (see Results). Assays for thrombin binding were, therefore, carried out for 120 min with *Serratia*-treated platelets and for 10 min with control platelets. Longer incubation resulted in a decrease in binding with control platelets (but not with *Serratia*-proteolyzed platelets) which can be attributed to degradation or processing of the binding sites since there is no detectable degradation of ^{125}I-α-thrombin between 10 and 150 min as measured by an increase in radioactivity not precipitable by 5% trichloroacetic acid. The time course of dissociation of labeled thrombin (0.07 nM) from *Serratia*-proteolyzed and control platelets on dilution was similar after steady state had been achieved.

To obtain thrombin binding isotherms, the concentration of labeled ligand in all assay tubes was maintained constant at approximately 0.1 nM while the concentration of unlabeled ligand was varied from 0.1 nM to 1 μM. The binding assay was initiated by the addition of platelets to assay tubes containing the appropriate labeled and unlabeled ligands in order to measure competition rather than the ability of the one ligand to displace the other. The amount of radioactive thrombin bound to the platelets was measured in a γ counter (Micromedic ME Plus, Horsham, PA).

Binding isotherms were obtained simultaneously on *Serratia*-proteolyzed platelets and on the same donor platelets which had not been proteolyzed (control). The model which best described the data was selected by F-test comparison of the sum of squares of the various models examined using a nonlinear least-squares curve-fitting program (LIGAND: Munson & Rodbard, 1984) as previously described (Harmon & Jamieson, 1985). The models examined included (1) a one-site model with nonspecific binding, (2) a two-site model with or without nonspecific binding, and (3) a three-site model with or without nonspecific binding.

The binding parameters for the two active-site-inhibited thrombin analogues, PPACK-thrombin and TLCK-thrombin, were obtained for *Serratia*-proteolyzed platelets by examining the binding isotherms and the cross-reactivity of each of these analogues for the α-thrombin binding sites, as previously described using nonproteolyzed platelets (Harmon & Jamieson, 1986b). The combinations studied were the following: (1) ^{125}I-α-thrombin/α-thrombin; (2) ^{125}I-α-thrombin/PPACK-thrombin; (3) ^{125}I-α-thrombin/TLCK-thrombin; (4) ^{125}I-PPACK-thrombin/α-thrombin; (5) ^{125}I-PPACK-thrombin/PPACK-thrombin; (6) ^{125}I-TLCK-thrombin/α-thrombin; (7) ^{125}I-TLCK-thrombin/TLCK-thrombin.

Serotonin Secretion. Washed platelets (1×10^9/mL), either control or *Serratia*-proteolyzed, were incubated with [^{14}C]-serotonin (0.25 μCi/mL, 1.6 μM) for 1 h at 37 °C, and serotonin release induced by α-thrombin was measured in a total volume of 0.1 mL of modified Tyrode's buffer with 1% bovine serum albumin containing 4×10^8 platelets/mL as previously described (Harmon & Jamieson, 1986). The [^{14}C]serotonin in the assay medium in the absence of α-thrombin was considered as background and subtracted from the results. Under these conditions, both control and proteolyzed platelets took up equivalent amounts of [^{14}C]serotonin (90%) and released about 90% of this serotonin upon addition of 10–20 nM α-thrombin. Inhibition of serotonin release from *Serratia*-proteolyzed platelets induced by α-thrombin (2 nM) was measured in the absence and presence of increasing concentrations of either PPACK-thrombin or TLCK-thrombin. Neither analogue alone was able to induce serotonin release at 1 μM concentration.

Results

Serratia Protease Treatment. The ability of platelets to bind thrombin decreased rapidly following treatment with increasing concentrations of *Serratia marcescens* protease. The total amount of thrombin bound at high-, moderate-, and low-affinity sites was reduced to about 15% of control values with 30-min treatment at 1 μg/mL but was only reduced a further 5% by increasing the *Serratia* concentration to 2.5 μg/mL (Figure 1, closed circles). Since study of the thrombin binding isotherms (see below) showed that high-affinity binding was lost under these conditions (2.5 μg/mL for 30 min), they were routinely used for the removal of high-affinity binding. Examination of autoradiographs of periodate–borotritide-labeled platelets treated under these conditions, following polyacryl-

Table I: Binding Parameters for α-Thrombin to Control and *Serratia*-Proteolyzed Platelets (n = 4)

	high affinity		moderate affinity		low affinity	
	K_1[a]	R_1[b]	K_2	R_2	K_3	R_3
control	1.1 ± 0.5	230 ± 110	28 ± 20	2600 ± 1500	1300 ± 400	107000 ± 17000
Serratia proteolyzed[d]	n.d.[c]	n.d.	16 ± 5	930 ± 300	4600 ± 3000	170000 ± 90000

[a] The affinity parameters (K) are given as the dissociation constants in nanomolar ± standard errors. [b] The concentrations of binding sites (R) are given as sites per platelet ± standard errors. [c] n.d., not detected. [d] Conditions of *Serratia* proteolysis were as described under Materials and Methods (2.5 μg/mL; 30 min).

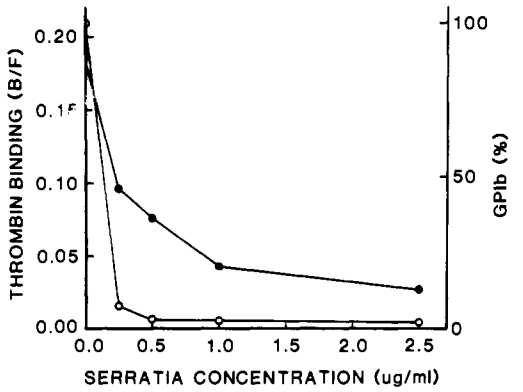

FIGURE 1: Thrombin binding and GPIb content in *Serratia*-treated platelets. Platelets were treated with increasing concentrations of *Serratia* protease as described under Materials and Methods. Total binding of ^{125}I-α-thrombin (bound/free) was measured at tracer concentrations (0.12 nM; closed circles). GPIb content as percent of control was measured by scanning densitometry of the autoradiogram (open circles).

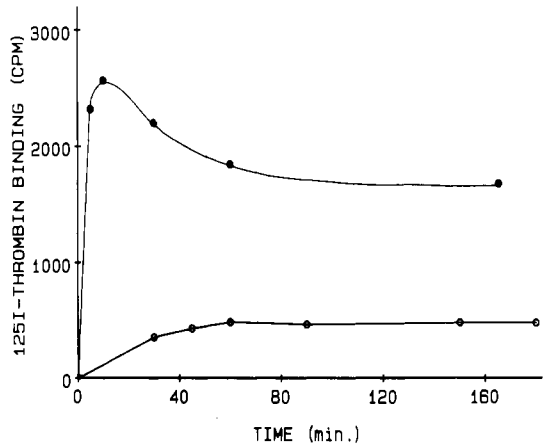

FIGURE 2: Time course of binding to control (closed circles) and *Serratia*-proteolyzed platelets (open circles) at tracer concentrations of ^{125}I-α-thrombin (0.08 nM). The *Serratia* protease treatment was conducted for 30 min with 2.5 μg/mL protease. The points represent the mean of triplicate determinations.

amide gel electrophoresis in sodium dodecyl sulfate, showed that 95–97% of GPIb had been cleaved (Figure 1, open circles).

α-Thrombin Binding. The time course of binding to reach steady-state conditions was determined both for intact control platelets and for *Serratia*-proteolyzed platelets. Binding of α-thrombin (~0.1 nM) to control platelets reached steady state in 10 min as previously observed, but 60–120 min was required for this concentration to reach steady state with *Serratia*-proteolyzed platelets (Figure 2).

We next examined the α-thrombin binding isotherms under steady-state conditions for control and platelets proteolyzed with 2.5 μg/mL *Serratia marcescens* protease. The data from four separate experiments performed in triplicate are shown in Figure 3. As expected from previous studies (Harmon & Jamieson, 1985), the model which best described the data obtained with control platelets contained three independent binding sites of high, moderate, and low affinities without nonspecific binding: the values obtained (Table I) were in good agreement with those previously obtained. On the other hand, high-affinity binding could not be detected in *Serratia*-proteolyzed platelets (Figure 3), and the model which best described the data obtained was one of two independent binding sites of moderate and low affinity without nonspecific binding.

There was good agreement in the affinity constants for α-thrombin at the moderate-affinity sites for control (K_d = 28 ± 20 nM) and *Serratia*-proteolyzed platelets (K_d = 16 ± 5 nM) (Table I). There were no significant differences in the data for the low-affinity sites in the two preparations.

Response to α-Thrombin. We next measured the responsiveness of control and *Serratia*-proteolyzed platelets to α-thrombin as measured by release of serotonin. In this context, it was important to establish that both control and *Serratia*-proteolyzed platelet preparations were equivalent: the ratio of radioactivity taken up per 4 × 10^8 *Serratia*-proteolyzed platelets to the radioactivity taken up per 4 × 10^8 control

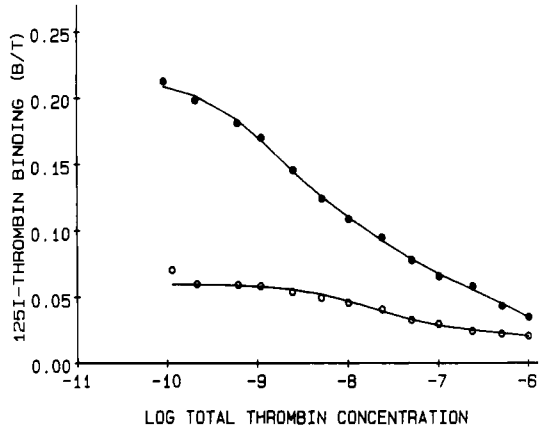

FIGURE 3: Thrombin binding isotherms obtained on control platelets (closed circles) and *Serratia*-proteolyzed platelets (open circles). Platelets from the same donor were used for controls and for treatment with 2.5 μg/mL *Serratia* protease (30 min, room temperature). After several washings, the platelets were assayed for their ability to bind thrombin. The binding data were generated with a constant amount of ^{125}I-α-thrombin (0.1 nM) and increasing amounts of unlabeled α-thrombin. The data points represent the mean of four separate experiments in each of which triplicate determinations were made. The lines represent the predicted result obtained by computer analysis of the binding data for a model of three independent binding sites with the control platelet experiments and for a model of two independent binding sites for the *Serratia*-proteolyzed platelet experiments.

platelets was 1.1 ± 0.1 (n = 4). Likewise, in both platelet preparations, the percent of [^{14}C]serotonin taken up but subsequently released in the absence of α-thrombin was the same: 26 ± 4% for control platelets and 27 ± 5% for *Serratia*-proteolyzed platelets. The percent of total radioactivity released at thrombin concentrations greater than 10 nM was also the same in the two preparations: 92 ± 15% for control platelets and 86 ± 21% for *Serratia*-proteolyzed platelets. Thus, the serotonin release data on the two platelet preparations are directly comparable.

Table II: Binding Parameters for α-, PPACK-, and TLCK-Thrombins to *Serratia*-Proteolyzed Platelets ($n = 2$)[a]

	moderate affinity		additional site		
	K_1[b]	R_1[c]	K_2	R_2	nonspecific
α	3.3 ± 1	81 ± 30	n.d.[d]	n.d.	0.025 ± 0.0008
PPACK	2.8 ± 1		n.d.		0.072 ± 0.02
α	38 ± 20	1000 ± 700	0	45 ± 30	0.028 ± 0.002
TLCK	630 ± 300		0.25 ± 0.2		0.22 ± 0.007

[a] Conditions of *Serratia* proteolysis were as described under Materials and Methods (2.5 μg/mL; 30 min). [b] The affinity parameters (K) are given as the dissociation constants in nanomolar ± standard errors. [c] The concentrations of binding sites (R) are given as sites per platelet ± standard errors. [d] n.d., not detected.

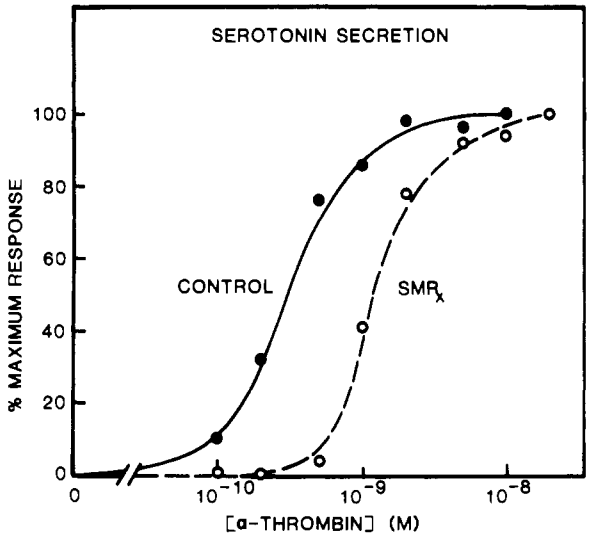

FIGURE 4: Serotonin release from control (closed circles) or *Serratia*-proteolyzed platelets (open circles) in response to α-thrombin. Platelets from the same donor were used for control and for *Serratia* protease treatment (2.5 μg/mL, 30 min, room temperature). Both platelet populations were loaded with [^{14}C]serotonin and exposed to increasing concentrations of α-thrombin for 10 min at room temperature. The results indicate that the concentrations of α-thrombin necessary to elicit a 50% maximum response were 0.28 nM in control platelets and 1.2 nM in *Serratia*-proteolyzed platelets. The points are the mean of triplicate determinations from a single experiment and are representative of four separate experiments.

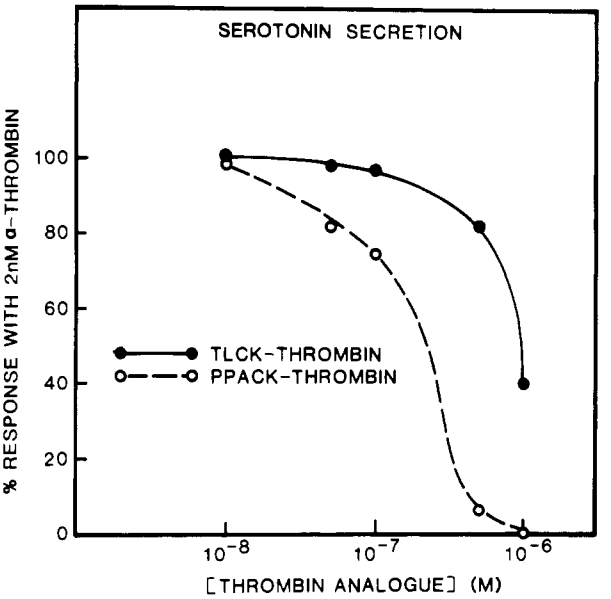

FIGURE 5: Inhibition of α-thrombin response by active-site-inhibited thrombin analogues in *Serratia*-proteolyzed platelets. *Serratia*-proteolyzed platelets were loaded with [^{14}C]serotonin and challenged with α-thrombin (2 nM) in the presence of increasing concentrations of TLCK-thrombin and PPACK-thrombin. The points represent the mean of triplicate determinations from a single experiment and are representative of four separate experiments.

Serratia proteolysis of platelets caused a decreased responsiveness to α-thrombin which was discerned as a rightward shift in the dose–response curve (Figure 4). In four separate experiments, maximum secretion was obtained with control platelets at 5.8 ± 4.0 nM α-thrombin with a 50% maximum response occurring at 0.28 ± 0.02 nM. Following *Serratia* treatment of these platelets, they responded maximally to thrombin at 13 ± 4 nM with a 50% maximum response occurring at 1.2 ± 0.4 nM. Thus, there was an approximate 4-fold shift to the right in the dose–response curve of *Serratia*-proteolyzed platelets versus control platelets.

Effect of Blocked Thrombins on Platelet Activation by α-Thrombin at the Moderate-Affinity Site. The above studies indicated that in *Serratia*-proteolyzed platelets, in the absence of detectable high-affinity binding, the moderate-affinity binding site was capable of effecting platelet activation by α-thrombin. The characteristic of the moderate-affinity binding site in intact platelets is that it binds both TLCK-thrombin and PPACK-thrombin. Hence, both of these should be able to compete with α-thrombin for platelet activation at the moderate-affinity site in *Serratia*-proteolyzed platelets.

To test this, *Serratia*-proteolyzed platelets were loaded with [^{14}C]serotonin and incubated with 2 nM α-thrombin to measure secretion in the absence and presence of increasing concentrations of either PPACK-thrombin or TLCK-thrombin (Figure 5). Both analogues inhibited the α-thrombin response: PPACK-thrombin caused a 50% inhibition at 240 ± 50 nM, and TLCK-thrombin caused a 50% inhibition at 880 ± 360 nM. Neither analogue alone induced serotonin release at concentrations up to 1 μM. Thus, both PPACK-thrombin and TLCK-thrombin act as antagonists of α-thrombin via the moderate-affinity thrombin receptor on *Serratia*-proteolyzed human platelets.

Binding of Thrombin Analogues. To verify that PPACK-thrombin and TLCK-thrombin competed with α-thrombin on the moderate-affinity site in *Serratia*-proteolyzed platelets, we examined the cross-reactivity of these analogues on *Serratia*-proteolyzed platelets.

(*A*) *PPACK-Thrombin.* Binding data were collected using a constant concentration of ^{125}I-α-thrombin or ^{125}I-PPACK-thrombin and increasing concentrations of unlabeled α-thrombin or unlabeled PPACK-thrombin to obtain the four ligand combinations (^{125}I-α/α, ^{125}I-α/PPACK, ^{125}I-PPACK/PPACK, and ^{125}I-PPACK/α). LIGAND analysis of these data, obtained on two separate occasions in triplicate, indicated that the best fit was described by a model of one binding site with nonspecific binding (Figure 6, Table II). With this model, α-thrombin and PPACK-thrombin both bind with similar affinities ($K_d \sim 3$ nM) to the same site on *Serratia*-proteolyzed platelets.

A model of two binding sites without nonspecific binding was also examined but was discarded since it caused an increase in the sum of squares for the fit. However, it should be noted that there was no significant difference in the binding parameters for the moderate-affinity site irrespective of

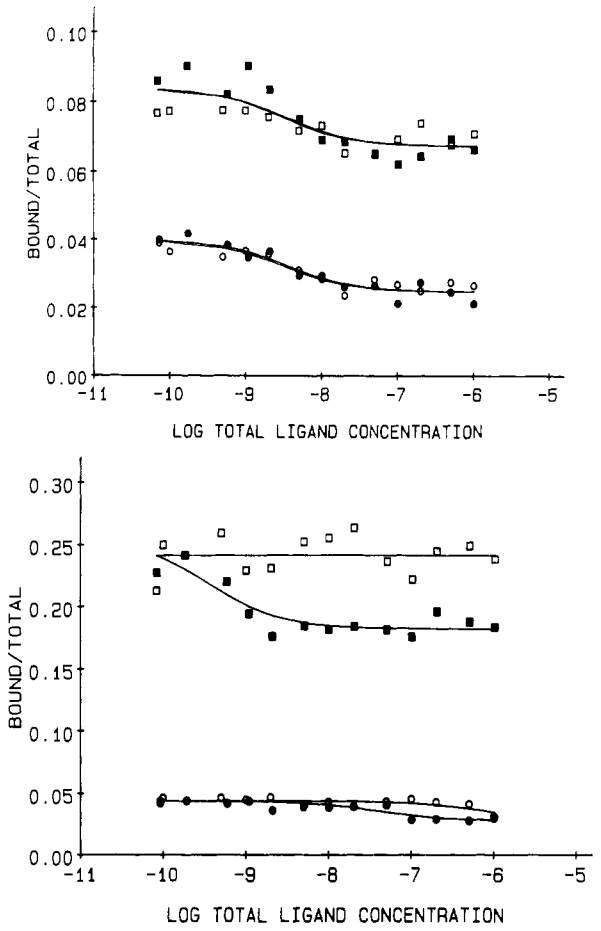

FIGURE 6: Isotherms for competition of α-thrombin binding by active-site-inhibited thrombins. *Serratia*-proteolyzed platelets were incubated with low concentrations (~0.1 nM) of either ^{125}I-α-thrombin or the ^{125}I-active-site-inhibited thrombins in the presence of increasing concentrations of competing ligand as detailed below. Data were analyzed by the LIGAND program for two separate experiments each carried out in triplicate. The binding parameters generated from these analyses are listed in Table II. (Upper) α-Thrombin and PPACK-thrombin: ^{125}I-α-thrombin and α-thrombin, closed circles; ^{125}I-α-thrombin and PPACK-thrombin, open circles; ^{125}I-PPACK-thrombin and PPACK-thrombin, closed squares; ^{125}I-PPACK-thrombin and α-thrombin, open squares. The lines represent predicted results from the simultaneous fit of all data sets using a model of one binding site with nonspecific binding: α-thrombin and PPACK-thrombin compete equally for this site. (Lower) α-Thrombin and TLCK-thrombin: ^{125}I-α-thrombin and α-thrombin, closed circles; ^{125}I-α-thrombin and TLCK-thrombin, open circles; ^{125}I-TLCK-thrombin and TLCK-thrombin, closed squares; ^{125}I-TLCK-thrombin and α-thrombin, open squares. The lines represent the predicted results from the simultaneous fit of all data sets using a model of two independent sites with nonspecific binding: α-thrombin binds to only one of these sites while TLCK-thrombin binds to both sites.

whether the very low-affinity site was described as a low-affinity binding site or as nonspecific binding (Table II). Inclusion of an additional binding site to which only PPACK-thrombin bound increased the number of parameters to be fit (decreased the degrees of freedom) without significantly improving the fit ($p = 0.63$). Therefore, this model was also discarded in favor of the simpler model in which both α-thrombin and PPACK-thrombin bound to the same site, and to only one site, on *Serratia*-proteolyzed platelets.

(*B*) *TLCK-Thrombin.* By a protocol identical with that used for PPACK-thrombin, the binding data of the four ligand combinations were obtained (^{125}I-α/α, ^{125}I-α/TLCK, ^{125}I-TLCK/TLCK, and ^{125}I-TLCK/α). Analysis of these data (Figure 6) indicated that the model which best described the data was that of two sites with nonspecific binding. α-Thrombin and TLCK-thrombin competed at the same binding site, albeit with a 17-fold difference in affinity (K_1 = 38 and 630 nM, respectively), but TLCK-thrombin also bound to an additional site (K_2 = 0.25 nM) to which α-thrombin did not bind. The binding parameters obtained for this model are given in Table II. No other models examined offered a significant improvement in the fitting parameters.

Discussion

α-Thrombin binds to platelets at high-, moderate- and low-affinity sites (Harmon & Jamieson, 1985). We have recently shown that the high-affinity binding of thrombin results in platelet activation by a receptor-mediated mechanism (Harmon & Jamieson, 1986b). Whether the moderate-affinity binding site also constituted a receptor, and its possible contribution to platelet activation, could not be established since intact platelets were activated by α-thrombin at concentrations below that at which TLCK-thrombin, the specific antagonist at the moderate-affinity site, would be effective.

There are several pieces of evidence that indicate that GPIb is the high-affinity thrombin receptor: (i) there is a concomitant decrease in thrombin binding, responsiveness, and GPIb content in Bernard–Soulier platelets (Jamieson & Okumura, 1978), in platelets from patients with myeloproliferative disorders (Bolin et al., 1977; Ganguly et al., 1978), and in platelets treated with chymotrypsin (Okumura et al., 1978; Tam et al., 1980) or elastase (Brower et al., 1985; Wicki & Clemetson, 1985); (ii) monoclonal antibodies to GPIb block thrombin-induced platelet activation (Kunicki et al., 1983; Yamamoto et al., 1985); and (iii) thrombin can be cross-linked to GPIb in intact platelets (Larsen & Simons, 1981; Jung & Moroi, 1983; Takamatsu et al., 1986). *Serratia marcescens* metalloprotease specifically cleaves GPIb from intact platelets with a high degree of specificity (Cooper et al., 1981, 1982; McGowan & Detwiler, 1985), and the resulting GPIb-depleted platelets show defects in thrombin responsiveness similar to those outlined above. For these reasons, we measured the effects of *Serratia marcescens* protease on the binding of α-thrombin following cleavage of GPIb. Under the conditions of *Serratia* proteolysis used here (2.5 μg/mL, 30 min), about 97% of the detectable GPIb was cleaved from platelets labeled by the periodate–borotritide procedure: high-affinity binding was not detectable, but moderate- and low-affinity binding was essentially unaffected.

The amount of thrombin bound at infinite dilution to platelets is the product of the association constant and the capacity of the site, in molar terms: data obtained in the present study (Table I) indicate that high-affinity binding in intact platelets constitutes about 50% of the total amount bound at infinite dilution while moderate- and low-affinity binding each contribute about 25%. Where high-affinity binding has been removed by protease treatment, it would be expected that the moderate- and low-affinity sites should bind approximately equal amounts of α-thrombin if they correspond to the sites in intact platelets with similar affinity. This was found to be the case: the bound/free ratios at infinite dilution of the ligand for the binding of α-thrombin to the moderate- and low-affinity sites in *Serratia*-treated platelets were 0.038 and 0.024, respectively. The corollary to this is that any proposal that possible receptors in *Serratia*-treated platelets are not being detected in the binding studies would require that they be of low affinity (in the micromolar range) and low in number (less than 100 sites per platelet): these conditions do not conform with what is known of the requirements for platelet activation by α-thrombin in either intact or *Serratia*-proteolyzed platelets.

The major characteristic differentiating high- and moderate-affinity binding in intact platelets is that the binding of α-thrombin at the moderate-affinity site is blocked by both TLCK-thrombin and PPACK-thrombin while only PPACK-thrombin binds to the high-affinity site for α-thrombin (Harmon & Jamieson, 1986b). In the present experiments, in the absence of high-affinity binding, both PPACK-thrombin and TLCK-thrombin competed with α-thrombin in binding to platelets, suggesting that the moderate-affinity binding properties of *Serratia*-proteolyzed platelets were qualitatively similar to intact platelets. Furthermore, quantitatively good agreement was observed for the affinity constants for the ligands, particularly for α-thrombin and PPACK-thrombin, at the moderate-affinity sites in intact and *Serratia*-proteolyzed platelets. For α-thrombin, the values obtained in the present studies were K_d = 28 ± 20 nM (n = 4) and 16 ± 5 nM (n = 4), respectively, as compared with values for moderate-affinity binding in platelets from normal individuals of 51 ± 20 nM (n = 15) (Harmon et al., 1986). For PPACK-thrombin, the value obtained in the present studies was 2.8 ± 1 nM (n = 2) as compared with 2.7 ± 0.3 nM (n = 3) in prior work using intact platelets (Harmon & Jamieson, 1986b). However, greater differences were seen in values for the binding of TLCK-thrombin to the moderate-affinity site in the two groups of platelets: 630 ± 300 nM (n = 2) in the present studies with *Serratia*-proteolyzed platelets as compared with a value of 1.5 ± 0.2 nM (n = 3) using intact platelets (Harmon & Jamieson, 1986b).

A comment should be made regarding the differences in time required to reach steady-state conditions for binding to the high-, moderate-, and low-affinity sites for α-thrombin in control platelets (10 min) and to moderate- and low-affinity sites in *Serratia*-proteolyzed platelets (120 min). To compare binding in the presence and absence of the high-affinity receptor, it is required that it be measured under steady-state conditions at the lowest concentration of ligand being studied (0.1 nM) (Munson & Rodbard, 1984). *Serratia*-proteolyzed platelets, which lack the high-affinity receptor, do not respond to 0.1 nM thrombin and require 120 min to reach steady state. At higher α-thrombin concentrations, where *Serratia*-proteolyzed platelets do respond (~1 nM), the binding of α-thrombin would rapidly have achieved steady state due to the law of mass action. However, binding of α-thrombin and competition by PPACK- and TLCK-thrombins could not have been adequately compared under these two different sets of conditions.

The fact that the rate of dissociation of α-thrombin from the binding site is the same in both control and *Serratia*-proteolyzed platelets once steady state has been achieved suggests that there must be a difference in the association rate constants, and therefore, in the affinity constants at the high- and moderate-affinity sites of at least 10-fold: this is indeed the case since the dissociation constant for α-thrombin at the high-affinity receptor in intact platelets is 1.1 ± 0.5 nM while the dissociation constant at the moderate-affinity receptor was determined as being 28 ± 20 nM in intact platelets and 16 ± 5 nM in *Serratia*-proteolyzed platelets. This similarity in the rate of dissociation also shows that processing of the ligand has not occurred during the prolonged incubation necessary to achieve steady-state binding at the moderate-affinity site although processing of α-thrombin to a nondissociable form does occur at the high-affinity site on prolonged incubation (Yeo & Detwiler, 1985).

A model for the binding of thrombin to *Serratia*-proteolyzed platelets can be developed from the present studies (Figure 7): this model is consistent with the model developed previously for the binding of thrombin to intact platelets (Harmon & Jamieson, 1986b). In the present studies, binding of α-thrombin itself to *Serratia*-proteolyzed platelets conforms with a model of two independent binding sites of moderate and low affinity without nonspecific binding. In the crossover studies with α-thrombin and PPACK-thrombin, the favored model for the binding of PPACK-thrombin was one of a single site of moderate-affinity binding with nonspecific binding. However, there was no significant difference in the binding parameters if the nonspecific binding was considered as a low-affinity site corresponding to that seen with α-thrombin, although there was an increase in the sum of squares. In the crossover studies with α-thrombin and TLCK-thrombin, the favored model for the binding of TLCK-thrombin was one of a single site of moderate affinity with nonspecific binding together with an additional high-affinity site (K_d = 0.25 nM) to which α-thrombin did not bind. That is, the additional binding site on intact platelets that was accessible to PPACK-thrombin but not to α-thrombin was apparently lost in *Serratia*-proteolyzed platelets while the site that was accessible to TLCK-thrombin but not to α-thrombin continued to be present in the proteolyzed platelets. The model presented here (Figure 7) is consistent with these findings and reflects the fact that it is not possible to describe the low-affinity binding (B_3) as being unequivocally due to a discrete low-affinity binding site or to nonspecific binding.

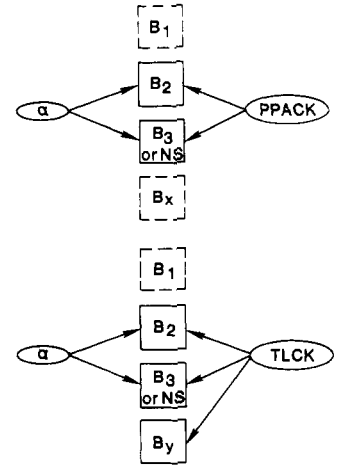

FIGURE 7: Models for the interaction of α-thrombin and active-site-inhibited thrombin analogues with *Serratia*-proteolyzed human platelets. The diagrams depict the models which offer the best fit for the binding data presented in Figure 6. This model is based on our earlier work using intact platelets (Harmon & Jamieson, 1986a). The binding sites represented by boxes enclosed by dashed lines are not detectable in *Serratia*-proteolyzed platelets. B_3 or NS indicates that it is not possible to determine unequivocally whether this component of total binding is to a specific low-affinity site or represents nonspecific binding.

These binding studies were confirmed by examining the ability of *Serratia*-treated platelets to respond to α-thrombin and the ability of both TLCK-thrombin and PPACK-thrombin to inhibit that response. Platelets treated with *Serratia marcescens* protease were fully responsive to α-thrombin as measured by their ability to secrete serotonin, but there was a rightward shift in the dose–response curve so that 50% of maximum secretion was obtained at about 1.2 nM thrombin concentration as compared with a value of about 0.3 nM in control platelets. Both blocked thrombins inhibited this response: 50% inhibition of secretion with 2 nM thrombin was obtained at about 250 nM PPACK-thrombin concentration and about 900 nM TLCK-thrombin concentration. In con-

trast, TLCK-thrombin has no effect on platelet response through the high-affinity receptor at a concentration of 1 μM (Harmon & Jamieson, 1985).

These studies show that platelet activation modulated by the moderate-affinity binding is also a receptor-mediated event; that is, occupancy of the moderate-affinity site is required for platelet activation by α-thrombin in the absence of the high-affinity receptor, and activation is inhibited when occupancy is blocked by TLCK- or PPACK-thrombins. Activation through this moderate-affinity receptor may be of significance in platelets from Bernard–Soulier platelets, in platelets from patients with myeloproliferative disease, and in other conditions of reduced platelet responsiveness.

We have recently shown that both high- and moderate-affinity binding sites for α-thrombin are located in the glycocalicin portion of GPIb (Harmon & Jamieson, 1986a). The structural relationship of this moderate-affinity binding site to the moderate-affinity receptor in *Serratia*-proteolyzed platelets is under active investigation.

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