Equilibrium Binding of Thrombin to Platelets†

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ABSTRACT: Binding of human [^{125}I]thrombin to washed human platelets was studied in order to analyze the nonenzymic aspects of the thrombin stimulation of platelets. Highly purified α-thrombin that was iodinated with lactoperoxidase retained full clotting and esterase activities and full activity toward platelets, was not distinguished from native thrombin by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or gel chromatography, and bound to platelets the same as unlabeled thrombin. Bound and free [^{125}I]thrombin were measured after rapid separation of platelets from the suspending medium by centrifugation through oil. Maximum binding was within 15 s, the shortest time measured. At concentrations of thrombin sufficient to cause less than maximal platelet stimulation, 90% of the total thrombin was free in the suspending solutions. Equilibrium binding was established, with both free thrombin and free platelets retaining activity, and with rapid reequilibration after dilution or addition of unlabeled thrombin. The equilibrium was complex, with the apparent number of binding sites and dissociation constants dependent on thrombin concentration. Analysis of bound thrombin as a function of thrombin concentration by double-reciprocal and Scatchard plots indicated 300–400 high affinity sites (K_{diss} = 1.8–2 nM); these correlate with thrombin stimulation of Ca^{2+} secretion, which shows half maximal effect at 1.5 nM thrombin and maximal effect with 500–600 thrombins bound per platelet.

The specific stimulation of platelets by thrombin is an interesting and unusual reaction that may represent an example of a more general reaction of proteases with cell surfaces. While thrombin is a proteolytic enzyme, its reaction with platelets has some characteristics inconsistent with an enzyme-catalyzed reaction (Detwiler and Feinman, 1973; Martin et al., 1975; Detwiler et al., 1975). For example, the *extent* of the reaction is dependent on enzyme concentration (Detwiler and Feinman, 1973), an observation that led to the hypothesis of tight binding of thrombin to the platelet, preventing enzyme turnover. It was subsequently reported by Tollefsen et al. (1974) that thrombin did indeed bind tightly to platelets, but that the binding was reversible. From this apparent contradiction (thrombin binding was reversible but there was apparently no turnover) and from studies of the effects of various perturbing conditions on the kinetics of thrombin stimulation of platelets, it was proposed that the simplest mechanism consistent with all observations was a reversible modification of receptors by thrombin (Martin et al., 1975; Detwiler et al., 1975), with the extent of stimulation dependent on the equilibrium concentration of the complex of thrombin and modified receptor.

Essential to this proposal is an equilibrium between free thrombin and platelet-bound thrombin. Equilibrium binding was suggested by the data of Tollefsen et al. (1974), but their method for measuring binding precluded an unequivocal interpretation. They measured platelet-bound [^{125}I]thrombin after separation of the platelets from the suspending medium by a procedure that involved a tenfold dilution of the suspension followed by filtration and washing of the filtered platelets with 10 volumes of buffer. Since this should have led to a substantial loss of bound thrombin due to reequilibration (unless the equilibration was exceptionally slow), the existence of a true equilibrium was uncertain and, since they measured binding after prolonged incubation (15 min), the platelets were presumably extensively modified. In addition, we considered it possible that the unbound radioactivity might have been associated with thrombin that had been modified or inactivated by reaction with platelets, thus accounting for the lack of turnover.

We have, therefore, studied the binding of [^{125}I]thrombin to platelets by measuring bound thrombin after separation of platelets from the suspension by rapid centrifugation through oil as described by Roblee et al. (1973). A rapid equilibrium of thrombin with platelets is demonstrated. The equilibrium is complex, with the estimated binding constants and number of sites dependent on thrombin concentration. With concentrations of thrombin that cause only partial stimulation, less than 15% of the thrombin is bound and both free thrombin and platelets remain reactive, consistent with the proposed equilibrium model for thrombin stimulation of platelets.

Materials and Methods

Thrombin. Human α-thrombin was prepared from Cohn fraction III paste, generously supplied by Dr. Robert M. Silverstein of Armour Pharmaceutical Company, Kankakee, Ill. Detailed methods of the purification will be described elsewhere. The procedure was essentially as outlined by Fasco and Fenton (1973), except that barium citrate adsorbed prothrombin was dissociated with a 10% molar excess of $(NH_4)_2SO_4$. In order to obtain thrombin derived solely from the activation process, prothrombin was passed through a column of Amberlite CG-50 resin (Mallinckrodt) to remove thrombin-like impurities (Fasco and Fenton, 1973). This material was then rapidly and quantitatively activated with human brain thromboplastin (Miller and Copeland, 1965). In the final step, thrombin was adsorbed onto a second column of CG-50 resin and was eluted at protein concentrations of 2–5 mg/ml in 0.75 M NaCl at approximately pH 6 (Fenton et al., 1971). These preparations were stored frozen in 0.5–2-ml aliquots at −50 °C or colder, where they retained their enzymic activities for well over a year.

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Thrombin was characterized by (i) fibrinogen clotting activity by the method of Fenton and Fasco (1974), (ii) active site titration with NphOGndBz[1] (Nutritional Biochemical Corp.) by the method of Chase and Shaw (1970), (iii) sodium dodecyl sulfate-polyacrylamide gel electrophoresis, essentially as described by Weber and Osborn (1969), and (iv) the distribution of [^{14}C]iPr$_2$P-F (New England Nuclear) labeled protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as similarly described by Mann et al. (1973). Calculations of specific activities were based on an absorption coefficient in 0.10 N NaOH of 1.68 ml mg^{-1} cm^{-1} at 280 nm and an assumed molecular weight of 36 500.

All experiments were done with thrombin preparations that had specific clotting activities greater than 2.3 U.S. clotting units/μg, that were more than 85% active by active site titration, and that electrophoresed essentially as a homogeneous protein. Major experiments were confirmed with a single high purity preparation that had a specific clotting activity of 2.6 U.S. units/μg, was 97% active by active site titration, and incorporated 96% of [^{14}C]iPr$_2$P-F into the α-thrombin electrophoretic component with 4% into the slight β-thrombin component. From these data, this preparation was calculated to be 93% active α-thrombin (active site titration times the percent of active protein that is α).

Since tyrosine contributes to the absorbance at 280 nm and iodination modifies tyrosine, protein concentrations of [^{125}I]thrombin were measured by the microbiuret procedure of Zamenhof (1957). In this paper active molar concentrations of thrombin are based on active site titrations.

Iodination of Thrombin. The following procedure for iodination of thrombin was developed empirically from the method of Morrison and Bayse (1970). It gave a reproducible product that was not detectably different from native thrombin and that was stable for at least 10 days. The high salt (0.75 M NaCl) with which thrombin is routinely stored interfered with the iodination reaction, but equilibrium dialysis against low salt (0.15 M NaCl) caused thrombin to precipitate and lose activity. Therefore, a *brief* dialysis was used in the following procedure. The iodination was carried out at room temperature in the presence of a competitive inhibitor, benzamidine, to protect the active site.

Aliquots of frozen thrombin preparations were rapidly thawed and dialyzed against 500 ml of 0.10 M NaCl, 50 mM sodium phosphate, pH 7.0, for *20 min* at 4 °C. Iodination was carried out in a 1.5-ml conical polypropylene tube in a reaction mixture that contained 5 μl of 1.0 M benzamidine hydrochloride (Aldrich), 5 μl of 100 mM EDTA (Sigma), 10 μl of 10 mM NaI (Fisher), 5 mCi (about 50 μl) of carrier-free Na^{125}I (Schwarz/Mann), 10 μl of 0.7 mg/ml lactoperoxidase (Worthington), and 150–600 μg of dialyzed thrombin. The final volume was brought to 0.50 ml with 50 mM sodium phosphate, pH 7.0, and iodination was initiated by addition of 5 μl of 5 mM H_2O_2. After 1 to 3 min, the iodinated protein was passed through a column (0.5 i.d. × 23 cm) of Sephadex G-25 (Pharmacia) equilibrated with 0.75 M NaCl–50 mM sodium phosphate (pH 7.0). The sample was eluted with this buffer, and the effluent was monitored by absorbancy at 280 nm and by radioactivity. The void volume (e.g., 6 through 8 ml) contained [^{125}I]thrombin, with benzamidine and free ^{125}I in subsequent fractions. Thrombin fractions were pooled and

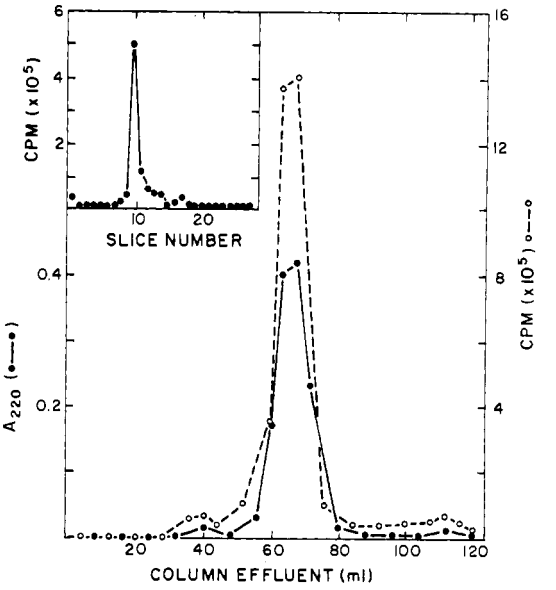

FIGURE 1: Gel chromatography and electrophoresis of[^{125}I]thrombin. A 0.5-ml mixture of 200 μg of unlabeled thrombin and 30 μg of [^{125}I]-thrombin was chromatographed on a 0.95 i.d. × 90 cm Sephadex G-100 column with 1 M NaCl–50 mM sodium phosphate, pH 7.0. The inset shows the distribution of radioactivity after electrophoresis of 9 ng of [^{125}I]thrombin on a 10% acrylamide–sodium dodecyl sulfate gel; the peak of radioactivity corresponds to the major band of Coomassie brilliant blue stained material on similar gels with 10 μg of unlabeled thrombin.

dialyzed for 45 min at 4 °C against three changes of column buffer to remove traces of free iodine. The final concentration of [^{125}I]thrombin was from 1–6 μM; the solutions were stored in 50-μl aliquots at −70 °C.

The specific radioactivity of the [^{125}I]thrombin was about 25 Ci/mmol and the mole ratio of iodine to thrombin was from 0.6 to 1. All radioactivity was precipitatable by 10% trichloroacetic acid, indicating covalent binding. The labeled thrombin was routinely compared with native thrombin by (i) gel chromatography and electrophoresis (Figure 1), (ii) fibrinogen clotting activity (see above), (iii) active site titration with NphOGndBz as described by Chase and Shaw (1970), except that absorbance was measured at 390 instead of 410 nm to avoid interference by lactoperoxidase, and (iv) platelet stimulating activity by quantitating the ability of thrombin to stimulate Ca^{2+} secretion in experiments of the type shown in Figure 5. Of the five preparations used in the experiments described here, activities per milligram of protein of iodinated thrombin as percentages of native thrombin were 100 ± 6.1 for clotting activity and 100 ± 2.9 for active site titration, and differences between native and iodinated thrombin could not be detected in platelet stimulation experiments of the type shown in Figure 5. In addition, no differences were found between native and [^{125}I]thrombin in platelet binding experiments (see Results, Figure 4).

Binding Measurements. Suspensions of washed human platelets were prepared as described by Detwiler and Feinman (1973). These suspensions contained from 2 to 5 × 10^9 platelets/ml in 136 mM NaCl, 25 mM Tris-HCl, pH 7.4, and 5 mM glucose; they were kept at 0–5 °C (ice bath) and were used within 3 h of preparation. Binding experiments were at room temperature in 0.25 ml of 136 mM NaCl, 25 mM Tris-HCl, pH 7.4, and 5 mg/ml of albumin (Sigma, essentially fatty acid free). Platelets at a final concentration of 2–5 × 10^8/ml were incubated in this buffer for 1 min for temperature equilibration before addition of thrombin. Incubation with thrombin was

[1] Abbreviations used are: NphOGdnBz, *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride; iPr$_2$P-F, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

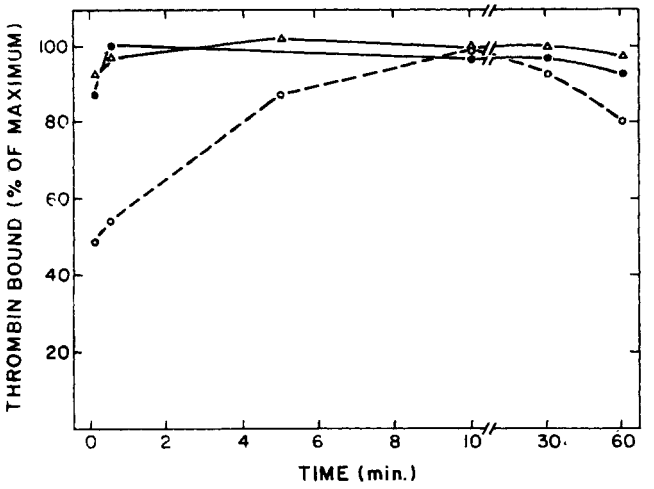

FIGURE 2: The effect of albumin on the rate of binding of thrombin to platelets. Approximately 7×10^8 platelets/ml and 30 nM [^{125}I]thrombin were mixed, and after the times indicated the platelets were centrifuged through oil for measurement of bound thrombin as described in Materials and Methods. (●—●) The suspension contained no albumin; (○- - -○) the suspension contained 5 mg/ml Sigma "twice crystallized and lyophilized" bovine serum albumin; (△—△) the suspension contained 5 mg/ml Sigma "essentially fatty acid free" bovine serum albumin. Neither albumin had any effect on the maximum amount of thrombin bound.

for 30 s unless specified. Platelets were separated from the suspending solution by centrifugation through oil by the method described by Roblee et al. (1973). The entire suspension was layered on 0.5 ml of a 2.5:1 mixture of Apiezon (specific gravity, 0.8788; J. Biddle Co.) and Dow 710 (Dow Chemical Co.) oils in a 1.5-ml conical polypropylene centrifuge tube and centrifuged at 12 000*g* for 2 min in an Eppendorf centrifuge. An aliquot of the aqueous phase (above the oil) was transferred to a counting vial, the remaining aqueous phase and oil were carefully removed, and the platelet pellet was transferred to counting vials in two 0.1-ml aliquots of incubation buffer. To estimate the amount of thrombin trapped (not bound) in the pellet, separations were done with [^{14}C]-inulin (New England Nuclear) added to the suspension. Of the added [^{14}C]inulin, 0.02% was recovered in the pellet. The maximum error due to trapping would be at high levels of thrombin, where the ratio bound/free is low. At the highest levels of thrombin used, the error due to 0.02% trapping would be less than 4%, so that corrections were not made.

Thrombin-Induced Secretion of Ca^{2+}. Stimulation of platelets by thrombin was measured by monitoring the thrombin-induced secretion of Ca^{2+} by the murexide spectrophotometric method of Detwiler and Feinman (1973). The parameters of the reaction reported in this paper are (i) the amount of Ca^{2+} released, expressed as percent of Ca^{2+} released with a saturating amount of thrombin, and (ii) t_i, the time to the inflection point of the progress–time curve (t_i is an inverse measure of the rate of thrombin stimulation). The interpretation of these parameters is described in detail by Detwiler and Feinman (1973).

Results

Rate of Binding. Initial experiments were carried out to establish the time required for maximum binding (Figure 2). In contrast to the report of Tollefsen et al. (1974) that maximum binding required 10 to 15 min, we found maximum binding within 15 s, the shortest time we could reliably measure. Other than the method of separating platelets from the incubation mixture, the major difference in our procedures was that initially we did not include albumin in our incubation solutions. When we included the same type of albumin used by Tollefsen et al. (Sigma, twice crystallized and lyophilized), maximum binding required 10 min (Figure 2). However, this albumin also greatly decreased the rate of thrombin-induced secretion of Ca^{2+}, such that concentrations of thrombin even 100-fold greater than control did not give a rate of stimulation (t_i for thrombin-induced secretion of Ca^{2+}, see Materials and Methods) approaching the control (without albumin). The same concentration of "essentially fatty acid free albumin" (Sigma) caused no delay in the rate of binding (Figure 2) or the rate of stimulation. (We have not further analyzed this intriguing observation. To attribute the difference simply to fatty acids is not warranted since there are other differences in these preparations.) In the experiments described in this paper, "essentially fatty acid free" albumin was used and 30-s incubations were selected as the shortest time that could be accurately and routinely reproduced.

TABLE I: Apparent Number of Binding Sites and Apparent Dissociation Constants Calculated from Different Ranges of Thrombin Concentrations.[a]

Range of Thrombin Concn (nM)	Sites/Plt.	K_{diss} (nM)
0.1–300	1100 ± 500	12 ± 10
0.2–1	290 ± 70	2.2 ± 0.2
1–10	1100 ± 400	10 ± 5
20–300	3300 ± 2300	120 ± 100

[a] The number of sites and K_{diss} were calculated by weighted least-squares analysis of double-reciprocal plots (as in Figure 3B) of six binding experiments in which wide ranges of thrombin concentrations were used. Calculations were made for the entire range (first row) and for segments of the entire range. Values are means ± standard deviations.

Binding as a Function of Thrombin Concentration. Thrombin bound to platelets was measured as a function of thrombin concentration, and the data were routinely plotted in three ways, shown in Figure 3. The binding data are plotted in the most direct form as bound vs. total thrombin (Figure 3A). From this and similar plots from many other experiments, the binding shows at least superficial characteristics of an equilibrium and appears to approach saturation, although complete saturation was never reached. The same data are plotted in Figure 3B in the double-reciprocal form as described by Steck and Wallach (1965); for a simple equilibrium this method will give a straight line from which the number of binding sites and an apparent dissociation constant can be calculated from the intercepts with the ordinate and abscissa. The major disadvantages of the double-reciprocal plot are that it is not suitable for the very wide range of thrombin concentrations necessary to show the complete binding characteristics and it minimizes deviations from linearity. For example, while the data in Figure 3B appear linear, it may be noted that the slope and intercepts are not the same with the higher and lower (inset) ranges of thrombin. Results of six experiments of this type are summarized in Table I, which demonstrates that the calculated number of sites and K_{diss} are highly dependent on thrombin concentration. Nonlinearity is also apparent in a plot of (bound × free^{-1}) vs. bound (Figure 3C) according to Scatchard (1949). This method also permits estimation of apparent binding constants and number of sites and it is much more sensitive to deviations from linearity. The Scatchard plot

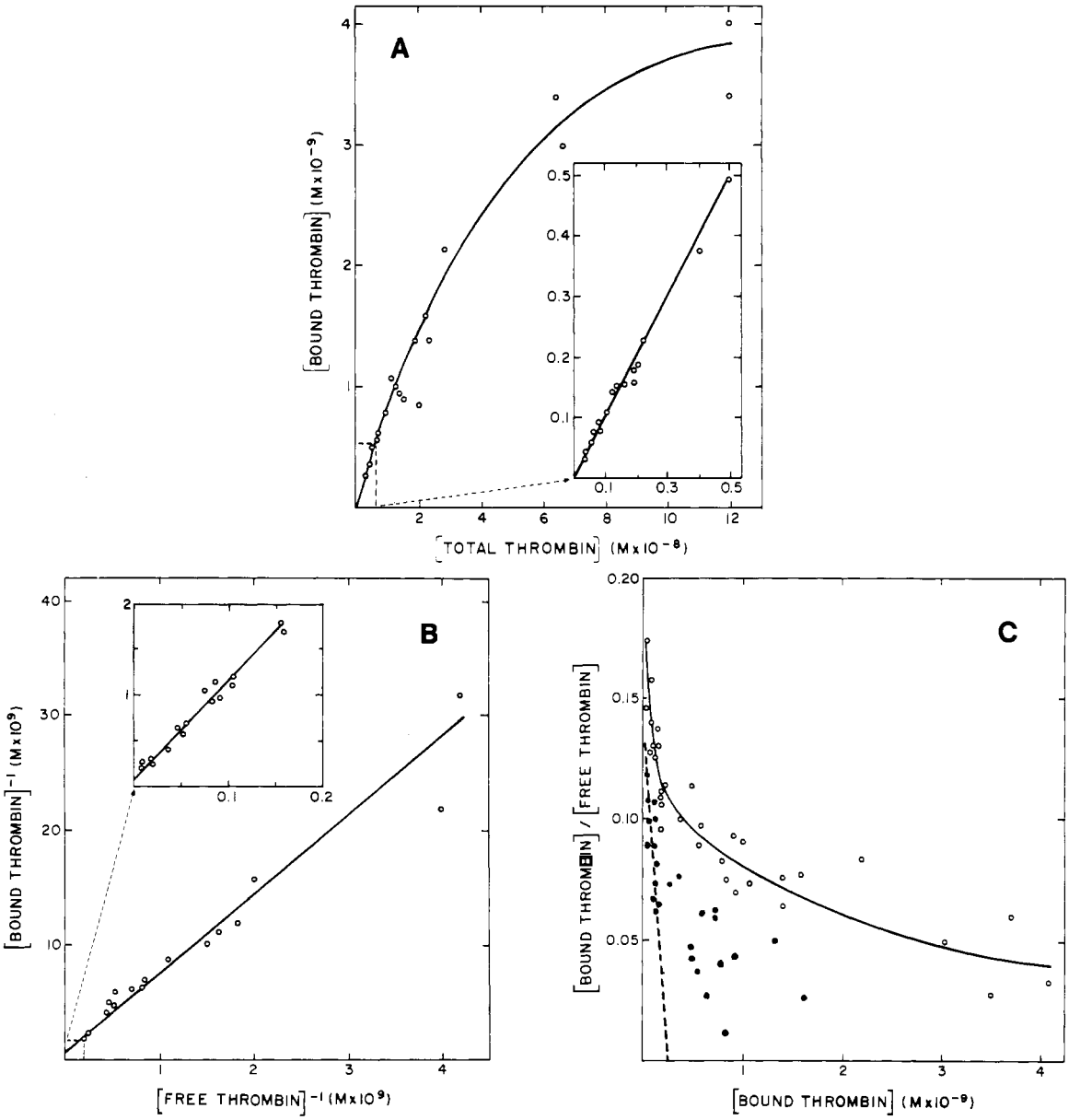

FIGURE 3: The binding of thrombin to platelets as a function of thrombin concentration. Bound and free thrombin were measured as described in Materials and Methods. The platelet concentration was 3.8×10^8 platelets/ml. The lines for the double-reciprocal plots in B were drawn using a least-squares program from Wang Laboratories, Tewksbury, Mass. Since with reciprocals the higher values have the greater error, the values were weighted according to the expression $w_i = \sigma_i^2/\sigma^2$, where w_i is the weight assigned to a datum point, σ_i is the deviation of the point from the least-squares line, and σ is the standard deviation (Guest, 1961). The line was then recalculated using the Wang program with the appropriate weights for each point. The line in the inset of B is calculated from the points in the inset only, while the other line is calculated from all points, including those in the inset. (Note: the points in the inset of B correspond to the points *not* in the inset of A.) In C, the actual data (open circles) are corrected for nonsaturable binding as described by Chamness and McGuire (1975), using 0.035 as the estimated limit of bound/free. The corrected data are shown with solid symbols.

approaches a limiting value of bound/free (0.035 in Figure 3C), indicating a component of nonsaturable binding. Correction for this nonsaturable binding gives a plot (Figure 3C, solid symbols) from which we calculate 390 high affinity sites/platelet (K_{diss} = 1.9 nM) with low affinity sites that appear to have an upper limit of about 2500/platelet (K_{diss} = 200 nM).

Labeled vs. Unlabeled Thrombin Binding. One possible cause of the pronounced deviation from a simple equilibrium is heterogeneity of thrombin resulting from iodination and reflected in altered binding properties. To test this, specific radioactivity of labeled thrombin was changed by mixing with unlabeled thrombin, and the bindings of the thrombins of different specific radioactivities were compared. Any appreciable differences in binding of labeled and unlabeled thrombin would be observed as differences in the binding curves. Figure 4 shows that there was no difference in the binding of these two thrombins. Thus, complex binding cannot be attributed to protein modification caused by labeling.

Binding vs. Stimulation. The binding of thrombin and the stimulation of secretion of Ca^{2+} under identical conditions are compared in Figure 5. Maximum stimulation was obtained with 4 nM thrombin, which represents only the very low concentrations of thrombin in experiments such as that shown in Figure 3. It is significant that, with less than maximum platelet stimulation, approximately 90% of the thrombin in the platelet suspension is unbound.

Do Platelets Modify Thrombin? Several possible expla-

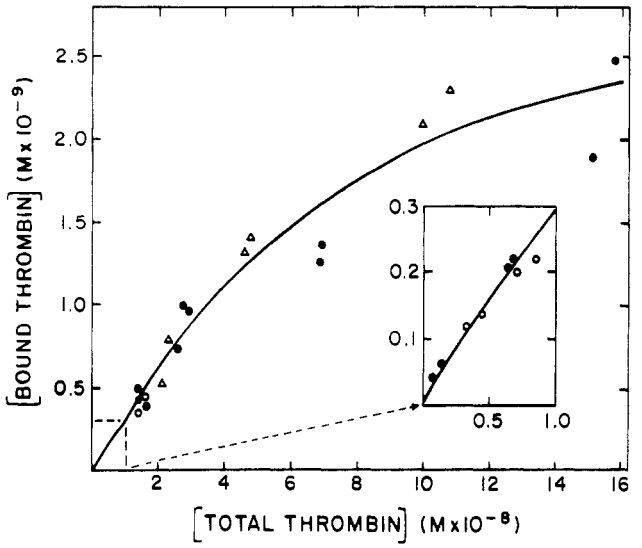

FIGURE 4: Comparison of binding of thrombin of different specific activities. [^{125}I]Thrombin was mixed with unlabeled thrombin to obtain solutions of different specific radioactivities and each thrombin solution was used to measure thrombin binding as a function of thrombin concentration. Only if labeled molecules bound exactly as unlabeled molecules would these solutions show identical binding characteristics. Binding was measured as described in Materials and Methods with 4.8×10^8 platelets/ml: (●) 36×10^{15} cpm/mol; (△) 6.1×10^{15} cpm/mol; (○) 2.9×10^{15} cpm/mol.

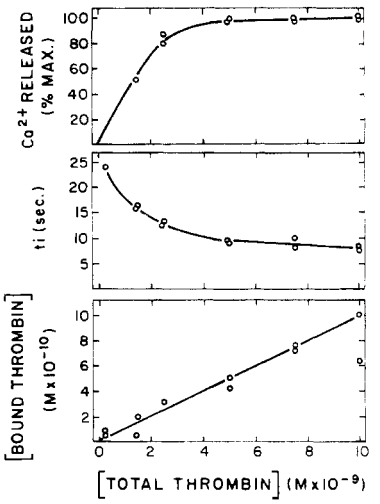

FIGURE 5: Comparison of thrombin binding and stimulation of Ca^{2+} secretion. Reactions were carried out in spectrophotometer cuvettes with Ca^{2+} release monitored by the murexide method of Detwiler and Feinman (1973). At completion of the secretion reaction (5 min), an aliquot of the reaction mixture was centrifuged over oil for measurement of bound thrombin as described in Materials and Methods. t_i, the time to the inflection of the progress-time curve, is an inverse function of the rate of stimulation. Platelet concentration was 4.2×10^8 platelets/ml.

nations for the failure of the free thrombin to react further with the platelets were considered. Had the free thrombin been modified by contact with platelets so that it was unreactive? Had the platelets released a thrombin or a platelet inhibitor? Were the platelets refractory to further stimulation? In experiments in which high concentrations of platelets ($2-7 \times 10^9$/ml) and just saturating levels of [^{125}I]thrombin (8–16 nM) were incubated for 5 min before removal of platelets by centrifugation, the supernatant thrombin and unreacted thrombin coelectrophoresed on sodium dodecyl sulfate–polyacrylamide gels and coeluted from a Sephadex G-100 column (as described in Figure 1). This rules out substantial degradation of thrombin or the presence of a complex of thrombin and another protein. A more critical test is whether the free thrombin retains activity toward platelets. This was tested by monitoring secretion of Ca^{2+} during repeated additions of platelets to a very low level of thrombin (Figure 6A). The amount of Ca^{2+} released was essentially the same for each addition of platelets, demonstrating that, in a platelet suspension in equilibrium with a concentration of thrombin that caused less than 35% of maximum stimulation, the thrombin retained its full activity toward platelets. Conversely, addition of thrombin to a platelet suspension that had reached equilibrium after partial stimulation by a low concentration of thrombin demonstrated that the platelets were still responsive to thrombin (Figure 6B).

Perturbation of Equilibria. The best test of an equilibrium is to perturb equilibrium conditions and observe reequilibration. Figure 7 shows the effect of dilution on the thrombin–platelet equilibrium. Within 30 s of dilution, the system has reached essentially the same condition as observed if these final concentrations are mixed initially; that is, the system reequilibrates as expected for a true equilibrium reaction. The system does not reach the point calculated for a simple equilibrium, presumably a reflection of the deviation from a simple equilibrium evident in Figure 3 and Table I. Reequilibration was also observed after addition of unlabeled thrombin to a suspension of platelets in equilibrium with labeled thrombin (Figure 8). The amount of labeled thrombin bound to platelets quickly reached the same point as when the unlabeled thrombin was added first.

Discussion

Evaluation of Methods. In studies of the binding of labeled proteins to cells, there are three major problems that are seldom, if ever, completely resolved. Our evaluations of these problems are discussed below.

1. Does the label accurately reflect binding of the physiologically functional protein? Studies with thrombin are complicated by the existence of more than one enzymically active form (Mann et al., 1973; Fasco and Fenton, 1975); in humans, α-thrombin is believed to be the only form with major physiological significance. There is, however, no single criterion of purity of α-thrombin, in part because it is so labile, subject to loss of activity and autolysis. Of our four criteria of purity (see Materials and Methods), specific clotting activity has no established upper limit and electrophoretic homogeneity can be misleading, depending in part on sample load. On the other hand, active site titration and distribution of [^{14}C]iPr_2P-labeled protein have defined upper limits when expressed as percentages, and together they permit a quantitative expression of active enzyme purity. In the present studies, critical experiments were with a single preparation of 93% active α-thrombin; it contained 4% active β-thrombin and 3% inactive protein.

Considerable effort was spent in establishing an iodination procedure that did not alter enzymic properties of thrombin. Our [^{125}I]thrombin was not distinguished from the unlabeled enzyme by gel filtration chromatography or sodium dodecyl sulfate–gel electrophoresis (Figure 1), by active site titration or by clotting and platelet stimulating activities. All radioactivity was protein bound (precipitated by trichloroacetic acid) and was associated with thrombin on gel chromatography and electrophoresis (Figure 1). More importantly, the experiment shown in Figure 4 indicates no appreciable difference in binding of labeled and native thrombin. This experiment was based on the fact that the *amount* bound, calculated from the

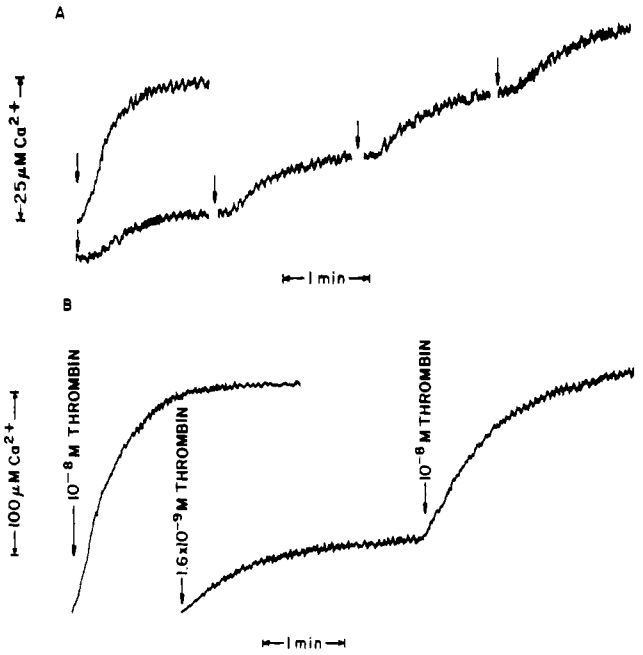

FIGURE 6: Release of Ca^{2+} after addition of platelets or thrombin to a mixture of platelets and less-than-saturating thrombin. Ca^{2+} secretion was measured as described by Detwiler and Feinman (1973). (A) Each addition of platelets, indicated by arrows, was 6×10^7 platelets/ml. The reactions were started by addition of thrombin, the upper curve was with 5 nM thrombin, which caused maximal secretion, and the lower series of curves was initiated with 0.2 nM thrombin (no additional thrombin was added through this entire series of curves). This demonstrates that reactive thrombin was available in a platelet suspension that was only partially stimulated. (B) Each reaction mixture contained 3.6×10^8 platelets/ml, with thrombin added as indicated. This demonstrates that reactive platelets were available in a mixture that was only partially stimulated. Use of lower concentrations of thrombin for the second addition suggested that the partially stimulated platelets were somewhat less responsive than control platelets, but this has proved difficult to quantitate because the shape of the curves is different with repeated additions of thrombin.

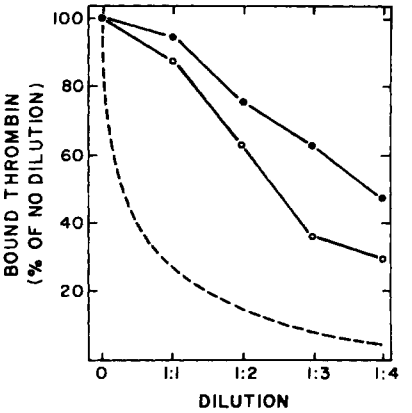

FIGURE 7: Effect of dilution on the thrombin–platelet equilibrium. Bound thrombin was measured as described in Materials and Methods. (O—O) [^{125}I]Thrombin (1 nM) was incubated with 2.4×10^8 platelets for 30 s, the suspension was then diluted with the incubation solution and, after an additional 30 s, platelets were separated for measurement of bound thrombin. (●—●) The experimental conditions were as above except that dilutions of thrombin and platelets were made *before* they were mixed. The broken line is the theoretical curve of the effect of dilution on a simple equilibrium system.

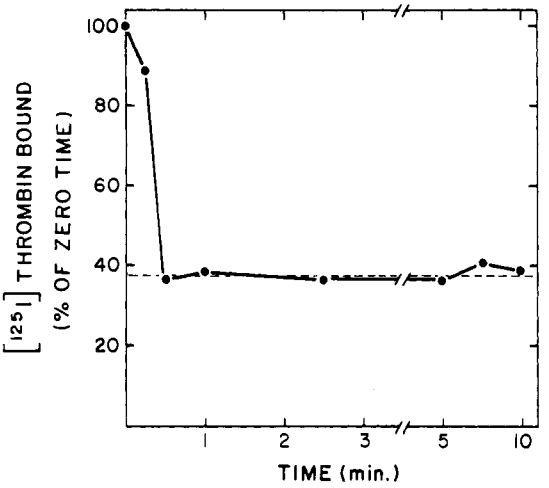

FIGURE 8: Displacement of bound [^{125}I]thrombin by an excess of unlabeled thrombin. Mixtures of 2.4×10^8 platelets/ml and 1 nM [^{125}I]-thrombin were allowed to equilibrate for 30 s before addition of 1.6 μM unlabeled thrombin. At the times indicated (after addition of unlabeled thrombin), platelets were separated from the suspension by centrifugation through oil and bound [^{125}I]thrombin was measured. The broken line shows the ^{125}I bound when the unlabeled thrombin was added prior to addition of [^{125}I]thrombin. Bound thrombin is expressed as percent of control, the amount bound in absence of the unlabeled thrombin.

radioactivity bound, would be independent of specific radioactivity only if labeled and unlabeled molecules bound identically. Tollefsen et al. (1974) and Shuman et al. (1976) used, as a similar criterion, the ability of unlabeled thrombin to inhibit (compete with) the binding of labeled thrombin. However, this would be strictly valid only in the case of a true hyperbolic binding curve, which neither they, nor we, observed.

2. Is the observed binding affected by the procedures for separation of the cells from the medium? There is no direct way of measuring bound thrombin before separating platelets from the medium so that it is not possible to establish the extent to which the separation procedure modifies the amount bound. Because free thrombin greatly exceeds bound thrombin, efficient removal of the suspending medium is essential, but for equilibrium binding washing of the cells is inappropriate, since it causes reequilibration.

The procedure we used, centrifugation of cells through oil, gives rapid and efficient separation of platelets from the medium and it allows convenient tests for such possible errors as contamination by unbound thrombin or lack of complete recovery of labeled thrombin.

3. Can "specific" binding (to a "receptor") be distinguished from "nonspecific" binding? There is no definitive way of determining whether observed binding is to specific, physiologically significant receptors. Tollefsen et al. (1974) defined "nonspecific" binding as binding of labeled thrombin in the presence of a large excess of unlabeled thrombin. It is implicit in this approach that (i) "specific" binding is saturated at such a low thrombin concentration that dilution with unlabeled thrombin competitively eliminates binding of labeled thrombin to the available sites, and (ii) "nonspecific" binding is an essentially linear function of thrombin to the highest total thrombin used, so that nonspecific binding of radioactive thrombin is independent of total thrombin concentration. While not unreasonable, there is no evidence that these conditions apply to the binding of thrombin to platelets.

One criterion of "specific" binding is that it is correlated with physiological effect. Only the binding observed at low concentrations of thrombin (0.1–4 nm) is correlated with stimulation of platelet secretion (Figure 5). There is, in fact, a striking correlation between platelet stimulation, which shows

half maximal effect at 1.5 nM thrombin and 500–600 thrombins per platelet bound for maximal effect (Figure 5), and the 300–400 high affinity sites, which have a dissociation constant of 1.8–2 nM (Table I, Figure 3C). This suggests that the high affinity sites are the specific "receptors". However, the binding at higher concentrations may be to identical receptors, with only a fraction of total sites required for maximal effect, and we cannot exclude that binding above that required for maximal secretion has an additional, yet to be identified, physiological function.

The evidence that thrombin binding to platelets is related to physiological function is (i) the binding appears to be specific to platelets (Tollefsen et al., 1974; Ganguly, 1974), (ii) prothrombin and intermediates in the conversion of prothrombin to thrombin do not stimulate platelets and do not bind (Tollefsen et al., 1975), (iii) the binding is markedly dependent on the anion composition of the medium and the ability to induce secretion shows the same dependence (Shuman and Majerus, 1975), and (iv) we have shown here that, when albumin slows the rate of binding, it also slows the rate of stimulation. On the other hand, these observations only indicate a relationship between binding and stimulation and do not show that binding is a *cause* of stimulation. In addition, Tollefsen et al. (1974) reported that active site blocked thrombin bound the same as native thrombin but did not stimulate, suggesting that binding can be distinct from stimulation.

Equilibrium Binding. Our results demonstrate that, in a mixture containing thrombin and suspended platelets, free thrombin is in equilibrium with platelet-bound thrombin. The equilibrium is established within 15 s, possibly much faster. The free thrombin is fully reactive toward platelets (Figure 6A) and the platelets retain sensitivity to thrombin (Figure 6B). Thus, the dependence of the amount of stimulation on thrombin concentration cannot be the reflection of a lack of thrombin turnover due to tight binding, as initially suggested by Detwiler and Feinman (1973), or to inhibition or degradation of thrombin, and the mechanism must involve some process that permits platelets to remain in the presence of free, active thrombin without being fully stimulated. This is in accord with the hypothesis of a reversible modification of a platelet receptor, with the degree of stimulation a function of the equilibrium concentration of modified receptor (Martin et al., 1975; Detwiler et al., 1975). While the results do not prove this or any mechanism, they clearly illustrate the need for consideration of something other than a simple catalytic reaction.

While the binding of thrombin to platelets is an equilibrium reaction, it is a complex equilibrium, as evidenced by the nonlinear double-reciprocal and Scatchard plots (Figures 3B and 3C). Some possible explanations for this complex equilibrium are: (i) different sites with different dissociation constants, (ii) sites that are progressively unmasked as available sites are occupied, (iii) negative cooperativity, such that, as some sites are occupied, dissociation constants increase, (iv) microheterogeneity of thrombin, (v) heterogeneity of platelets, and (vi) thrombin-induced morphological changes, which must be dependent on thrombin concentration. It is not possible with available information to determine which, if any, of these contribute to the nonlinear binding data.

Comparisons with Previous Binding Studies. In spite of substantially different methods, our results are remarkably similar to those of previous binding studies. Tollefsen et al. (1974) and Shuman et al. (1976) concluded that platelets had two classes of binding sites, 300–500 sites with a dissociation constant of about 0.2 nM and 30 000–50 000 sites[2] with a dissociation constant of 30–50 nM. These might be compared with our estimate of 290 high affinity sites (K_{diss} = 2 nM) and 3300 low affinity sites (K_{diss} = 120 nM). However, we believe the data do not distinguish between two classes of sites as opposed to many types of sites. From experiments of the type shown in Figure 5, we estimate that half maximal stimulation requires only about 300 thrombins bound per platelet, also in surprising agreement with similar values calculated from data of Tollefsen et al. (1974) and Shuman and Majerus (1975).

Since submission of this manuscript, two new reports of the binding of thrombin to platelets have been published. The data of Mohammed et al. (1976) suggest that the binding of thrombin to platelets is irreversible since washing (by centrifugation and resuspension) ten times with a 10-min equilibration between washes did not decrease the amount bound. However, there is an obvious inconsistency in their data. While there was irreversible binding to an unlimited number of sites, it can be calculated that only about 10% of added thrombin was bound; if binding is irreversible, all added thrombin should be bound until sites are saturated. In a paper more relevant to this discussion, Tollefsen and Majerus (1976) reported measurements of the rates of association and dissociation of [^{125}I]diisopropylphosphorylthrombin in low (0.4 nM) and high (140 nM) concentrations. The data for low concentrations, which presumably represent high affinity binding, are especially relevant here. Their experiments were done without albumin and they found, as reported here, a rapid association (Tollefsen and Majerus, 1976, Figure 4). Under conditions analogous to those in Figure 8 (a high concentration of unlabeled thrombin added after a low concentration of labeled thrombin), they found a half-time for dissociation of about 1 min, consistent with our data that suggest a half-time between 10 and 30 s. However, under conditions analogous to those in Figure 7, they found a half-time for dissociation of 140 min, whereas we found equilibration within 30 s. It should be noted that their dilution prior to filtration precludes detection of rapid dissociation, and we believe our methods, which involved a shorter incubation (30 s in contrast to 30 min) and a more direct method for perturbing the equilibrium (dilution in contrast to centrifugation and resuspension), are less subject to error.

The suggestion of Tollefsen and Majerus (1976) that their apparent slow rate of dissociation explains the dependence of the extent of stimulation on thrombin concentration overlooks the important point, emphasized in this paper, that, even with less than maximal stimulation, most thrombin is free and active. Thus the rate of dissociation is irrelevant to the apparent lack of thrombin turnover.

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[2] From kinetic studies, Detwiler and Feinman (1973) estimated 20 000 thrombins per platelet for maximal stimulation, but this was based on the invalid assumption that the observed lack of thrombin turnover was due to tight binding.

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Proteolytic Activation of Protein C from Bovine Plasma†

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ABSTRACT: Protein C is a vitamin K dependent protein present in bovine plasma (Stenflo, J. (1976), *J. Biol. Chem. 251,* 355). It is a glycoprotein (mol wt approximately 62 000) composed of a heavy chain (mol wt 41 000) and a light chain (mol wt 21 000). The heavy chain has an amino-terminal sequence of Asp-Thr-Asn-Gln and contains nearly three-fourths of the carbohydrate. The light chain has an amino-terminal sequence of Ala-Asn-Ser-Phe. Incubation of protein C with either factor X activator from Russell's viper venom or trypsin resulted in the cleavage of an Arg-Ile bond between residues 14 and 15 of the heavy chain. Concomitant with this cleavage was the formation of a serine enzyme which was inhibited by diisopropyl phosphorofluoridate. Liberation of the tetradecapeptide decreased the molecular weight of the heavy chain from about 41 000 to 39 000 and resulted in the formation of a new amino-terminal sequence of Ile-Val-Asp-Gly in the heavy chain. No change in the molecular weight of the light chain was observed during the activation reaction. These results indicate that protein C, like the four vitamin K dependent coagulation proteins, exists in plasma in a precursor form and is converted to a serine protease by hydrolysis of a specific Arg–Ile peptide bond. The biological substrate for the enzymatic form of protein C and the physiological mechanism whereby protein C is converted to a serine enzyme are not known.

It is generally accepted that four coagulation proteins (prothrombin, factor VII, factor IX, and factor X) require vitamin K for their biosynthesis. Present evidence indicates that vitamin K functions in a posttranscriptional step which results in the carboxylation of specific glutamic acid residues to form γ-carboxyglutamic acid residues in the amino-terminal region of these proteins (Shah and Suttie, 1971; Johnson et al., 1972; Stenflo et al., 1974; Magnusson et al., 1974; Nelsestuen et al., 1974). The γ-carboxyglutamic acid residues are required for the binding of calcium to these proteins and their interaction with phospholipid during the coagulation process. The four vitamin K dependent coagulation factors are glycoproteins which exist in plasma as precursors of serine proteases (Davie and Fujikawa, 1975). Prothrombin, factor VII, and factor IX are single-chain proteins, while factor X has been isolated as a two-chain molecule.[1]

Recently, Stenflo (1976) has reported the isolation and characterization of a fifth vitamin K dependent protein from bovine plasma employing an immunochemical assay. This protein, designated protein C, was isolated by a combination of barium citrate adsorption and elution, ammonium sulfate fractionation, and DEAE-Sephadex A-50 chromatography. It is composed of a light chain and a heavy chain, and these chains are held together by a disulfide bond(s). The light chain was found to bind Ca^{2+} and showed considerable homology in its amino-terminal sequence with that of the four vitamin K dependent coagulation factors. In addition, γ-carboxyglutamic acid residues were also observed in the amino-terminal region of the light chain of protein C.

The goal of the present investigation was to determine if

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[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).