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Thrombin Binds to a High-Affinity ~900 000-Dalton Site on Human Platelets†

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ABSTRACT: The functional sizes of the binding sites for thrombin on human platelets and isolated membranes have been determined by the technique of radiation inactivation: similar results were obtained. Independent studies using different radiation doses (0, 3, and 48 Mrad) and different thrombin concentrations (10^{-10}, 10^{-8}, and 10^{-6} M) confirmed the presence of three binding sites with functional sizes of 900 000, 30 000, and 4000 daltons. The binding site of lowest apparent size (4000 daltons) probably corresponds to what has been termed nonspecific binding since its dissociation constant (2900 nM) is well outside the physiological range. The site of intermediate size (30 000 daltons) is also probably not involved in platelet activation since its dissociation constant (11 nM) is also beyond the concentration range required for activation, although it may be involved in other aspects of platelet–thrombin interaction. The sites with the largest functional size are probably important in platelet function since their dissociation constant (0.3 nM) is in the range required for platelet activation. The functional size of these sites (900 000 daltons) suggests that the high-affinity site for thrombin binding to platelets may involve a multimolecular complex of membrane components.

Thrombin (α-thrombin) is one of the most potent physiological activators of platelet function and can induce platelet aggregation and secretion at concentrations below 1 nM. The mechanism of its action on platelets is not understood but appears to embody aspects both of an enzyme-catalyzed reaction and of agonist–receptor equilibrium (Martin et al., 1975).

Thrombin binds to intact platelets (Detwiler & Feinman, 1973; Tollefson et al., 1974; Ganguly, 1974) and to isolated membranes (Tam & Detwiler, 1978). The nature of these binding sites is not known: glycoprotein I (GPI; M_r 185 000) has been suggested as being a binding site on the basis of the ability of its proteolytic product glycocalicin (Okumura et al., 1978) and GPI itself (Ganguly & Gould, 1979) to inhibit the binding of thrombin to platelets. The possible role for GPI in thrombin binding and activation has been supported by the correlation between decreased GPI and the reduction of thrombin reactivity in Bernard–Soulier syndrome (Jamieson & Okumura, 1978), in myeloproliferative disorders (Bolin et al., 1977a; Ganguly et al., 1978), and in other pathological

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conditions (Bolin et al., 1977b) in which GPI is reduced. Glycoprotein V (GPV; M_r 75 000) has also been proposed as a modulator of platelet function since it is a substrate for thrombin (Berndt & Phillips, 1981a) and may also have thrombin binding properties (Berndt & Phillips, 1981b). Cross-linking of bound thrombin to the platelet surface with glutaraldehyde has suggested a molecular weight of ca. 200 000 for the high-affinity receptor ($K_d \sim 1$ nM) (Tollefson & Majerus, 1976) while the use of a photoactivatable cross-linking derivative of thrombin has also suggested M_r 200 000 for the high-affinity sites and values of 400 000 and 46 000 as possible low-affinity thrombin binding sites ($K_d \sim 30$ nM) (Larsen & Simons, 1981). Other studies have proposed binding sites of 74 000 (Chelladurai et al., 1983; Shuman et al., 1981) as well as the formation of covalent complexes of thrombin with a binding site of ca. M_r 40 000 (Bennett & Glenn, 1980).

In an attempt to resolve this problem, we have used radiation inactivation analysis to determine the functional sizes of thrombin binding sites on intact platelets and isolated platelet membranes. When high-energy radiation causes ionization in a molecule, the molecule's functional capacity is destroyed. Since the target size is proportional to the mass of the functional unit (Kempner & Schlegel, 1979), the radiation-dependent loss of binding activity is a measure of its mass: the technique is accurate to within ±15%. A major advantage of the technique is that it can measure the functional size in intact cells and membranes without a requirement for solubilization, purification, or affinity-labeling procedures. Thus, the size of the functional unit can be obtained in situ. A disadvantage of this technique is that samples must be frozen or lyophilized at the time of radiation exposure so that the results are dependent on only the direct effects of radiation. Recently, this technique has been used to determine the functional size of the glucagon receptor (Schlegel et al., 1979) and the adenylate cyclase system in liver membranes (Schlegel et al., 1979) and turkey erythrocytes (Nielsen et al., 1981) and the insulin receptor in liver membranes (Harmon et al., 1980, 1981, 1983) and the functional sizes of factors VIII:C[1] and VIII:R_{CoF} in citrated and heparinized plasmas (Harmon et al., 1982).

Using the radiation inactivation technique, we have now established the functional size of the high-affinity binding site ($K_d = 0.3$ nM) for thrombin on human platelets as having a molecular weight of 900 000. We have also established functional molecular weights of 30 000 and 4000, respectively, for moderate-affinity ($K_d = 11$ nM) and low-affinity ($K_d = 2900$ nM) binding sites by the same technique.

Materials and Methods

Preparation of Platelets and Platelet Membranes. Whole blood anticoagulated with CPD-A1 was obtained within 3 h of venipuncture from the American Red Cross Washington Regional Blood Services, Washington, DC. Platelet-rich plasma was prepared as previously described (Okumura et al., 1978) and the pH adjusted to 6.5 with citric acid. Platelets were separated from plasma by centrifugation (1400*g*, 10 min) and gently resuspended in citrate–albumin buffer (11 mM glucose, 128 mM sodium chloride, 4.26 mM monobasic sodium phosphate, 7.46 mM dibasic sodium phosphate, 4.77 mM sodium citrate, 2.35 mM citric acid, and 0.35% bovine serum albumin, pH 6.5). Contaminating red cells were removed by recentrifugation (800*g*, 4 min), and the platelets were washed 3 times with citrate–albumin buffer. Finally, the platelets were resuspended in binding buffer [136 mM sodium chloride, 25 mM Tris, and 0.6% polyethylene glycol 6000, pH 7.4] to a concentration of 1×10^9 platelets/mL.

For the preparation of a platelet membranes, 20 units of platelet concentrates was obtained within 24 h of venipuncture from Washington Regional Blood Services. The concentrates were pooled, their pH was adjusted to 6.5–6.7 with citric acid, and the platelets were removed from the plasma by centrifugation (1400*g*, 10 min). Platelet membranes were prepared by the glycerol–lysis technique (Barber & Jamieson, 1970), but the lysis buffer was modified by inclusion of 50 μg/mL leupeptin, 10 mM EGTA, and 1 mM PMSF as protease inhibitors. The membranes were collected after the first sucrose step gradient and washed once with binding buffer, then resuspended in binding buffer to a protein concentration of 1–2 mg/mL. The membranes were stored at −70 °C.

Preparation of Samples for Irradiation. Platelets were suspended at a concentration of 2×10^9/mL in binding buffer plus 50 μg/mL leupeptin, 10 mM EGTA, and 1 mM PMSF. For the irradiation, either 300 μL of platelets (1×10^9 platelets/mL) or 500 μL of platelet membranes (0.5–1 mg) was rapidly frozen in 2-mL glass ampules by immersion in liquid nitrogen. The ampules were sealed and stored at −70 °C prior to irradiation.

Irradiation Procedures. The ampules were irradiated with 13-MeV electrons from a linear accelerator (Armed Forces Radiobiology Research Institute, Bethesda, MD). The temperature of the samples during the irradiation was maintained at −135 ± 5 °C by a stream of nitrogen gas generated from a Dewar flask of liquid nitrogen.

Thrombin Binding Assay. α-Thrombin was purified by the procedure of Fenton et al. (1977) with the single modification that the α-thrombin was eluted from the Amberlite CG-50 column with a linear gradient of 0.15–0.75 M sodium chloride. The specific activity in a clotting assay was between 2700 and 3400 NIH units/mg of protein.

The α-thrombin was iodinated at 4 °C by an Iodogen (Pierce Chemical Co., Rockford, IL) procedure. The iodination reaction was performed in a 12 mm × 75 mm glass test tube that was coated with 5 μg of Iodogen and that contained 50 μL of low-salt buffer (0.1 M NaCl, 50 mM sodium phosphate, and 10 mM benzamidine, pH 7.0), 50 μg of α-thrombin, and 2 mCi of $Na^{125}I$. ^{125}I-Labeled thrombin was separated from $Na^{125}I$ on a Sephadex G-25 column with a high-salt buffer (0.75 M sodium chloride, 50 mM sodium phosphate, pH 7.0). The specific radioactivity was 24–32 μCi/μg (0.4–0.5 mol of iodine/mol of thrombin). The ^{125}I-labeled thrombin retained 100% of its clotting activity, and contained <1% impurities of lower molecular weight (probably β- or γ-thrombin) as determined by SDS–PAGE electrophoresis by the method of Laemmli (1970). The ^{125}I-labeled thrombin was diluted with an equivalent volume of 25% bovine serum albumin and stored at −70 °C. Under these conditions, the ^{125}I-labeled thrombin was stable for 3–4 weeks as judged by the maintenance of trichloroacetic acid precipitation of radioactivity and by binding activity in a radioreceptor assay. The labeled and unlabeled thrombins demonstrated equivalent affinities for the platelet thrombin receptor as measured by competition binding assays (Tandon et al., 1983).

The binding assay was performed for 15 min at room temperature in a total volume of 100 μL. The final concentration

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; VIII:C, antihemophilic factor procoagulant activity; VIII:R_{CoF}, ristocetin cofactor and the VIII complex; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Glycoproteins I, V, and IX are defined as per the recommendations of the International Committee on Thrombosis and Haemostasis (1981).

of reactants was (0.9–2) × 10^{-10} M ^{125}I-labeled thrombin, 10 mg/mL bovine serum albumin, and either 4 × 10^8 platelets/mL or 200 μg of platelet membrane protein/mL. Unlabeled thrombin was added in increasing concentrations up to 10^{-6} M to measure competitive binding with the labeled thrombin. All reagents were prepared in binding buffer. Bound thrombin was separated from free thrombin by a 10-min centrifugation in a Beckman airfuge (Beckman, Palo Alto, CA). The supernatant (free ligand) was removed by aspiration, and the tube containing the cell or membrane pellet was counted in a LKB 80000 gamma sample counter at an efficiency of 69%. Thrombin degradation was measured in the supernatant of the binding assay as the amount of radioiodine soluble in 5% trichloroacetic acid. Under these conditions, specific binding reached a steady state, and thrombin degradation during the assay was less than 4%.

Data from competition-binding experiments were subjected to computer (VAX 11/780) analysis by weighted nonlinear least-squares curve fitting, with total ligand concentration as the independent variable and by assuming a constant percentage error in the concentration of bound ligand (Munson & Rodbard, 1980). Results were displayed graphically as competition curves. Objective statistical criteria (F test, extra sum of squares principle) were used to evaluate goodness of fit and for discriminating between different models. Curves from multiple experiments were analyzed both individually and simultaneously by constrained curve fitting to obtain improved precision of parameter estimates (Munson & Rodbard, 1980). Nonspecific binding was treated as a parameter subject to error and was fit simultaneously with other parameters.

Protein determinations were made by the fluorescamine assay (Udenfriend, et al., 1972) with bovine serum albumin as the standard. Specific thrombin binding was found to be directly proportional to platelet number over the range of (1–7) × 10^8/mL and to platelet membrane protein concentrations over the range of 0.1–0.6 mg/mL. The platelet number or membrane protein concentration was maintained within this range in all the experiments described.

Calculations. The thrombin binding activity (bound/free = B/F) was calculated for each irradiated sample. The radiation inactivation curve was obtained by plotting the logarithm of the fraction of remaining binding activity, $(B/F)/(B/F)_0$, vs. the radiation dose, where $(B/F)_0$ was the binding activity of the nonirradiated sample. The functional unit is defined as the smallest component necessary to elicit the measured activity. If a single functional unit is responsible for the binding activity, then the inactivation curve will be monoexponential:

$$\frac{B/F}{(B/F)_0} = e^{-MD}$$

where M is the mass of the functional unit and D is the dose of radiation in megarads (Kempner & Schlegel, 1979). The size of the functional unit is determined from the slope of the inactivation curve: the steeper the slope, the higher the mass.

Multiexponential curves may also be obtained that suggest the presence of at least two components of differing sizes (Hutchinson & Pollard, 1961), each of which contributes to the total initial activity. To obtain the functional sizes of each component, the radiation inactivation data for total binding were subjected to weighted least-squares analysis (RS/1, BBN Research Systems, Cambridge, MA) to give the best fit to the function

$$\frac{B/F}{(B/F)_0} = Ae^{-MD} + Be^{-ND} + Ce^{-PD}$$

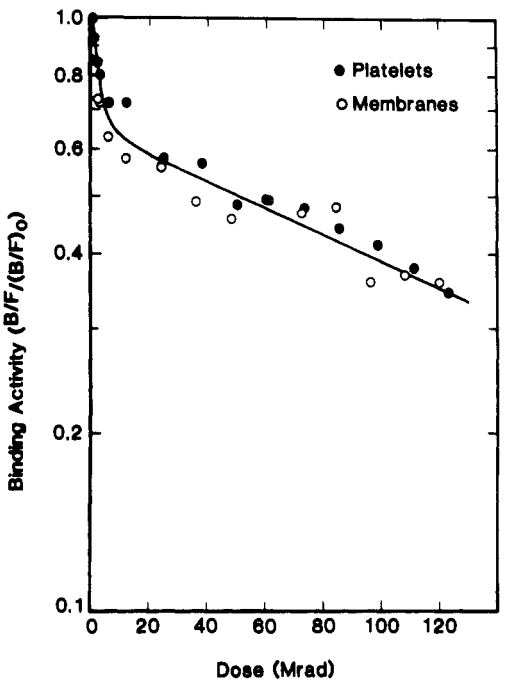

FIGURE 1: Binding of ^{125}I-labeled thrombin to platelets and platelet membranes following radiation inactivation.

where A, B, and C are the relative activity contributions (from analysis of the binding isotherms) of the high-, medium-, and low-affinity sites, respectively, and M, N, and P are the slopes of the inactivation curves for the high-, medium-, and low-affinity sites, respectively. The functional sizes can be calculated from the slopes of the individual inactivation curves by

$$M_r = (6.4 \times 10^5)(\text{slope}) \times 2.8$$

where the slope is given in megarads and 2.8 is the temperature correction factor (Schlegel et al., 1979). Computer-assisted statistical analysis was used for the binding data (LIGAND, Munson & Rodbard, 1980) and the radiation inactivation data (RS/1, BBN Research Systems, Cambridge, MA).

RESULTS

Effect of Radiation on Thrombin Binding to Platelets and Platelet Membranes. In unirradiated samples, 15–30% of total counts added were bound to platelets or platelet membranes that had been carried through all of the steps with the exception of being exposed to radiation, and 70–75% of the total binding could be competed by 10^{-6} M unlabeled thrombin. The presence of proteolytic inhibitors (see Materials and Methods) was essential to prevent total loss of binding activity on freezing and thawing of platelets. However, the presence of inhibitors was not required to maintain the thrombin-binding activity of membrane preparations that had themselves been prepared in the presence of inhibitors.

When platelets were exposed to radiation, a biphasic decrease in total thrombin binding was observed: similar results were obtained with isolated platelet membranes (Figure 1). Exposure to very low doses of radiation (0–6 Mrad) resulted in a sharp decrease of 30–40% in the amount of thrombin bound. In contrast, exposure to higher doses of radiation (6–120 Mrad) resulted in a more gradual further loss of thrombin binding activity such that approximately 65% of the initial binding activity was destroyed by exposure to 120 Mrad. These results suggested that thrombin bound to at least two components: one component that had a large functional size

Table I: Binding Constants Obtained with Platelets Exposed to Various Radiation Doses[a]

dose (Mrad)	K_1 (nM)	K_2 (nM)	K_3 (nM)	R_1[b]	R_2[b]	R_3[b]	NS[c]
0	0.30 ± 0.2	11 ± 7	2900 ± 800	53 ● 50	1700 ± 700	590000 ± 13000	0
3	0.33 ± 0.2	11 ± 5	2500 ± 500	14 ± 7	1000 ± 100	510000 ± 13000	0
48		10 ± 4	1900 ± 300		650 ± 200	330000 ± 3600	0

[a] Errors are given as ± standard deviation. [b] Sites per platelet. [c] Nonspecific binding.

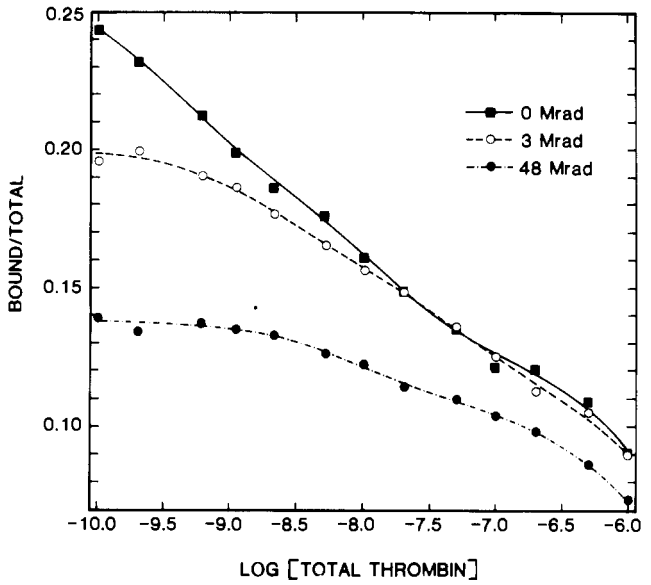

FIGURE 2: Effect of radiation on thrombin binding isotherm. Thrombin binding was measured on platelets exposed to either 0 (control), 3, or 48 Mrad. The concentration range for thrombin in the assay was from 10^{-10} to 10^{-6} M. The data points represent the mean of triplicate determinations obtained in four separate experiments in which platelets were irradiated on different days. The lines represent the best-fitting line generated by computer analysis (see text) assuming a model of three independent binding sites for data obtained after exposure to 0 or 3 Mrad and a model of two independent binding sites for data obtained after exposure to 48 Mrad. Similar results were obtained with platelet membranes.

and thus was destroyed by low levels of radiation and another component with a smaller functional size that was only partially destroyed by very high doses of radiation.

Effect of Radiation on Thrombin Binding Isotherm. Usually, the effect of radiation is to decrease the receptor number without affecting the affinity of the binding site for the ligand (Kempner & Schlegel, 1979). However, changes in affinities have also been reported (Harmon et al., 1980). Therefore, we examined the thrombin binding isotherm with platelets that had been exposed to 0, 3, or 48 Mrad. The models used to evaluate our results were (i) a one-site model with nonspecific binding, a two-site model (ii) with and (iii) without nonspecific binding, and a three-site model (iv) with and (v) without nonspecific binding. The results of these statistical analyses indicated that the best fit was obtained when a model of three independent binding sites without nonspecific binding was used to analyze the data both in unirradiated samples and in samples exposed to 3 Mrad of radiation (Figure 2): similar results favoring a three-site model have been obtained for binding data on unfrozen platelets and platelet membranes (Harmon and Jamieson, unpublished results). A comparison of the fit of this model vs. the two-site model with nonspecific binding indicated that the three-site model was strongly preferred ($p = 0.003$, F test comparison).

As can be seen, radiation caused a decrease in the number of receptor sites without having a significant effect on the affinities of the sites to bind thrombin. The highest affinity sites (K_1 = 0.3 nM) were most susceptible to radiation destruction as demonstrated by the total loss of these sites by exposure of platelets to 48 Mrad. The moderate affinity sites (K_2 = 11 nM) were less susceptible to radiation in that 40% of the sites remained after platelets had been exposed to 48 Mrad. The lowest affinity sites (K_3 = 2900 nM) are the least susceptible to radiation with 60% of the sites remaining after 48 Mrad. After exposure to this dose of radiation, it should be noted that statistical analysis of the binding data fit best with a model of two independent binding sites with no nonspecific binding (Figure 2). Thus, these results indicate that there are three thrombin binding sites on platelets that differ in their sensitivity to radiation. This radiation sensitivity indicates that the highest affinity sites have the largest functional size and that the moderate affinity sites are larger than the lowest affinity sites. The best fit parameters from these analyses are given in Table I.

Table II: Functional Sizes of Thrombin Binding Sites on Platelets and Platelet Membranes[a]

prepn	highest affinity	moderate affinity	lowest affinity
platelets (n = 3)	720000 ± 160000	13000 ± 41000	8000 ± 16000
membranes (n = 6)	990000 ± 210000	38000 ± 10000	3600 ± 2000
all (n = 9)	880000 ± 140000	30000 ± 9000	3800 ± 1800

[a] Errors are given as ± standard deviation.

Changing Thrombin Concentrations. As an independent approach to obtaining the functional sizes for these three binding sites, radiation inactivation curves were generated from binding data acquired at three different thrombin concentrations (10^{-10}, 10^{-8}, and 10^{-6} M). Thus, binding studies were conducted at these concentrations on platelets that had been exposed to increasing doses of radiation (from 0 to 120 Mrad) (Figure 3a). The difference in the binding between 10^{-10} and 10^{-8} M was operationally defined as "high-affinity" binding and the difference in binding between 10^{-8} and 10^{-6} M was operationally defined as "low-affinity" binding. As shown in Figure 3b, there is a biphasic decrease in the high-affinity binding, suggesting that at least two components of different molecular weights are contributing to the binding measured at low thrombin concentrations (10^{-10}–10^{-8} M). However, there is a linear decrease in the low-affinity binding suggesting that this binding activity is attributable to one component that is relatively insensitive to radiation; i.e., this component has a very low molecular weight. This experimental protocol gives direct evidence for three thrombin binding sites.

Determining the sizes of these three sites requires determining the difference between two large numbers, especially after the destruction of approximately 90% of the high-affinity binding activity. Therefore, to increase the precision of the molecular weight estimates for the three binding components, the radiation inactivation data for total binding (10^{-10} M thrombin) were subjected to weighted least-squares analysis as described under Materials and Methods. Attempts were made to fit the data with a one-site model, a two-site model, and a three-site model. Again, the best fit was obtained with a three-site model ($p < 0.001$ by F test comparison). The functional sizes obtained by this analysis were 900000 ±

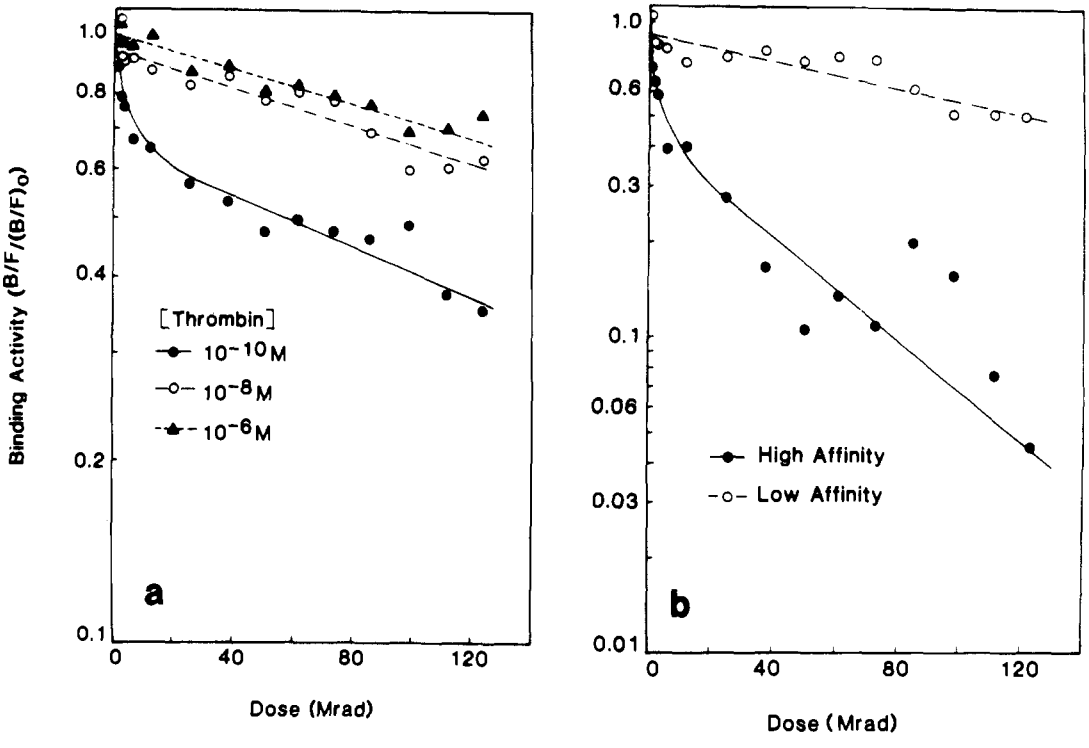

FIGURE 3: (a) Effect of radiation on binding of different concentrations of thrombin to platelets. Radiation inactivation curves were obtained with thrombin concentrations of 10^{-10}, 10^{-8}, and 10^{-6} M. Note that linear inactivation curves were obtained at 10^{-8} and 10^{-6} M thrombin while a curvilinear inactivation curve was obtained at 10^{-10} M. (b) Effect of radiation on the binding of thrombin to high-affinity and low-affinity sites. Radiation inactivation curves were generated from binding data measured over the thrombin concentration range of 10^{-10}–10^{-8} M (high affinity) or 10^{-8}–10^{-6}M (low affinity). The data obtained between 10^{-8} and 10^{-6} M thrombin generated a linear radiation inactivation curve. The data obtained between thrombin concentrations of 10^{-10} and 10^{-8} M generated a curved line that was analyzed as being composed of two components of different sizes. Similar results were obtained with platelet membranes. The data points indicate the mean of triplicate determinations obtained in two separate experiments.

140 000 (n = 9), 30 000 ± 9000 (n = 9), and 4000 ± 2000 (n = 9) (Table II). These sizes agree well with those obtained by the direct experimental approach and, together with the competition results, indicate that the highest affinity binding component (K_1 = 0.3 nM) has a functional size of 900 000, the moderate affinity binding component (K_2 = 11 nM) has a functional size of 30 000, and the lowest affinity binding component (K_3 = 2900 nM) has a functional size of 4000.

Discussion

Radiation inactivation is a very powerful technique useful in determining the functional size of any active component, even in a complex, heterogeneous system such as a membrane or whole cell. The sizes obtained by this procedure relate to the sum of the structural components required to elicit the measured activity. Therefore, if a given activity is embodied in a single polypeptide, radiation inactivation will indicate a functional size equivalent to that of the molecular weight of the peptide chain. However, if the activity requires the interactions of multiple components, then the functional size determined by radiation inactivation will be related to the sum of the molecular weights of each of the interacting peptides.

In the case of the thrombin binding sites on human platelets and platelet membranes, the radiation inactivation results indicate the presence of at least three binding sites of molecular weights 900 000, 30 000, and 4000. It should be noted that graphical analysis of previous binding studies (Detwiler & Feinman, 1973; Tollefson et al., 1974; Ganguly, 1974; Tam & Detwiler, 1978) has been interpreted as indicating two types of binding sites: about 300 binding sites of high affinity (K_d ~ 3 nM) and about 30 000 binding sites of relatively low affinity together with nonsaturable binding that can be as high as 50% of the total amount bound, depending on assay conditions. The binding studies of fresh, unfrozen platelets (Harmon and Jamieson, unpublished results) and the present combination of binding studies and target analysis all favor a three-site model.

Since exposure of platelets to radiation occurs prior to the initiation of the binding assay, the proteolytic activity of α-thrombin cannot be responsible for these results. The effect of proteolysis after irradiation has been elegantly examined with prothrombin and thrombin (Aronson & Preiss, 1962). When prothrombin (M_r 70 000) was irradiated and then proteolytically cleaved to α-thrombin (M_r 37 000) for the measurement of activity, the functional size of prothrombin was determined to be 70 000. When α-thrombin was irradiated, the functional size was determined to be 37 000. Thus, proteolytic cleavage after irradiation does not interfere with an accurate determination of a functional molecular weight because the functional size is determined at the time of irradiation.

Upon examination of the equilibrium parameters, the lowest affinity site (M_r 4000) appears to represent what would generally be considered nonspecific binding: i.e., the dissociation constant is approximately 3 μM, which is well outside the probable range of thrombin concentrations occurring under physiological conditions. However, the presence of a very low affinity site does explain the difficulty of other investigators, as well as ourselves, in being able to demonstrate complete competition of labeled α-thrombin (10^{-10} M) with 10^{-6} M unlabeled thrombin. To compete for a site with a dissociation constant of 3 μM, one should be using an α-thrombin concentration of approximately 30 μM. Under the conditions of the binding assay, e.g., ionic strength, α-thrombin is insoluble at these concentrations. The binding site with moderate af-

finity has a dissociation constant (K_d = 11 nM) that is also beyond the minimum thrombin concentration required to induce platelet activation, but it is possible that these binding sites could have other functions such as acting as a platelet antithrombin (Watanabe et al., 1977).

The site with the highest affinity has a dissociation constant (K_d = 0.3 nM) in the range of thrombin concentrations required for platelet activation. The functional size calculated for this component (900 000) is far beyond the size of any of the previously proposed receptors for thrombin binding as summarized in the introduction. This high-affinity component may represent a complex of several individual molecules whose mutual interaction is required for the effective binding of thrombin to platelets. This could be analogous to the adenylate cyclase system, which is known to be composed of at least three components: a catalytic component, a nucleotide regulatory component, and a hormone binding component. Thus, the size of the hormone binding component can be obtained either directly by a receptor assay or indirectly by the difference in size of adenylate cyclase activity stimulated by hormone and guanyl-5′-yl imidodiphosphate and adenylate cyclase activity stimulated by guanyl-5′-yl imidodiphosphate alone. The size of the β-adrenergic receptor in turkey erythrocytes is identical whether it is determined by the direct or indirect approach (Nielsen et al., 1981) while the size of the glucagon binding component in rat liver membranes is 670 000 by receptor assay and about 100 000 by the indirect approach (Schlegel et al., 1979). It has been postulated that this difference suggests that the binding component and nucleotide regulatory component form a complex that is necessary for the activation of adenylate cyclase.

The initial proposal for the molecular basis of platelet activation (Okumura et al., 1978) suggested that GPI could function as the binding component but that a second molecule was required as an effector on the basis of the dissociation of these two events in chymotrypsin-treated platelets. These results were confirmed in later studies (McGowan et al., 1983). The defects in thrombin binding and activation in Bernard–Soulier platelets have previously been attributed to their lack of GPI (Jamieson & Okumura, 1978). However, recent studies have shown that Bernard–Soulier platelets also lack GPV (M_r 75 000) and GPIX (M_r 17 000) (Berndt et al., 1983). The combined absence of these from Bernard–Soulier platelets suggests that they may exist as a complex in the membrane of normal platelets although their combined molecular weights (280 000) would be only one-third of that determined for the high-affinity receptor in the present work. Similarly, a thrombin-reactive complex of M_r 74 000 (Chelladurai et al., 1983) would not itself meet the size requirements of the high-affinity complex defined in the present study, although one of its components has a molecular weight (M_r 27 000) similar to that detected here for the moderate affinity receptor.

The highest affinity site, which is the one responsible for the binding of thrombin in the very low concentration range required for platelet activation, has the highest functional size since it is destroyed by low doses of radiation. Further studies require the isolation of this binding complex to ascertain whether the high-affinity–high-mass binding component contains within it the effector system necessary for platelet responsiveness to thrombin.

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Registry No. Thrombin, 9002-04-4.

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Inactivation of Chymotrypsin by 5-Benzyl-6-chloro-2-pyrone: ^{13}C NMR and X-ray Diffraction Analyses of the Inactivator–Enzyme Complex†

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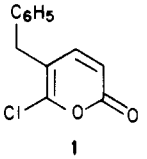
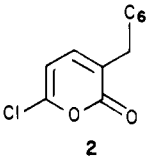
ABSTRACT: The inactivation of chymotrypsin by 5-benzyl-6-chloro-2-pyrone has been studied. Chloride analysis of the inactivated enzyme suggests that chlorine is no longer present in the complex. ^{13}C NMR spectroscopy of chymotrypsin inactivated with 5-benzyl-6-chloro-2-pyrone-*2,6*-$^{13}C_2$ shows the presence of two new resonances from the protein-bound inactivator. The chemical shift values of these resonances are consistent with an intact pyrone ring on the enzyme as well as the replacement of the C-6 chlorine by a different heteroatom. X-ray diffraction analysis at 1.5-Å resolution of the inactivator–enzyme complex demonstrates that the γ-oxygen of the active site serine residue (serine-195) is covalently attached to C-6 of the inactivator and that the pyrone ring is intact. The 5-benzyl group of the inactivator is bound to the enzyme in the hydrophobic specificity pocket. The conformational changes that occur in the protein as a result of complexation with the inactivator are discussed.

The inactivation of chymotrypsin and other serine proteases by substituted 6-chloro-2-pyrones has recently been reported (Westkaemper & Abeles, 1983). Inactivation of chymotrypsin by 5-benzyl-6-chloro-2-pyrone (**1**) and 3-benzyl-6-chloro-2-pyrone (**2**) occurs in a time-dependent manner.



1

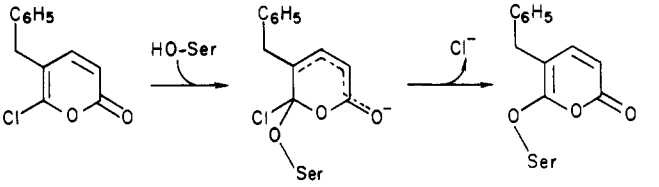


2

The benzyl group is important since the unsubstituted chloropyrones are not inactivators of chymotrypsin. The mechanism of inactivation depends on the position of the benzyl group. Scheme I shows a proposed mechanism for inactivation of chymotrypsin by **1**.

This mechanism was proposed based on the observation that the UV–vis spectrum of chymotrypsin inactivated with **1** shows a distinct absorbance at 320 nm (Westkaemper & Abeles, 1983). This long wavelength band is characteristic of an intact pyrone. It was suggested that **1** reacts with the active-site serine residue (serine-195) (Westkaemper & Abeles, 1983) although no direct evidence was presented in the earlier report.

Scheme I



In contrast, no near-visible band is seen in the spectrum of chymotrypsin inactivated with **2**, suggesting that the pyrone ring is no longer intact. Thus, the benzyl group adjacent to the carbonyl group in **2** directs the attack of the active-site serine onto the haloenol–lactone resulting in destruction of the pyrone ring.

This paper reports our structural characterization of the adduct formed between chymotrypsin and **1**. We have prepared **1** enriched with ^{13}C and have obtained the ^{13}C NMR spectrum of the inactivated enzyme complex. Crystalline chymotrypsin was inactivated with **1** and the structure of the complex determined by X-ray diffraction methods. These studies show that the pyrone ring remains intact in the complex with chymotrypsin. C-6 of **1** forms a covalent bond with the

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