Preparation and Application of a Photoreactive Thrombin Analogue: Binding to Human Platelets†

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ABSTRACT: α-Thrombin has previously been shown to bind to specific, saturable glycoproteins on the platelet surface. Modification of the thrombin active site with tosyllysyl chloromethyl ketone ($TosLysCH_2Cl$) does not alter thrombin's binding characteristics. Interaction of α-thrombin with high-affinity binding sites ($K_D = 10^{-9}$ M) initiates the platelet response which involves proteolytic hydrolysis of this glycoprotein. Although $TosLysCH_2Cl$–thrombin binds to and competes for the same sites as α-thrombin, it cannot induce platelet stimulation because it is enzymatically inactive. In this study, we describe the preparation and application of photoreactive tritium-labeled thrombin analogues. The α-thrombin derivative retains its platelet-stimulating and enzymatic activities and, upon photoactivation, covalently binds to specific platelet membrane components. When freshly washed human platelets are exposed to less than saturating doses (≤2 nM) of the thrombin derivatives in the dark and photoactivated, a single labeled complex is detected. The same experiment with greater than saturation doses (≥20 nM) of the thrombin derivative yields a similar complex as well as two additional ones. Molecular weight estimates of these thrombin-bound complexes were obtained by gel filtration and $NaDodSO_4$–polyacrylamide gel electrophoresis. The low dose (high affinity) complex with $TosLysCH_2Cl$–thrombin has an approximate molecular weight of 200 000, while that with active α-thrombin is smaller, approximately 120 000, due to enzymatic cleavage. The additional complexes detected with the high thrombin dose had estimated molecular weights of 400 000 and 46 000, respectively, and appeared to be the same for $TosLysCH_2Cl$–thrombin and for the α-thrombin coupled platelets. These isolated complexes appear to correspond to the two previously detected populations of thrombin binding sites on the platelet.

Thrombin has long been known to stimulate platelets and to induce the platelet reaction which leads, eventually, to secretion of granule contents and to platelet aggregation (White et al., 1977). The specific mechanism of the induction, although not yet completely elucidated, requires the binding of thrombin to specific platelet membrane receptors[1] (Detwiler & Feinman, 1973a,b; Tollefsen et al., 1974; Ganguly, 1974; Martin et al., 1975, 1976; Ganguly & Sonnichsen, 1976; Tollefsen & Majerus, 1976; Workman et al., 1977; Seegers, 1977; Tam & Detwiler, 1978), binding which is unimpaired by diisopropyl fluorophosphate (iPr_2P-F) (Ganguly, 1974; Seegers, 1977) or tosyllysyl chloromethyl ketone ($TosLysCH_2Cl$)[2] (Ganguly & Sonnichsen, 1976) blockage of the thrombin active site. Stimulation of the platelet requires active thrombin and involves proteolysis of the receptor, as suggested by the disappearance of a specific band in the acrylamide gel electrophoresis pattern of the membrane proteins of activated platelets (Phillips & Agin, 1973, 1974, 1977; Mosher et al., 1979). These studies have demonstrated that platelets possess a low number (500–1000) of very high affinity ($K_D = 10^{-9}$ M) thrombin receptors and a larger number (50 000–75 000) of sites with 100-fold lower affinity ($K_D = 10^{-7}$ M) (Tollefsen et al., 1974; Workman et al., 1977). An alternative interpretation of these data, namely, a single site exhibiting negative cooperativity, has been suggested (Tollefsen & Majerus, 1976).

A model incorporating the known facts about thrombin stimulation has been proposed (Detwiler & Feinman, 1973a,b; Martin et al., 1975) which combines the enzyme–substrate characteristics of the thrombin–platelet interaction with the agonist–receptor behavior of the thrombin binding. According to this model, thrombin binds to the high-affinity receptor, forming a thrombin–receptor complex. If active, the thrombin enzymatically modifies the receptor to yield an activated thrombin–receptor complex which leads to platelet stimulation (Detwiler & Feinman, 1973a; Detwiler & Wasiewski, 1977; Martin et al., 1975, 1976). Neither complex has yet been isolated.

We report here the selective labeling of a protein by means of a photoreactive thrombin derivative whose biological properties (proteolysis, esterolysis, and platelet stimulation) are not greatly altered by the derivatization. The application of photoreactive probes to the study of biochemical processes has recently been reviewed (Chowdhry & Westheimer, 1979). They have been used to investigate acetylcholine (Frank & Schwyzer, 1970; Kiefer et al., 1970; Waser et al., 1970) and hormone (Gorski & Gannon, 1976; Yamamuto & Alberts, 1976; Gospodarowicz & Moran, 1976; Reichlin et al., 1976) receptors as well as to demonstrate the existence of a membrane ADP binding protein (Bennett et al., 1978). We have chosen to derivatize thrombin by means of a recently synthesized (Huang & Richards, 1977) aryl azide, DNCO [*N*,*N*′-bis(2-nitro-4-azidophenyl)cystamine *S*,*S*-dioxide], which contains a cleavable S–S bond.

Short incubation of the biologically active thrombin derivative DNCO–α-thrombin or of the active site blocked DNCO–$TosLysCH_2Cl$–thrombin with washed platelets, fol-

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[1] The literature has referred to the platelet's highly specific thrombin binding sites as receptors.

[2] Abbreviations used: $TosLysCH_2Cl$, N^{α}-*p*-tosyl-L-lysyl chloromethyl ketone; TosArgOMe, N^{α}-tosyl-L-arginine methyl ester; $NaDodSO_4$, sodium dodecyl sulfate; Bis, *N*,*N*′-methylenebis(acrylamide); TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; F-NAP, 4-fluoro-3-nitrophenyl azide; DTT, dithiothreitol; DNCO, *N*,*N*′-bis(2-nitro-4-azidophenyl)cystamine *S*,*S*-dioxide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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lowed by photoactivation, leads to the formation of a thrombin–protein complex. With very low thrombin concentrations, the high-affinity binding site is the only one covalently coupled to thrombin while two additional proteins are also labeled at much higher thrombin concentrations, confirming previous reports (Tollefsen et al., 1974; Workman et al., 1977) that separate and different high- and low-affinity binding sites exist.

Experimental Procedures

Reagents. N^{α}-*p*-Tosyl-L-lysyl chloromethyl ketone (TosLysCH_2Cl), N^{α}-tosyl-L-arginine methyl ester (TosArgOMe, Sigma), sodium dodecyl sulfate (Na$DodSO_4$), acrylamide, and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Fisher, dithiothreitol (DTT) was from Eastman, Sepharose 2B, SP-Sephadex C-50, and Sephacryl S-200 were from Pharmacia, 4-fluoro-3-nitrophenyl azide (F-NAP) was from Pierce, sodium borotritide (50 Ci/mmol, ~500 mCi/mg) was from Amersham, Protosol and Econofluor were from New England Nuclear, Ultrafluor and Soluscint-O liquid scintillation cocktail were from National Diagnostics, and fibrinogen was from Kabi. All other chemicals were reagent grade.

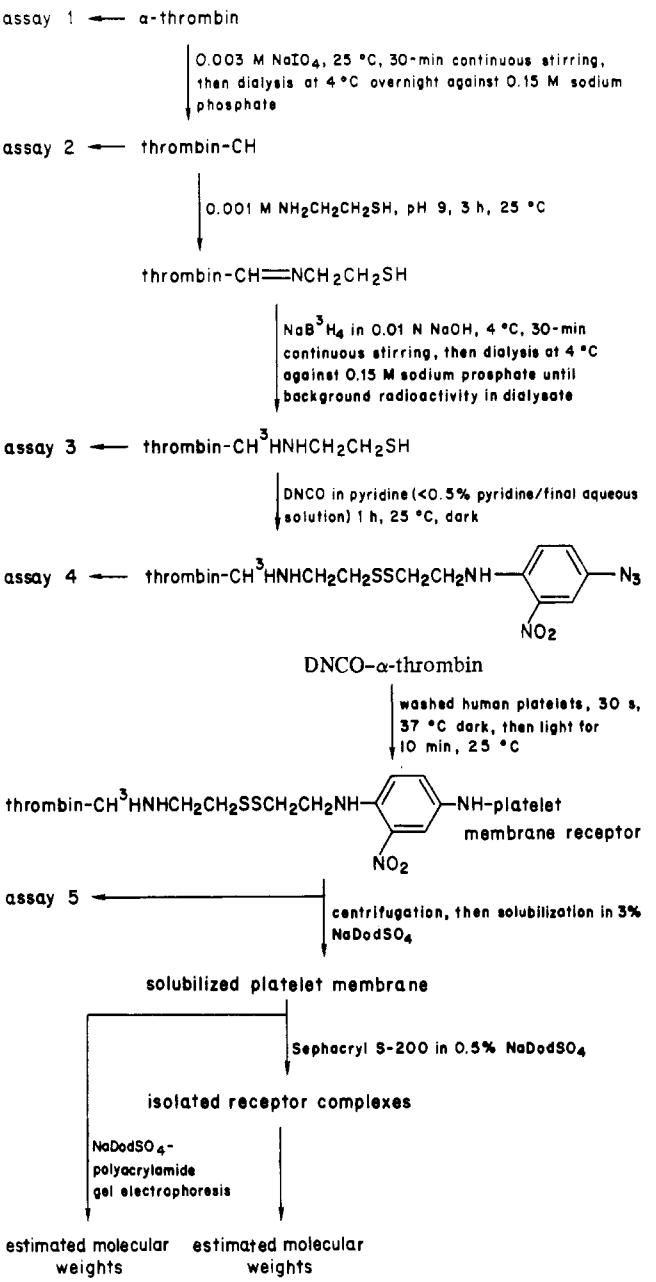
Thrombin Purification. Parke-Davis topical thrombin was purified according to Lundblad et al. (1975) by ion-exchange chromatography on SP-Sephadex C-50 and affinity chromatography through a Sepharose 2B–lysine column to remove contaminating plasminogen. Fibrinogen clotting activity was determined by measuring the amount of time it took 0.1 mL of thrombin to clot 0.2 mL of fibrinogen (5 mg/mL of 0.15 M NaCl) at 37 °C, 1 U/mL being defined as the concentration (181 nM) which is capable of clotting 1 mg/mL fibrinogen in 0.25 min (Lundblad, 1971). The ability to hydrolyze the small ester, TosArgOMe, was determined according to Hummel (1959). Protein concentrations were determined by the method of Lowry et al. (1951), using human serum albumin for the standard curve.

Active-Site Blocking: TosLysCH$_2$Cl–Thrombin. As described by Workman et al. (1977), purified α-thrombin, pH 8.0, was reacted with 10 mg/mL TosLysCH_2Cl for 2 h at room temperature and then overnight at 4 °C with constant stirring. TosLysCH_2Cl–thrombin was then dialyzed against 4 L of 0.15 M sodium phosphate buffer, pH 6.5, for 24 h. The modified enzyme was tested for activity by both the TosArgOMe esterase and the fibrinogen clotting assays, and none could be detected.

DNCO Synthesis. The photolabel DNCO, *N,N′*-bis(2-nitro-4-azidophenyl)cystamine *S,S*-dioxide, was synthesized according to Huang & Richards (1977), using 4-fluoro-3-nitrophenyl azide (F-NAP) as the starting material. All handling of the azidophenyl compounds was in the dark or under a red safe light. DNCO was recrystallized from warm pyridine as red needles: observed mp 124–125 °C, reported mp 127–128 °C; λ_{max} 459 nm. DNCO was stored in crystal form at 4 °C in the dark. For thrombin derivatization, a stock solution of 5–15 mg/mL DNCO in pyridine was used, with the final pyridine concentration in the thrombin sample less than 0.5% (v/v). A portion of the DNCO used in these experiments was the generous gift of Dr. Frederic Richards. All reactions with DNCO are carried on in the dark or with weak red photolights.

Thrombin Derivatization. The thrombin analogue was prepared by derivatizing α-thrombin via the carbohydrate residues on its B chain. As reported earlier (Hageman et al., 1975) and confirmed in this study, these carbohydrate residues are not involved in any of the three above-mentioned activities of thrombin. For detection of the thrombin and, hence, the thrombin–membrane protein complex, the photoreactive derivative had to contain a tritium label of high specific activity. In order to couple DNCO to thrombin, a free SH group had to be available. Both of these requirements were met by incorporating β-mercaptoethylamine in the derivative via Schiff's base formation with oxidized carbohydrate residues followed by reduction with sodium borotritide (Scheme I).

Scheme I: Schematic Diagram of Typical Thrombin Derivatization



For preparation of the thrombin derivative, 60 U/mL purified α-thrombin was oxidized with 0.1 mL of 0.02 M $NaIO_4$ per mL under continuous stirring at room temperature for 30 min. Excess periodate was removed by overnight dialysis at 4 °C against 0.15 M sodium phosphate, pH 6.5. Full conversion of carbohydrates to aldehydes was verified by means of the *N*-methylbenzothiazolone hydrazone (MBTH) assay (Paz et al., 1965). Fibrinogen clotting, TosArgOMe hydrolysis, and platelet stimulation assays showed that the oxidized α-thrombin had retained its biological activity (Table I).

The oxidized α-thrombin, ~60 U/mL of sodium phosphate (pH 6.5), was then reacted with 10 μL of 0.1 M β-mercap-

Table I: Representative Activities of Thrombin Derivatives

assay[a]	derivative	rate of TosArgOMe hydrolysis (U/mg of protein)	fibrinogen clotting time (s)	platelet membrane potential change (relative change in fluorescence, %)	platelet aggregation
1	α-thrombin	24.4	4	25	+
2	$NaIO_4$-treated thrombin	24.4	4	25	+
3	$NH_2CH_2CH_2SH$ and NaB^3H_4		7-8		+
4	DNCO-α-thrombin	[b]		20	+
5	α-thrombin derivative coupled platelets; washed and treated with additional α-thrombin	[b]		none[c]	
				20[d]	

[a] Point at which assay aliquot has been taken is shown on Scheme I. [b] Cannot be measured due to DNCO absorbance. [c] When saturating dose had been used for complex formation. [d] When 80% saturation dose had been used for complex formation.

toethylamine per mL of thrombin solution at pH 9.0 for 3–4 h to form the Schiff base. A 5-fold molar excess of sodium borotritide (50 Ci/mmol), 1×10^{-4} M in cold 0.01 M NaOH, was then added and the reduction allowed to proceed in an ice bath with constant stirring for 30 min. Extensive dialysis at 4 °C against 0.15 M sodium phosphate, pH 6.5, followed until only low background counts remained. Tritiated α-thrombin was tested for fibrinogen clotting and TosArgOMe esterase activity and counted to determine specific activity (2.32×10^7 cpm/mg). It could be stored at −20 °C without detectable loss of enzymatic activity (Table I).

Thrombin–Platelet Coupling. Immediately before use, 20 mL of a 60 U/mL solution of the tritiated mercaptoethylamine modified α- or $TosLysCH_2Cl$–α-thrombin was reacted with 0.100 mL of DNCO (10 mg/mL of pyridine) for 1 h at room temperature. The resulting DNCO–α-thrombin was tested for fibrinogen clotting, TosArgOMe hydrolysis, and platelet stimulating activity. Since $TosLysCH_2Cl$–thrombin competes with thrombin for the platelet binding sites (Larsen et al., 1979) but cannot activate the platelet, this competition was used as an assay to ensure identity of binding characteristics of DNCO–$TosLysCH_2Cl$–α-thrombin to those of $TosLysCH_2Cl_2$–α-thrombin.

Fresh human platelets, prepared and washed on Sepharose 2B as previously described (Larsen et al., 1979; Horne & Simons, 1978), were used at a concentration of 2×10^8/mL of modified Tyrodes. For example, 200 mL (4×10^{10} platelets was reacted with the desired quantity of DNCO–α-thrombin or of DNCO–$TosLysCH_2Cl$–thrombin (0.01 U/mL final concentration) in the dark and incubated for 30 or 60 s, respectively. The platelets were then poured into shallow Petri dishes and exposed to light for 10 min. In a control experiment performed in the absence of platelets, DNCO–α-thrombin exhibited no loss in biological activity as a result of this coupling procedure.

The photoactivated platelet–thrombin mixture was then centrifuged and washed repeatedly with 0.02 M Tris-HCl, 0.15 M NaCl, and 0.001 M Na_2EDTA, pH 7.5. The pellet was solubilized in 3% $NaDodSO_4$ at 100 °C (boiling H_2O) for 10 min to inhibit proteolysis. We have compared this solubilized total platelet pellet with that obtained by solubilization of platelet membranes, isolated according to Barber & Jamieson (1970) after photocoupling. There was no detectable difference in the thrombin–protein complex (as detected by column chromatography or electrophoresis; cf. below), but a smaller yield. In either case, the solubilized material was used immediately or stored at −85 °C under nitrogen.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the method of Weber & Osborn (1969). Gels were scanned at 550 nm for protein and 560 nm for carbohydrate. For detection of radioactive bands, the gels were sliced into 1.0-mm sections and incubated overnight at 37 °C in 7 mL of 3% Protosol in Econofluor or Soluscint-O. The gels were then counted for tritium on a Packard liquid scintillation counter for 5 min per vial.

Gel Filtration of Complexes between Thrombin and Platelet Membrane Proteins. A total of 2×10^{10} solubilized platelet pellets were counted, concentrated, mixed with blue dextran (1 mg/mL), and applied to a Sephacryl S-200 column (65 × 2.5 cm) (void volume 95 mL). The column was preequilibrated with 0.04 M sodium phosphate–0.0 M NaCl–0.002 M Na_2EDTA–0.5% $NaDodSO_4$–0.002% sodium azide, pH 7.2, and eluted with the same buffer at room temperature at approximately 25 mL/h. The fractions were collected with a Gilson microfractionator. Radioactivity was detected by counting 50 μL of each fraction in 5 mL of Ultrafluor for 1 min per vial (80–90% recovery). Protein can be detected by the absorbance at 280 nm, but A_{280} of the radioactive peak(s) was too low to be detectable until the corresponding eluate fractions were concentrated. The protein content of these concentrates was estimated by their extinction coefficient ($\epsilon^{1\%}_{280\ nm} = 10$) (Ganguly & Gould, 1979) and their radioactivity (2.32×10^4 cpm/μg of thrombin).

Results

DNCO–Thrombin. In order to utilize a photoactivatable derivative of a stimulus for reaction with and eventual coupling to its specific membrane binding protein or receptor, it must retain the biological activity (in this case, the fibrinolytic, esterolytic, and platelet-stimulating activities) and specificity of the unmodified stimulus. The following experiments show that DNCO–α-[^{3}H]thrombin fulfilled these criteria.

The analogue was assayed at each step of the derivatization described in Table I and found to have retained the fibrinogen clotting time, TosArgOMe hydrolysis kinetics, platelet aggregation, and platelet stimulation characteristics (all described under Experimental Procedures) of the parent compound to an extent of at least 75% (Table I).

The fully labeled derivative was photoactivated in the presence of a large excess of cystamine dihydrochloride which, in this case, acts as a scavenger for the highly reactive nitrene generated upon exposure to light. This thrombin derivative, no longer photoreactive, was shown to possess fibrinolytic, esterolytic, and platelet-stimulating activity. Therefore, photoactivation of the thrombin derivatives apparently does not perturb normal activities.

For verification that specificity is retained, i.e., that platelet binding occurs at the same site with DNCO–[^{3}H]-$TosLysCH_2Cl$–α-thrombin as with the unlabeled molecules,

the platelet coupling experiment was conducted separately with 0.01 and 0.05 U/mL DNCO-[^{3}H]TosLysCH$_2$Cl derivative, previously shown to lead to approximately 80% and 100% stimulation (Larsen et al., 1979). If specificity of binding to the platelet receptor has been retained by the thrombin derivative, platelets exposed and coupled to less than saturating doses of TosLysCH$_2$Cl–DNCO-α-thrombin or DNCO-α-thrombin should exhibit residual sensitivity to α-thrombin stimulation, while platelets coupled to saturating doses should exhibit no residual stimulatability. Thus platelets were reacted with each of the DNCO derivatives (0.1 or 0.05 U/mL, respectively) as described above and photoactivated. They were then passed through a Sepharose 2B column to remove excess derivative and tested for residual platelet stimulatability by the membrane potential change assay. Those photoreacted with 0.01 U/mL showed approximately 20% residual stimulatability with added saturating thrombin, while those photoreacted with the saturating 0.05 U/mL were unresponsive to further stimulation. These observations held whether the DNCO compound was the derivative of active thrombin, which can stimulate platelets, or of inactive TosLysCH$_2$Cl-α-thrombin, which cannot. The lack of residual platelet activatability, therefore, cannot be attributed to the formation of thrombin-refractory platelets since TLCK thrombin cannot lead to such refractoriness. These controls indicate that DNCO derivatization did not affect the platelet binding specificity of thrombin. The slight decrease in thrombin activity (Table I) did not lead to erroneous binding.

Covalent Coupling of DNCO Derivatives to Platelets. The affinities of both types of platelet thrombin receptors, K_D = 10^{-9} and 10^{-7}, respectively (Tollefsen et al., 1974; Workman et al., 1977), are sufficiently high to satisfy the literature criteria for utilization of photoaffinity labels, K_D = 10^{-5} (Chowdhry & Westheimer, 1979); i.e., the binding to the receptors should be so strongly favored that nonproductive binding is insignificant. In order to couple primarily to the high-affinity sites (K_D = 10^{-9}), platelets were exposed to 0.01 U/mL thrombin derivative, a less than saturating dose. Since the time course of platelet stimulation by thrombin shows it to be complete within 1 min (Larsen et al., 1979; Horne & Simons, 1978), platelets and the derivative were incubated in the dark for 30 s for DNCO-α-[^{3}H]thrombin or 60 s for DNCO-[^{3}H]TosLysCH$_2$Cl-α-thrombin. When identification of the low-affinity sites was desired, the same experiments were performed at several times the saturation dose, 0.125–0.25 U/mL derivative (Scheme I). After the incubation, photoreaction was initiated by exposure to light for approximately 10 min. The platelets, or the platelet membrane preparations, were then isolated by centrifugation, washed, and solubilized in 3% sodium dodecyl sulfate at 100 °C, as described under Experimental Procedures. Total thrombin coupling, as calculated from the radioactivity in the solubilized membranes divided by that in the DNCO-[^{3}H]thrombin derivative, was approximately 4%. An aliquot of this solubilized material was retained for electrophoresis while the remainder was chromatographed by gel filtration.

Identification of Thrombin–Receptor Complexes. The solubilized pellets described above were subjected to gel filtration on a Sephacryl S-200 column equilibrated and developed with a phosphate–NaDodSO$_4$–EDTA buffer (cf. Experimental Procedures).

When a low concentration of the thrombin derivative had been used, only one of the eluted peaks was radioactive (Figure 1), suggesting a single thrombin–membrane protein complex was formed upon exposure to either thrombin analogue.

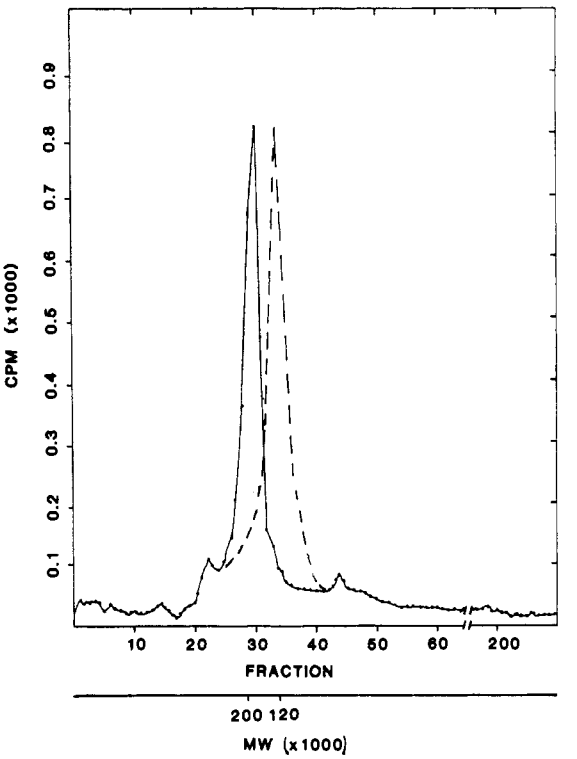


FIGURE 1: Gel filtration of high-affinity platelet–thrombin binding protein complex. Radioactivity of Sephacryl S-200 column eluate in 0.04 M sodium phosphate, 0.1 M NaCl, 0.002 M EDTA, and 0.5% NaDodSO$_4$, pH 7.2, of solubilized platetlets after photoactivated covalent coupling to a low (0.01 U/mL) concentration of DNCO-[^{3}H]TosLysCH$_2$Cl-α-thrombin (—); corresponding peak for DNCO-α-[^{3}H]thrombin (---). See Experimental Procedures for exact procedure.

Detection of this complex was made possible by the high specific activity of the thrombin derivatives since its absorbance at 280 nm is too low: the maximal total amount of thrombin complex from 2 × 10^{10} platelets was 8 μg, as calculated from the specific activity, which in 8 mL of eluate would exhibit $A_{280} \simeq 0.001$ ($\epsilon^{1\%}_{280\,nm}$ 10; Ganguly & Gould, 1979). Rechromatography of the single peak on a separate Sephacryl column again yielded a single radioactive peak, in each case.

To label the corresponding low-affinity (K_D = 10^{-7}) thrombin binding protein complexes, the same procedure was used, with the exception that 0.250 U/mL DNCO-[^{3}H]-TosLysCH$_2$Cl-thrombin or 0.125 U/mL DNCO-α-[^{3}H]-thrombin was used. In each case, three radioactive peaks were isolated (Figure 2). The one of intermediate size was identical in mobility with the previously described high-affinity complex with DNCO-TosLysCH$_2$Cl-α-thrombin or with DNCO-α-thrombin, respectively. The other two peaks, one very large and the other very small, were identical whether the complex contained TosLysCH$_2$Cl or α-thrombin and thus were not cleaved by active enzyme.

The eluates containing each of the complexes described above were concentrated and subjected to gel electrophoresis (Weber & Osborn, 1969). Each complex gave rise to a single band whose molecular weight was estimated by using erythrocyte and platelet membranes, solubilized in 3% sodium dodecyl sulfate, as mobility standards. Each complex gave rise to a single band, as detected by ^{3}H activity, by Coomassie blue and by periodic acid–Schiff (PAS) stain. The intact high-affinity site complexed with DNCO-TosLysCH$_2$Cl-α-thrombin is a glycoprotein with an approximate molecular weight of 200 000 while the cleaved high-affinity protein complex with DNCO-α-thrombin is a protein (PAS negative)

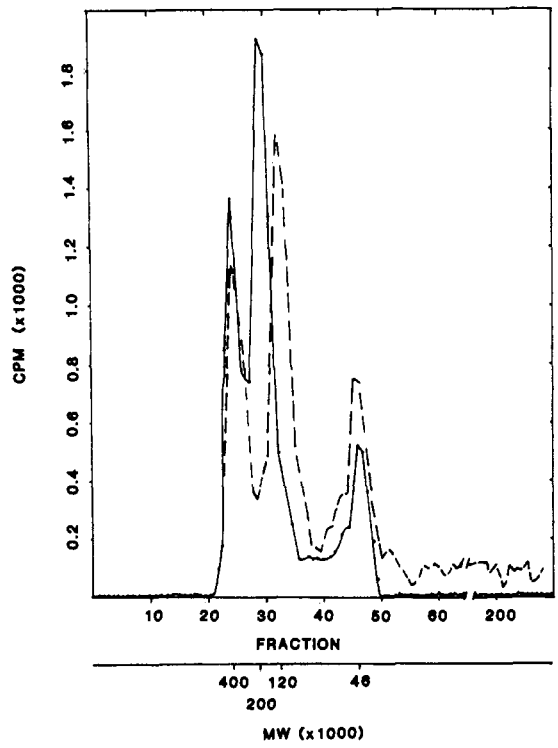


FIGURE 2: Gel filtration of high- and low-affinity platelet–thrombin binding protein complexes. Radioactivity of Sephacryl S-200 column eluate in 0.004 M sodium phosphate, 0.1 M NaCl, 0.002 M EDTA, and 0.5% $NaDodSO_4$, pH 7.2, of solubilized platelets after photoactivated covalent coupling to a high concentration of DNCO–[^{3}H]$TosLysCH_2Cl$–α-thrombin (—) or of DNCO–α-[^{3}H]-thrombin (---). See Experimental Procedures for exact procedure.

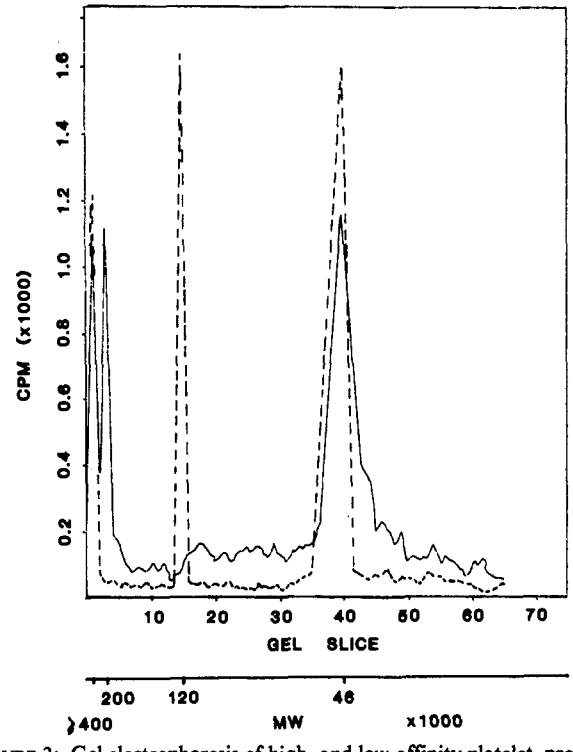


FIGURE 3: Gel electrophoresis of high- and low-affinity platelet–protein thrombin complexes. Radioactivity of sodium dodecyl sulfate–polyacrylamide gel electrophoresis slices from electrophoresis of solubilized platelets after photoactivated covalent coupling to a high concentration of DNCO–[^{3}H]$TosLysCH_2Cl$–α-thrombin (—) or of DNCO–α-[^{3}H]thrombin (---). Molecular weights estimated from calibration with erythrocyte and with platelet membrane components.

with a molecular weight of 120000 (Figure 3). The low-affinity entities complexed with either DNCO–$TosLysCH_2Cl$–α-thrombin or DNCO–α-thrombin are glycoproteins whose approximate molecular weights are $\simeq$400000 and 46000, respectively (Figure 3).

Discussion

It has previously been shown by binding studies that platelets possess at least two distinct populations of thrombin receptors, binding sites of high and low affinity ($K_D = 10^{-9}$ and 10^{-7}), respectively (Tollefsen et al., 1974; Workman et al., 1977; Tam et al., 1980). It has heretofore not been possible to couple the thrombin to its receptor, and identification has hence been indirect. Since change of the receptor by thrombin is involved in platelet stimulation (Detwiler & Feinman, 1973a,b; Martin et al., 1975), the receptor function has been attributed to specific membrane glycoproteins whose $NaDodSO_4$–polyacrylamide gel electrophoresis bands disappear or appear to shift to lower molecular weights upon platelet stimulation by thrombin (Phillips & Agin, 1973, 1974, 1977; Lawler et al., 1977; Mosher et al., 1979). Alternatively, specific glycoproteins have been implicated by competitive binding studies (Okumura & Jamieson, 1976a; Okumura et al., 1978; Ganguly & Gould, 1979), although there remains some disagreement on this identification (Tam et al., 1980). It has seemed to us that direct covalent coupling of thrombin, at low concentration, to a high-affinity binding site on the intact functional platelet would be a logical first step in the identification of the platelet thrombin receptor. We report here that such coupling has now been achieved.

The derivatization of thrombin to yield a photoreactive radiolabeled product retaining the biological activity and specificity of the parent compound has made it possible to isolate the specific coupled complex. By coupling platelets with less than saturating doses of thrombin, we have been able to show residual stimulatability of the platelets compatible with the amount of thrombin required to achieve saturation. These findings extend and corroborate previous reports that thrombin binding to the high-affinity receptors is responsible for platelet stimulation (Detwiler & Feinman, 1973a,b; Tollefsen et al., 1976; Martin et al., 1975; Ganguly & Sonnichsen, 1976; Workman et al., 1977). They also allow us to conclude that covalent binding of DNCO–thrombin or of DNCO–$TosLysCH_2Cl$–thrombin to a certain percentage of the high-affinity binding sites precludes further activation of the same percentage by additional thrombin. Because the affinity constant is so high ($K_D = 10^{-9}$), a similar conclusion was reached when $TosLysCH_2Cl$–thrombin was bound noncovalently (Workman et al., 1977). It should be noted that the thrombin concentrations used for these high-affinity binding studies, 1–3 nM, correspond to physiological concentrations in, for example, actively clotting blood and have been shown (Horne & Simons, 1978; Larsen et al., 1979) to correspond to maximal platelet response within 1 min.

When low concentrations (1 nM) of the DNCO derivatives were equilibrated with platelets (in the dark) for only 30 or 60 s before covalent band formation via photoactivation, a single ^{3}H-labeled complex was formed. It could be detected in either the column eluate or the polyacrylamide gel because of its high specific activity, but was present in too low a quantity to be detectable by A_{280} or Coomassie blue staining without concentration. Tam et al. (1980) have also recently suggested the possibility that the high-affinity thrombin receptor of the human platelet is present in too low a concentration to be detected or identified with the usually reported platelet membrane glycoprotein pattern (Phillips & Agin, 1973, 1974). Molecular weights of the concentrated complexes were estimated by $NaDodSO_4$ gel electrophoresis as 200000

for the uncleaved TosLysCH$_2$Cl–thrombin coupled moiety and 120 000 for the cleaved α-thrombin coupled moiety. Since thrombin has a molecular weight of 38 000, we therefore estimated that the intact high-affinity entity has a molecular weight of ~160 000 while the residual membrane protein after thrombin cleavage has one of ~80 000.

There have been a number of studies of the platelet membrane glycoproteins and of the disappearance of some of these upon addition of thrombin. Ganguly & Gould (1979) have implicated glycoprotein Ib (GPIb) in high-affinity thrombin binding while Jamieson and his colleagues (Okumura & Jamieson, 1976a,b; Okumura et al., 1978) have shown that glycocalicin, which is also of similar size (M_r 160 000), binds thrombin tightly. However, neither membrane protein is cleaved by thrombin nor does digestion of either one by chymotrypsin affect response (Tam et al., 1980). Thus neither glycocalicin nor glycoprotein Ib corresponds to the platelet thrombin binding protein described here. In contrast, others (Baenziger et al., 1971; Phillips & Agin, 1973, 1974; Mosher et al., 1979) have shown that a minor glycoprotein isolable from platelet membranes decreases in quantity upon thrombin stimulation. A fragment whose size varies from an M_r of 68 000 to 98 000 is liberated into the medium (Phillips & Agin, 1977; Lawler et al., 1977; Mosher et al., 1979; Tam et al., 1980). Estimates of the original size of the glycoprotein also differ [190 000 in Baenziger et al. (1971) and Ganguly (1971); 118 000 in Phillips & Agin (1973); 89 000 in Phillips & Agin (1977); 68 000–85 000 in Mosher et al. (1979)]. This may be because molecular weights of glycoproteins are notoriously difficult to determine accurately but may also be due to variation in poststimulation thrombin cleavage due to the long (usually 10–50 min) incubation times and high doses used in the various studies mentioned above. Our covalent coupling is activated after very short exposure and is therefore less likely to be subject to these problems. The high-affinity complexes have been isolated as single radioactive peaks. Rechromatography again yielded a single radioactive peak and electrophoresis a single radioactive band. Therfore we must conclude that we are dealing either with a single moiety or with several of identical mobility under these conditions and with identical affinity for thrombin. Previous reports have dealt with the presence of a single high affinity receptor species to which α-thrombin and TosLysCH$_2$Cl–thrombin bind equally well (Tollefsen et al., 1974; Martin et al., 1975; Ganguly & Sonnichsen, 1976; Workman et al., 1977; Phillips & Agin, 1974; Tam & Detwiler, 1978). Very recently (Tam et al., 1980), it has been shown that binding of thrombin and activation of thrombin cleavage are separable functions. It has therefore been postulated that either (a) a single high-affinity receptor species binds thrombin or TosLysCH$_2$Cl–thrombin in one region and then reacts with the free active site (of thrombin but not of TosLysCH$_2$Cl–thrombin) in a different region of the same molecule or (b) different receptors fulfill the separate binding site and thrombin cleavage site functions. Our data would fit the first possibility, as would the previously published data mentioned above, but might be more difficult to reconcile with the second hypothesis.

As previously mentioned here and discussed recently by Tam et al. (1980), the concentration of the high-affinity receptor is very low, and it may not correspond to any of the previously identified platelet membrane proteins. The low-affinity complexes are present in somewhat larger quantity but still need to be concentrated before their detection by protein A_{280} or Coomassie blue staining, rather than by their radioactivity, is possible.

At higher doses of thrombin, the enzyme has been shown to bind platelets with lower affinity, but it has been unclear whether to a separate binding site or to the same one but with decreased affinity, i.e., negative cooperativity. The results of photocoupling experiments at high doses of thrombin clearly support the first alternative and are incompatible with the single-site hypothesis. Three covalently coupled radioactive thrombin derivative–platelet complexes were identified after short exposure (30–60 s) to higher (0.125–0.250 U/mL) thrombin doses. The one of intermediate size (M_r 200 000 and 120 000 for the TosLysCH$_2$Cl–thrombin and the active thrombin coupled entities, respectively) appeared identical in size and in susceptibility to thrombin cleavage to the high-affinity receptor discussed above. The two other complexes bind to but are not hydrolyzed by thrombin and thus are isolated whether the low-affinity sites have been coupled to the TosLysCH$_2$Cl or to the active derivative of thrombin. These two low-affinity binding proteins have not been previously identified as such in the studies (Phillips & Agin, 1973, 1974, 1977; Mosher et al., 1979) in which changes in membrane proteins resulting from platelet thrombin stimulation are reported since thrombin sensitivity was defined in these studies as thrombin lysis. These low-affinity sites may, however, correspond to some of the thrombin binding proteins which have been previously identified, such as glycocalicin or the glycoprotein I complex (Okumura & Jamieson, 1976a,b; Okumura et al., 1977; Ganguly & Gould, 1979; Nachman et al., 1979), which are not cleaved by thrombin. Under nonreducing conditions, concentrates of each of these low-affinity thrombin-bound complexes give rise to a single radioactive peak or band upon gel chromatography or electrophoresis. The larger of the two has an estimated molecular weight of 400 000. Thus the binding moiety is ~360 000, larger than either glycocalicin (150 000) or glycoprotein Ia (210 000) or glycoprotein Ib (150 000), but could be the glycoprotein I complex (400 000) identified by Nachman (Nachman et al., 1979) as a multisubunit thrombin binding complex on the platelet membrane. We cannot, as yet, definitively identify the protein isolated after covalent thrombin coupling with any of the proteins or glycoproteins described in the literature as low-affinity binding proteins.

It should be noted that isolation and identification of all the thrombin-bound complexes discussed here have been made possible by detecting the radioactive complex after careful chromatography or electrophoresis and, therefore, by using a photoreactive thrombin derivative which has a very high specific ^{3}H activity. Identification by protein or carbohydrate content of the unconcentrated complex is, as has also been noted by Tam, not possible. We have no evidence as yet that each complex is purer than these techniques would allow us to judge. We do, however, show here a method for establishing covalent coupling between a stimulus, thrombin, and a specific binding protein on the platelet membrane. Furthermore, we show here that, in platelets, two different types of such proteins of different thrombin affinities exist.

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