Properties of the Factor X₈ Binding Site on Human Platelets

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The affinity (Kₐ) of human coagulation Factor X₈ for thrombin-treated (to stimulate the release reaction) platelets has been determined to be 3 to 4 × 10⁻¹⁶ M⁻¹ by equilibrium binding studies using ¹²⁵I-labeled X₈. The binding of Factor X₈ to platelets results in an increase of 50,000-fold in the apparent enzymatic activity of X₈ in the conversion of prothrombin to thrombin. The activity of platelet surface X₈ is approximately 16-fold greater than that observed with optimum concentrations of bovine Factor V and phospholipids in place of platelets. Ca²⁺ is required for the X₈-bound interaction; the optimum concentration is 2.5 mM. Related coagulation factors, including Factor X, Factor IX, α-diisopropylphosphoryl Factor X₈, and prothrombin do not compete with Factor X₈ for the X₈ binding sites. The rate of thrombin formation at saturating amounts of X₈ is directly proportional to the number of platelets from 1 × 10⁸ to 5 × 10⁸ platelets/ml. Factor X₈ bound to platelets is not inactivated by antithrombin III. An antibody that inhibits both human and bovine coagulation Factor V activity blocks both X₈ binding to released platelets and the rapid thrombin formation associated with this binding, suggesting that Factor V from platelets is involved in the X₈-platelet interaction.

Most of the plasma coagulation proteins have been purified to homogeneity in recent years (1). Understanding the specific functions of these proteins in the sequence(s) of reactions that lead to fibrin formation has been the object of intense study. Several of these reactions, most notably the activations of X₈ and prothrombin by IX₈ and X₉, respectively, are thought to occur on phospholipid surfaces in highly efficient complexes between the enzymes, their substrates, and accessory factors VIII and V, respectively (2).

Primary hemostasis, which occurs in response to injury, depends on the adhesion, aggregation, and release reactions of blood platelets. Platelets also shorten the clotting time of plasma in vitro and it has been suggested that platelets provide the phospholipid surface for several reactions of coagulation (3). Based on experiments comparing the clot-promoting activity of platelets and extracted platelet phospholipid, Marcus et al. (4) proposed that more than phospholipid is involved. The hypothesis that platelets actively participate in the reactions leading to fibrin formation has been extended by Walsh (5) who postulates that platelets adhering to collagen or other substances at sites of injury can initiate the intrinsic sequence of coagulation and that all of the subsequent reactions take place on the platelet surface at accelerated rates protected from plasma proteinase inhibitors.

To identify and quantitate interactions of platelets with individual plasma coagulation proteins we have used platelets washed free of plasma and extensively purified coagulation factors. We previously described the binding of thrombin to a specific platelet surface receptor (6). Recently we reported that Factor X₈ binds to a small number of platelet receptors (about 200/platelet). The Factor X₈ binding sites are distinct from the thrombin binding sites since: 1) Ca²⁺ is required for the X₈-platelet interaction; 2) the receptor for X₈ binding is available only after platelets have undergone the release reaction; and 3) X₈ does not compete for thrombin binding sites nor does thrombin compete for X₈ binding sites (7). Factor X₈ was also shown to be more active with respect to prothrombin activation in the presence of platelets than X₈ free in solution or mixed with phospholipid. We now describe the effects of varying the concentrations of Factor X₈, prothrombin, Ca²⁺, or platelets, as well as changing the pH, on X₈ binding and thrombin formation. A more detailed study of the enzymatic activity of X₈ in various reaction mixtures is presented and compared with previously reported (8) results using bovine Factor X₈. We also report the effect of antithrombin III, a major plasma inhibitor of X₈, on the activity of X₈ bound to platelets. Finally, we describe experiments with an antibody to Factor V that suggest that this factor is, at least in part, the platelet X₈ receptor.

EXPERIMENTAL PROCEDURES

Materials

Materials were obtained from the following sources: carrier-free [¹²⁵I]iodide from New England Nuclear; N-succinimidyl 3-(4-hydroxyphenyl)propionate from Pierce; bovine fibrinogen (90% clotting) from Miles Laboratories; Russell's viper venom in crude cephalin, bovine serum albumin, Factors VII- and X-deficient bovine plasma, rabbit brain cephalin, and heparin (H-3125) from Sigma; Quaternary aminoethyl (QAE)-Sephadex A-50 (40 to 120 μm) from Pharmacia; DEAE-cellulose (DE52) from Whatman; Taipan snake venom, Ci-Trol coagulation control human plasma, level I, from Dade; Apiezon oil from J. B. Biddle Co.; all other chemicals were reagent grade products of Sigma, Fisher, or Mallinckrodt.

Methods

Coagulation Assays

All assays were done at 37°C in a Fibrometer (Becton Dickinson). Prothrombin was measured by a two-stage method following activation to thrombin by 10 μg/ml of Taipan snake venom (30 min). Thrombin activity was determined by the method of Penton and Fasco (9) with the following modifications: the final volume was 0.3 ml, the final concentration of fibrinogen was 4 mg/ml, and aliquots of samples to be assayed were added directly to polypropylene cups.
containing appropriate amounts of the polyethylene glycol clotting buffer (9). This last modification eliminated time-consuming dilutions and prolonged sampling intervals at the time of sampling. The final dilution of sample in the assay was always greater than 50-fold when rates of thrombin were being measured, and no significant continued prothrombin activation occurred. This point was established by diluting samples 50-fold in buffer and then serially assaying for thrombin concentration over 10 min.

Factor X, Factor IX and X, were diluted in the following buffer for assays: 0.15 M sodium chloride containing 10 mg/ml of bovine serum albumin, 5.1 mM sodium citrate, 7.1 mM sodium acetate, and 7.1 mM sodium diethyl barbiturate, adjusted to pH 7.4 with hydrochloric acid.

Factor X was assayed by a standard method (10) in which 75 μl of 25 mM calcium chloride is added to a mixture of 75 μl of sample, 75 μl of Russell's viper venom in crude cephalin, and 75 μl of Factors VII- and X-deficient bovine plasma. Dade Ci-Trol control human plasma, level one, was used to construct the standard curve. Factor X, was determined similarly except that 75 μl of sample, 75 μl of 25 mM calcium chloride, and 75 μl of 400 μg/ml of rabbit brain cephalin were incubated at 37°C for 30 s and the reaction was initiated by addition of 75 μl of Factors VII- and X-deficient bovine plasma (at 37°C). A standard curve was constructed by assaying dilutions of the sample with the highest specific activity.

Factor IX activity was determined from the kaolin-partial thromboplastin time using human Factor IX-deficient plasma. In this assay 60 μl of the IX-deficient plasma (~1%), 60 μl of rabbit brain cephalin (400 μg/ml), 60 μl of kaolin suspension (20 mg/ml), and 60 μl of sample were incubated at 37°C for 10 min. The mixture is started with the addition of 60 μl of 20 mM calcium chloride. A standard curve was constructed with the Dade control human plasma.

Factor IX, was not determined by one-stage coagulation assay since IX is not the rate-determining enzyme when as little as 1% (by weight) Factor IX is present. In a one-stage assay, 10 ng of IX/ml incubated with 1 μg of/ml in the presence of 100 μg of phospholipid/ml, 8 mM calcium chloride, and 10 μg (0.15 units of procoagulant activity) of human Factor VIII/ml resulted initially in the formation of 15 ng of X/ml/min. The Factor VIII used in these experiments was provided by Dr. Patrick McKee (Duke University Medical Center, Durham, N. C.) and had a specific activity of 15 units/mg/ml when assayed against a standard preparation of factor VIII (Hvland Reference Plasma Lot 3403C0034).

In our experiments we define 1 unit/ml of factor V activity as the amount that results in formation of 1 unit of thrombin/ml/min when incubated in 0.15 M sodium chloride containing 2.5 mM calcium chloride and 0.02 M Tris-HCl, pH 7.4, with 10 ng of X/ml, 75 μg of prothrombin/ml, and 12 μg of rabbit brain cephalin (10). The rate of thrombin formation is linear from 0 to 2 units of Factor V/ml. The maximal rate of thrombin generation when V is saturating is 3 units of thrombin/ml/min. If the unit of Factor V is defined as the amount present in 1 ml of plasma, then approximately 100 of the above defined units equals 1 plasma V unit.

**Protein Purifications**

Protein concentrations were estimated from absorbance at 290 nm using the following values for E290: prothrombin, 13.8 (11); thrombin, 16.2 (12); Factor X, 11.6 (13); Factor IX, 13.3 (13); antithrombin III, 5.7 (14); human IgG, 14.3 (15); bovine Factor V, 11.9 (1).

Human IgG was precipitated at 0°C by slow addition of 1 volume of saturated ammonium sulfate, adjusted to pH 7.5 with ammonium hydroxide, to 2 volumes of anticoagulated plasma and was collected by centrifugation at 10,000 x g for 10 min (at 0°C). The pellet was washed with 5% saturated ammonium sulfate, collected again, dissolved in 1 volume of 0.01 M sodium phosphate, pH 7.0, and dialyzed against 2,000 volumes of the same buffer for 16 h at 4°C. A precipitate that formed was removed by centrifugation at 10,000 x g for 10 min and the supernatant was applied to a column (10 volumes) of DEAE-cellulose. IgG is not adsorbed and was eluted with the 0.01 M sodium phosphate buffer.

Antithrombin III was prepared by Dr. Marc A. Shuman (University of California, San Francisco, Medical Center) by a modification of the method of Yin et al. (16) as previously reported (17). The activity of 10 ng of human Factor X, was inhibited by 12.3 ng of antithrombin III; 0.5 unit of human thrombin (~150 ng) was inactivated by 190 ng of antithrombin III. In the presence of 1 unit of heparin (6.6 μg/ml) both reactions were complete in less than 1 min. Thrombin activity was inhibited at these concentrations in the presence of platelets (10 μg/ml) at a slightly slower rate.

The X-coagulant protein of Russell's viper venom was isolated (18) and further purified by QAE-Sephadex chromatography (19). The bovine Factor V used in experiments involving formation of human thrombin by human Factor X, was isolated by the same procedure as the preparation employed in the analysis of bovine prothrombin activation products (20). In the Factor V assay described above the specific activity of this preparation was 29,800 units/mg. When diluted by 0.5 unit/ml this preparation, stored at 0.5 mg/ml in 0.4 M NaCl, 0.1 M CaCl2, and 0.02 M Tris buffer, pH 7.5, was stable at 4°C for 6 months.

Human prothrombin, Factor X, and Factor IX were isolated as follows.

**Barium Citrate Adsorption**—Fresh-frozen, citrated human plasma from normal donors was provided by Dr. William Miller of the Missouri-Illinois Regional Red Cross Blood Program, was thawed at 37°C and stored at 4°C in a plastic container. Barium chloride (1.0 M) was added dropwise to a final concentration of 50 mM over 30 to 45 min. The barium citrate precipitate was centrifuged at 3000 x g for 20 min at 4°C. A Tek-mar tissue homogenizer was used to resuspend the precipitate in 1 liter of 0.1 M sodium chloride containing 0.01 M barium chloride. The precipitate was again collected by centrifugation and resuspended in 400 ml of 0.1 M sodium chloride containing 0.01 M barium chloride.

**Ammonium Sulfate Elution**—The barium citrate suspension was stirred at 0°C and 6.9 g of ammonium sulfate/liter of starting plasma was slowly added. After 60 min, the mixture was centrifuged for 10 min at 10,000 x g and the supernatant was adjusted to contain approximately 5 mM DFP (1.0% v/v) and stirred in a ture hool for 30 min. The pH remained at 7.4. The solution was dialyzed overnight at 4°C against 100 volumes of 0.15 M sodium chloride containing 1 mM benzamidine and 0.02 M Tris-HCl, pH 7.4. It became cloudy, but this did not interfere with subsequent steps.

**QAE-Sephadex A-50 Chromatography**—The sample was applied at room temperature to a column (2 x 20 cm) of QAE-Sephadex A-50 equilibrated in the buffer used for dialysis. The column was washed with 1.5 volumes of 0.25 M sodium chloride containing 1 mM benzamidine and 0.02 M Tris-HCl, pH 7.4. Prothrombin and Factors IX and X were eluted at 0.33 M sodium chloride gradient (0.25 to 0.50 M in the same buffer). Appropriate fractions were pooled and dialyzed overnight at 4°C against 50 volumes of 0.01 M sodium phosphate containing 1 mM benzamidine, pH 7.0. This dialysis and the subsequent step are not essential to the Factor IX and Factor X purification but remove small amounts of contaminating proteins that otherwise elute with prothrombin in the heparin-agarose chromatography (see below). The pooled protein solutions were dialyzed at least 16 h at 4°C against several changes of 50 volumes of 0.05 M imidazole-HCl, pH 6.0, containing 1 mM benzamidine.

**Heparin-Agarose Chromatography**—A column (2.5 x 25 cm) of Whatman DE52 prepared according to manufacturer's instructions in the buffer used for dialysis. The flow rate was adjusted to 250 ml/h with a pump and 2.0 liters of buffer with a linear sodium chloride gradient (0 to 0.40 M) was used to develop the column. The protein peak, which is 90% heparin-Agarose (see below), although we have no indication that this is necessary. The pooled protein solutions were dialyzed at least 16 h at 4°C against several changes of 50 volumes of 0.05 M imidazole-HCl, pH 6.0, containing 1 mM benzamidine.

**Heparin-Agarose Chromatography**—A column (2.5 x 25 cm) of heparin-agarose was equilibrated at room temperature in 0.05 M imidazole-HCl, pH 6.0, containing 1 mM benzamidine and 2.5 mM calcium chloride. The samples were also adjusted to contain 2.5 mM calcium chloride just before application of the column. Fractions were collected into 0.25 M EDTA (adjusted to pH 7.0) to give a final concentration of 0.4 M. A 1.5-liter linear gradient of sodium chloride (0 to 0.40 M) in the starting buffer was used to develop the column. Prothrombin elutes first (~0.05 M NaCl) followed by Factor X (0.20 M NaCl) and then Factor IX (~0.35 M NaCl). The fractions of highest specific activity of each protein were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane. The proteins (1 to 2 mg/ml) were stored at -70°C in 0.60 mM sodium chloride containing 1 mM benzamidine and 0.01 M sodium phosphate, pH 6.8.

1 The abbreviations used are: DFP, diisopropyl fluorophosphate; 125I-X, 125I-labeled Factor X; 125I-X, 125I-labeled Factor X.
Platelet Binding Site for Factor Xa

Preparation of Thrombin, Xa, and IXa

Human thrombin was isolated by adsorption on Amberlite CG-50 ion exchange resin as described by Fenton et al. (12) after activation of 0.1 mg of purified prothrombin (in 0.15 M sodium chloride and 0.02 M Tris-HCl, pH 7.4) by incubation with either Taipan snake venom (10 μg/ml) or Factor Xa (100 ng/ml) and bovine Factor V (800 ng/ml) in the presence of calcium (2.5 mM) and phospholipid (120 μg/ml of rabbit brain cephalin) for 15 min.

Factor Xa was formed by incubating Factor Xa with 0.1% by weight of the Xa-coagulant protein of Russell’s viper venom at 37°C for 15 min in 0.15 M sodium chloride, 7 mM calcium chloride, and 0.02 M Tris-HCl, pH 7.4. Greater than 90% of the Xa was converted to Xa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Xa was isolated by chromatography on QAE-Sephadex A-50, but eliminating this step had no effect on either the stability of the Xa, or its interaction with platelets. At a concentration of 1 ng/ml in the coagulation assay factor Xa gave a clotting time of 35 min.

Factor IXa was prepared by incubating Factor IXa (1 mg/ml) with human Factor XI (20 μg/ml), provided by Dr. Earl Davie (University of Washington, School of Medicine, Seattle), at 37°C in 0.15 M sodium chloride, 2.5 mM calcium chloride, and 0.02 M Tris-HCl, pH 7.4. The extent of conversion of IXa to IXa was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol and was complete in 5 min.

Protein Iodination

125I-labeled N-succinimidyl (3-(4-hydroxyphenyl)propionate (1.1. atoms of iodine/molecule of coat) was prepared by the method of Bolton and Hunter (21). Fifty micrograms of human Factor Xa (400 μg/ml) in 0.1 M borate buffer, pH 8.5, was added to 150 μCi of 125I-labeled iodogen (60 μCi/μg) for 5 min. The mixture was added to 0.1 M borate buffer, pH 7.4. After determination of protein the 125I-Factor Xa was diluted to a concentration of 50 μg/ml in the same buffer with 5 mg/ml of bovine serum albumin and stored at −70°C. Approximately 30% of the labeled ester was incorporated into protein, corresponding to 2000 cpn/ng of protein (−0.02 μmol iodine/molecule of Factor Xa). For some experiments, Factor Xa was labeled with 125Iiodide by a modified chloramine-T procedure (22).

Binding Assays

Except as stated otherwise, reaction mixtures contained 106 platelets/ml, 75 μg of prothrombin/ml, and 2.5 mM calcium chloride in the buffer used for resuspending the platelets (see below). All incubations were at room temperature (22°C) in 0.15 M sodium chloride containing 1.8% bovine plasma which do not separate widely from Factor X on a modified chloramin T procedure (22).

Thrombin Generation Rates

Thrombin concentrations of the reaction mixtures were determined at the time of sampling by direct addition of 2 to 60 μl to the clotting buffer (238 to 180 μl) followed immediately by addition of fibrinogen (60 μl). Since the thrombin standard curve is linear in a log-log plot from 0.04 to 1.0 unit/ml (160 s to 15 s, respectively), thrombin concentrations from 0.2 to 150 units/ml can be measured. Platelets have no detectable effect on the thrombin assay at thrombin concentrations greater than 1 unit/ml. The concentration of thrombin increased linearly with time from 12 to 80 units of thrombin/ml and rates were determined during this interval.

Other Methods

Platelets were isolated from human blood anticoagulated with EDTA and washed free of plasma as previously described (23). When suspended at 107/ml in the phosphate buffer used for isolation, the platelets were stable with respect to their interactions with thrombin or Xa for more than 16 h. Just prior to use the platelets were collected by centrifugation at 2000 × g for 10 min and resuspended in the buffer used for these experiments (0.15 M sodium chloride containing 1.0 mg/ml of glucose, 5 mg/ml of fat-free bovine serum albumin, and 0.02 M Tris-HCl, pH 7.4). Platelets should not be incubated for long times in this buffer prior to experiments because they produce acid metabolites and Tris is a poor buffer for hydrogen ions at this pH. Fat-free bovine serum albumin was prepared (24). Polyacrylamide gel electrophoresis at a final acrylamide concentration of 10% (w/v) was done in the presence of sodium dodecyl sulfate in the Tris/glycine buffer described by Laemmli (25).

Heparin-agarose was prepared by reacting 200 ml of Bio-Gel A-1.5 (200 to 400 mesh) with 1 M sodium chloride containing 400 units of heparin (Sigma) in 200 ml of 0.2 M sodium bicarbonate at pH 8.5. The slurry was agitated at 4°C overnight. The agarose was packed in a column (5 × 15 cm) and washed with 20 liters of 0.1 M sodium chloride containing 0.2% (w/v) sodium azide. This material was stored at 4°C.

Radioactive iodide was determined with a Beckman Bio Gamma II y counter at an efficiency of 80%.

RESULTS

Preparation of Factor Xa—A summary of the purification of Factor Xa is shown in Table I. Our final preparation is purified 18,000-fold compared to the starting plasma and has a specific activity of 225 units/mg of protein. This is very similar to the material recently reported by DiScipio et al. (13) using a different method. Polyacrylamide gel patterns of the final product following electrophoresis in the presence of sodium dodecyl sulfate are shown in Fig. 1. These gels were overloaded to increase the visualization of minor contaminants. Since Factor X is composed of two polypeptide chains joined by a disulfide bond, the gel pattern in the presence of β-mercaptoethanol is particularly useful in estimating contamination by the other vitamin K-dependent proteins of plasma which do not separate widely from Factor X on unreduced gels. We estimate that this protein is greater than 99% pure based on the gel electrophoresis pattern of material labeled with 125Iiodide (see “Methods” for details). On reduced sodium dodecyl sulfate-gels the apparent molecular weight was 49,000 for the heavy chain and 17,000 for the light chain when compared to human fibrinogen as a standard.

Radiolabeling of Factor Xa—In our earlier work we labeled Factor Xa, directly with 125Iiodide by a chloramine-T procedure (22). In our initial experience this material retained full enzymatic activity. With repeated use of this method, however, we noted considerable variability in different samples of the radiolabeled protein even though the labeling technique...

<p>| Table I: Purification of human Factor X |
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* Cryosupernatant was assumed to have 1 unit of Factor X activity/ml; activity in subsequent steps was determined by comparison to a standard control plasma.

* Protein concentration was estimated by absorbance at 290 nm assuming ε290 = 10.

* Protein concentration was estimated assuming ε290 = 13.8.

* Protein concentration was estimated assuming ε290 = 11.6.
A radioactive product formed by the reaction of Factor X with the 125I-labeled \( ^{125}\text{I}-\)XII ester of p-hydroxyphenyl propionic acid. Free amines of the protein react with this ester to form stable amides without the addition of any external oxidizing or reducing agents. The 125I-X was converted to 125I-XII with the X-coagulant protein of Russell's viper venom. Analysis of this mixture by Sephadex G-100 chromatography and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and in the presence and absence of \( \beta \)-mercaptoethanol showed that >90% of the incorporated radioactivity was in Factor X (the rest being in the peptides released by activation of X) and that 95% of this amount was in the heavy chain. No difference in the enzymatic activity by

coagulation assay of this 125I-X, and control samples could be detected, either initially or after storage at \(-70^\circ\text{C}\) for 2 months.

**Affinity of X, for Platelets**—The concentration dependence of steady state X, binding to thrombin-treated platelets in the presence of prothrombin is shown in Fig. 2. The time required before X, binding (and resultant thrombin generation) becomes maximal in this system depends on the added Xa concentration. At the lowest concentration of added 125I-Xa (0.4 ng/ml) this takes 50 min; at the highest concentration (50 ng/ml), only 10 min is required. Since Xa binding decreases when prothrombin is depleted (see below) it is not possible to measure steady state Xa binding in the presence of prothrombin at the same time for every Xa concentration.

As the concentration of added 125I-Xa is increased from 0 to 50 ng/ml, the percentage bound to platelets progressively decreases until a constant value of approximately 2% is reached (Fig. 2, the line of constant slope beyond 10 ng of added 125I-Xa/ml). Further increases of added 125I-Xa of up to 2 \( \mu \text{g}/\text{ml}\) result in a continuation of this line (data not shown). We conclude that there is a relatively small number (about 200/platelet) of high affinity Xa receptors as well as a larger number (not determined) of nonspecific Xa binding sites on the platelet surface. Since the amount of 125I-Xa bound nonspecifically increases linearly with added Xa concentration, addition of a large amount of unlabeled Xa has no effect on nonspecific binding. However, since the high affinity sites saturate at a low concentration of added Xa, the amount of 125I-Xa bound to high affinity sites in the presence of a large amount of unlabeled Xa has no effect on high affinity binding. Thus, the high affinity sites saturate at a low concentration of added Xa, the amount of 125I-Xa bound to high affinity sites in the presence of a large amount of unlabeled Xa is negligible. The difference between the amount of 125I-Xa bound to platelets in the absence and presence of excess unlabeled Xa is a measure of binding to high affinity receptors under these circumstances. Such a correction is shown in Fig. 2. In this experiment the high affinity receptors were nearly saturated by the addition of 5 ng of 125I-Xa/ml; the total binding was 2 ng of 125I-Xa/10^9 platelets of which 1.9 ng (95%) was to high affinity sites. Nonspecific binding was 2.3% of the amount added.

To circumvent this problem we reacted Factor X with the 125I-labeled \( \beta \)-hydroxy succinimide ester of \( \beta \)-hydroxyphenyl propionic acid. Free amines of the protein react with this ester to form stable amides without the addition of any external oxidizing or reducing agents. The 125I-X was converted to 125I-XII with the X-coagulant protein of Russell's viper venom. Analysis of this mixture by Sephadex G-100 chromatography and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and in the presence and absence of \( \beta \)-mercaptoethanol showed that >90% of the incorporated radioactivity was in Factor X (the rest being in the peptides released by activation of X) and that 95% of this amount was in the heavy chain. No difference in the enzymatic activity by

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**Fig. 1.** Sodium dodecyl sulfate-gel electrophoresis of human Factor X in 10% polyacrylamide gels stained with Coomassie brilliant blue. Left, 20 mg of unreduced Factor X; right, 20 mg of reduced Factor X. Arrows indicate tracking dye.

**Fig. 2.** Steady state binding of 125I-Xa to platelets in the presence of prothrombin. Reaction mixtures initially contained 0.5 unit of thrombin/ml, 75 mg of prothrombin/ml, 10^9 platelets/ml, and varying amounts of 125I-Xa. Bound 125I-Xa was determined as described under "Methods." Each point represents the average of two measurements.
addition of 1 μg of 125I-Xa/ml resulted in a total of 25 ng bound/10^9 platelets of which 23 ng (92%) was nonspecific (data not shown).

The double reciprocal plot (27) of bound versus free Xa for a typical experiment is shown in Fig. 3. The high affinity binding of Xa to platelets can be described as a noninteracting saturating process. The apparent dissociation constant for 125I-Xa was 3.0 × 10^{-11} M (1.3 ng of 125I-Xa/ml) with 160 high affinity binding sites/platelet (1.2 ng/10^9 platelets). When 1 ng of unlabeled Xa/ml was added to reaction mixtures with increasing amounts of 125I-Xa, competitive inhibition of high affinity binding was observed and the apparent inhibition constant was the same as the apparent affinity constant determined with 125I-Xa (data not shown). This is consistent with the requirement that the labeled material not be altered with respect to its binding to platelets.

The enzymatic activity of Xa on prothrombin is increased (300,000-fold, see below) when Xa is bound to the high affinity platelet receptors. The measured rate of thrombin formation by low concentrations of Xa in the presence of platelets is therefore independent of the concentrations of free Xa and reflects only the amount of Xa bound to high affinity sites (7). By measuring the thrombin generation rates at different concentrations of added 125I-Xa we determined the affinity of 125I-Xa for platelets. The results were similar to those obtained by direct binding experiments; i.e. the concentration of 125I-Xa required for half-maximal high affinity binding also resulted in a half-maximal rate of thrombin formation. We also determined an apparent dissociation constant for high affinity binding with unlabeled Xa, using this enzymatic assay (Fig. 4). The concentration of Xa required to give half-maximal thrombin generation in this experiment was 1.8 ng/ml. If the binding data shown in Fig. 3 are used to estimate the concentration of free Xa at an added concentration of 1.8 ng/ml, the dissociation constant for unlabeled Xa is calculated to be 2.7 × 10^{-11} M (1.2 ng of Xa/ml). When labeled and unlabeled Xa were directly compared with the same preparation of platelets, no difference in the rate of thrombin formation at a given Xa concentration was observed. These results are consistent with the requirement that the Xa preparation labeled with the iodinated ester not be altered with respect to its enzymatic activity when bound to platelets.

In contrast to high affinity Xa binding to platelets, nonspecific binding does not depend on the platelet release reaction (7). However, it is Ca^{2+} dependent and is 5 to 10 times greater than independently determined values for nonspecific binding measured with 125I-labeled ovalbumin (7) or for trapped water measured with [14C]inulin (28). It is possible, therefore, that nonspecific Xa binding may have some significance not yet appreciated. But nonspecific binding of Factor Xa to platelets does not produce the increase in enzymatic activity that results from Xa binding to high affinity sites. This is most apparent from the experiment shown in Fig. 4. The rate of thrombin formation saturates with added Xa. Nonspecific binding, on the other hand, does not saturate at any of the concentrations tested. In some experiments the amount of Xa bound nonspecifically was 2 to 3 times greater than was bound to high affinity receptors, but thrombin formation correlated only with the latter. This does not necessarily mean that Xa bound nonspecifically is inactive; but its specific activity with respect to prothrombin conversion must be considerably less than that of Xa associated with high affinity sites.

Specificity of Xa Binding—To determine the specificity of the Xa association with platelets we measured the binding of 125I-Xa, in the presence of increasing amounts of related coagulation factors as shown in Fig. 5. Enough 125I-Xa was added to half-saturate high affinity Xa binding sites at equilibrium in the absence of any added factors. Factor Xa competes with 125I-Xa for binding effectively at low concentrations. In contrast, Factor X competes only 0.2% as well as Factor Xa for the Xa sites. Since traces of Xa activity can be detected in the X preparation and since the slope of the competitive curve is approximately the same for Xa as for Xa, the observed competition may be due entirely to contaminating Xa. This specificity might be important in vivo if only a small fraction of Xa is converted to Xa and must interact with platelets in the presence of large amounts of X.

Factor Xa inactivated by DFP does not interact with the Xa-binding site, at least up to the highest concentration tested (100 ng/ml). DFP inactivated factor Xa also has no effect on thrombin generation by Xa added to platelets. We have found that human Xa is approximately 200-fold less sensitive than human thrombin to DFP, so that high concentrations (5 to 10 mM) of DFP are required to effectively inhibit Xa. Other investigators have reported a similar sensitivity of bovine Xa to DFP (29-31).

Factor IXa was also tested as an inhibitor of Xa binding (data not shown). The sensitivity of this experiment was limited by our ability to prepare IXa free of Xa. With a IXa preparation that had 1 to 2% Xa, by coagulation assay we observed displacement of bound 125I-Xa with the same slope as observed for Xa, but at a 50-fold higher concentration.
Fig. 5. Competition of various proteins with $^{125}$I-X$_a$ for X$_a$ binding sites on platelets. Platelets were incubated with 2.5 ng of $^{125}$I-X$_a$/ml and increasing amounts of related plasma coagulation factors for 30 min and then total $^{125}$I-X$_a$ bound was determined. Each point is the average of two measurements. Reaction mixtures only contained prothrombin where indicated. The specific radioactivity of the $^{125}$I-X$_a$ was 2510 cpm/ng. The 100% bound value was 0.9 ng of $^{125}$I-X$_a$/10$^9$ platelets. $\circ$, added X$_a$; $\square$, added DFP-inactivated factor X$_a$; $\blacksquare$, added X$_a$; $\Delta$, added prothrombin.

Since IX$_a$ is not inactivated by DFP (32) but X$_a$ is, and since DFP-inactivated factor X$_a$ does not compete with X$_a$ for binding (see above), we tested a DFP-treated IX$_a$ preparation. After a 6-h incubation with 12 mM DFP there was no detectable X$_a$ activity but IX$_a$ was unaffected in its clotting activity. Ten thousand nanograms per ml of this preparation did not displace any $^{125}$I-X$_a$ from platelets.

Prothrombin increases the binding of $^{125}$I-X$_a$ to platelets in a concentration-dependent manner (Fig. 5). This effect depends on prothrombin and not on the thrombin formed in the reaction mixture. Once platelets have undergone the release reaction, increasing the thrombin concentration has no effect on X$_a$ binding sites provided X$_a$ is included in the incubation mixture, although thrombin at high concentrations (50 units/ml) reduces the number of X$_a$ binding sites if incubated with platelets for prolonged times prior to the addition of X$_a$ (7). Also, once the prothrombin in the reaction mixture has been mostly converted to thrombin, there is a decrease in X$_a$ binding of similar magnitude (7) to the increase observed on addition of the prothrombin (Fig. 5). It is possible that prothrombin participates in a complex (involving X$_a$) at the platelet surface and can thus affect equilibrium to favor the formation of the complex (33). It is not possible from this experiment to determine the minimum concentrations of prothrombin required to maximally increase X$_a$ binding since the prothrombin was being converted to thrombin and the actual concentration remaining in each reaction mixture at the time binding was determined was not measured. Effects of intermediates in prothrombin activation on X$_a$ binding could also explain the enhanced binding. In other experiments (not shown) we demonstrated that at high concentrations of added X$_a$ prothrombin had no effect on the amount of X$_a$ bound. Thus, the apparent number of X$_a$ binding sites does not change in the presence of prothrombin.

Platelet Concentration—The effect of increasing the platelet concentration from 1 x 10$^7$ to 5 x 10$^9$/ml on the rate of thrombin formation by X$_a$ is shown in Fig. 6. The X$_a$ was saturating at every level. The time required to reach the steady state (maximal) rate of thrombin generation decreased as the platelet concentration increased. Maximum rates were observed after 6 min at 1 x 10$^7$ platelets/ml, 3 min at 5 x 10$^7$ platelets/ml, 2 min at 1 x 10$^8$ platelets/ml, and <1 min at 2 x 10$^9$ platelets/ml and at 5 x 10$^9$ platelets/ml. Bound $^{125}$I-X$_a$ was also determined at 5 x 10$^3$, 1 x 10$^6$, 2 x 10$^6$, and 5 x 10$^6$ platelets/ml, and showed a similar dependency (0.72, 2.97, 6.88, and 18.8 ng bound, respectively). At the lower platelet concentrations the measured amount bound/platelet was spuriously low because of decreased platelet recoveries in the oil centrifugation assay, and at the higher platelet concentrations the measured amount bound per platelet is high because of increased trapped water and nonspecific binding. We did not correct the binding data presented above to account for these problems.

Ca$^{2+}$ Dependence—In the absence of Ca$^{2+}$ no significant X$_a$ binding or thrombin formation occurs (7). As shown in Fig. 7, the rate of thrombin generation by X$_a$ increases from zero to a maximum as the calcium chloride concentration is increased from zero to 2.5 mM. Further increasing calcium has no effect. This Ca$^{2+}$ concentration dependence of thrombin formation is indistinguishable from the observed in the bovine model system with X$_a$, V$_a$, prothrombin, and phospholipid.$^2$ The amount of bound $^{125}$I-X$_a$ also increased to a maximum as the calcium chloride concentration was increased to 2.5 mM (data not shown). At calcium concentrations greater than 15 mM the amount of $^{125}$I-X$_a$ bound decreased somewhat (~20%), but this was not further investigated.

pH Dependence—The pH dependence of the X$_a$-platelet interaction was investigated over the range 6.1 to 8.5. A mixture of 10 mM 1,4-piperazineethanesulfonic acid (Pipes) and 10 mM Tris was chosen as the buffer since it results in a nearly constant buffering capacity from pH 6.1 to 8.5. Identical results were obtained when 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) was used instead, suggesting that none of these buffers have any stimulatory or inhibitory effects.

The platelet release reaction is also somewhat dependent on pH. Four times as much thrombin is required for 50% release at pH 6.1 as at pH 8.5. For these experiments reaction mixtures initially contained 1.0 unit of thrombin/ml which caused maximal release at every pH investigated.

The amount of $^{125}$I-X$_a$ added (2 ng/ml) was chosen to give 50% of maximal binding at pH 7.4. Nonspecific binding corrections at each pH are essential since they increase approximately linearly, being 4.5 times as high at pH 8.5 as at pH 6.1. Specific binding also increases, but not linearly, representative

$^2$ C. T. Easmon and C. M. Jackson, unpublished observations.
values (per 10^9 platelets) being 0.21 ng at pH 6.1, 0.45 ng at pH 6.6, 0.64 ng at pH 7.0, 0.68 ng at pH 7.3, 0.70 ng at pH 7.6, 0.78 ng at pH 8.0, and 0.77 ng at pH 8.5. Thrombin formation rates were also determined and found to be (in units/ml/min) 0.9 at pH 6.1, 2.8 at pH 6.6, 4.3 at pH 7.0, 4.7 at pH 7.3, 4.7 at pH 7.6, 5.1 at pH 8.0, and 6.1 at pH 8.5. We conclude that the Xr-platelet interaction is relatively insensitive to pH, but that the largest changes occur when solutions are more acidic than pH 7.0. This effect is mostly due to an actual decrease in the amount of Xr bound, although the specific activity of bound Xr is also decreased somewhat (e.g. 4.3 units/ml/min/ng bound at pH 6.1 and 7.9 units/ml/min/ng bound at pH 8.5).

**Enzymatic Activity of Platelet-bound Xr**—The thrombin generation rate resulting from 1 ng of Xr bound to the platelet Xr receptor was determined with platelets from six normal donors to be 4.2 ± 0.28 (S.D.) units of thrombin/ml/min. As shown in Table II this represents an increase in the enzymatic activity of the platelet-bound Xr, of 300,000-fold as compared to Xr free in solution. Also shown in Table II are the rates of prothrombin conversion by Xr in the presence of optimum amounts of phospholipid or phospholipid and bovine Factor V (thrombin-activated). For comparison, previously reported (8) data obtained using bovine Factor Xr and bovine prothrombin are also shown. Other workers have reported 20-fold (34) and 30-fold (35) stimulation of prothrombin activation by Factor V. The results from the two sets of experiments, although determined in reaction mixtures with minor differences, are in good agreement with respect to both the absolute activity of Xr and the relative enhancement observed with the various additions. We find that human Factor Xr, by itself is a relatively poor enzyme, converting only 0.006 molecule of prothrombin to thrombin/min/molecule of Xr, that phospholipid increases the rate of this reaction 50-fold, that the effect of Factor V is 400-fold, and that together they act multiplicatively to increase the rate 20,000-fold. Although the addition of phospholipid and Factor V to Xr as described provides a good model system for studying the enhancement of the activity of Xr on prothrombin, the enzymatic activity of Factor Xr is increased to an even greater extent in the presence of released platelets.

Since essentially all of the thrombin is formed at the platelet surface when Xr and prothrombin are added to platelets (see above), the rate of thrombin formation was calculated per µg of bound Xr. In the experiment with phospholipid and Factor V, the rate was calculated per µg of added Xr. Based on data for the Ca^{2+}-mediated lipid-protein associated of prothrombin,3 we estimate that nearly all of the Xr was bound to phospholipid and Factor V at the concentrations tested, i.e. that the reaction mixtures were saturating with respect to phospholipid and Factor V. This was supported experimentally by reducing the phospholipid or Factor V, or both, 5-fold with no effect on the rate of thrombin formation, which was 2.65 to 2.84 units of thrombin/ml/min/10 ng of added Xr (or 265 to 284 units of thrombin/ml/min/µg of Xr). Further the rate was linearly dependent on the amount of added Xr; 20 ng of Xr/ml resulted in a rate of 5.57 units of thrombin/ml/min at this higher concentration. The rate of thrombin formation by Xr in the presence of platelets can similarly be determined under conditions where platelets are saturating and the rate depends linearly on the Xr added. Since 10^6 platelets bind approximately 2 ng of Xr to high affinity sites (Fig. 2), the amount of added Xr must be less than 2 ng/ml at the platelet concentration used in most of our studies. The addition of 0.4 ng and 1.0 ng of Xr/ml resulted in rates of 1.35 and 3.05 units of thrombin/ml/min (3375 and 3050 units of thrombin/ml/min/µg of added Xr, respectively). Thus, the platelet surface is a more favorable environment for Xr activity than the phospholipid surface in the model system when the two are compared under comparable saturating conditions even when no corrections for actual amounts of Xr bound are made. The difference between the model system using bovine V and platelets cannot be explained by differences between human and bovine V since, in experiments using crude V released from platelets (not shown) with human Xr and phospholipid, rates of thrombin formation comparable to those seen with bovine V were obtained.

![Graph](image.png)

**FIG. 7. Effect of Ca^{2+} concentration on the rate of thrombin formation.** Reaction mixtures contained 5 ng of Xr/ml, 75 µg of prothrombin/ml, and 0 to 20 mM calcium chloride. Rates of thrombin generation were determined as described under "Methods."

*Final concentration of Factor Xr, 0.3 µg/ml.*

*Final concentration of Factor Xr, 1 µg/ml.*

*Final concentration of Factor Xr, 0.01 µg/ml.*

*Final concentration of Factor Xr, 0.001 µg/10^6 platelets.*
Inactivation of Xa by Antithrombin III—Marciniak (36) has shown that Xa activity is protected from inactivation by antithrombin III in a model system using phospholipid and Factor V, and Walsh and Biggs (37) have investigated the loss of Xa activity in plasma when phospholipid or platelets were present. We have demonstrated the inability of antithrombin III to neutralize the activity of Xa bound to platelets in our system. Factor Xa (110 ng/ml) was added to thrombin-treated platelets immediately before the addition of 210 ng/ml of antithrombin III and 1.9 unit/ml of heparin. This amount of the protease inhibitor inactivates both the Xa and the thrombin used to cause the platelet release reaction in less than 1 min at the concentration of heparin used (see “Methods”). After 20 min, prothrombin was added and the rate of thrombin generation was determined to be 0.4 unit/ml/min. In contrast, when Factor Xa was added to thrombin-treated platelets for 20 min followed by a 20-min incubation with the antithrombin III and heparin, a thrombin generation rate of 2.5 units/ml/min was observed after addition of prothrombin. Since the only difference in the two experiments is the length of time Xa can interact with platelets before the addition of antithrombin III and heparin, we conclude that the binding of Xa to platelets protects this coagulation factor from antithrombin III. When the Xa, antithrombin III, and heparin were incubated for 1 min before the addition of thrombin-treated platelets, no conversion of added prothrombin to thrombin was observed.

Effect of an Acquired Antibody to Factor V on Xa, Binding and Thrombin Formation—We obtained a plasma sample from a patient with an acquired antibody to Factor V* (38). The antibody was directed against V and did not inhibit Factors VIII, IX, X, XI or XII (38). As shown in Fig. 8, the IgG fraction of this plasma inhibited both Xa binding and thrombin generation at concentrations of less than 1 μg/ml when incubated with thrombin-treated platelets before the addition of Xa. The inhibition was reversible with time as might be expected from the high affinity of Xa for its binding site on platelets. Control human IgG had no effect on Xa binding or thrombin formation.

When similar concentrations of the inhibitor antibody were added to thrombin-treated platelets at the same time as Xa, little effect on Xa binding or thrombin generation was detected. Similarly, incubation of the antibody with platelets prior to the addition of thrombin, Xa, and prothrombin or with Xa, or prothrombin prior to the addition of thrombin-treated platelets produced no inhibition. We conclude that the antibody reacts with a platelet substance available only after thrombin treatment that is essential for Xa binding.

The inhibitor antibody was also tested with the bovine plasma Factor V preparation and human platelet Factor V obtained as follows: 106 platelets/ml were treated with 5 units of thrombin/ml in buffer with 25 mM calcium chloride for 2 min. The platelets were removed by centrifugation through oil and the supernatant was diluted 10-fold to reduce the concentration of thrombin and Ca2+. This platelet Factor V was labile with measurable losses of activity after 2 h and 80% inactivation after 16 h. One unit of V activity as determined in our Factor V assay (see “Methods”) was obtained from 2.5 to 5 × 107 platelets. The activities of both the bovine plasma and the human platelet V were decreased by the antibody, i.e. 1.8 units of V of the bovine material was reduced to 0.9 unit of V by 6.3 μg of antibody, while the platelet Factor V was decreased from 1.9 to 0.45 units of V by the same amount of anti-V antibody. In these experiments, higher concentrations of the antibody were used than in the studies with platelets because there was no preincubation of the antibody with the V preparations, i.e. the Factor V, inhibitor antibody, Xa, and phospholipid were mixed and the reactions were initiated by the addition of prothrombin. This was necessary because our Factor V assay is based on the rate of thrombin formation; although much lower concentrations of the antibody produced inhibition of thrombin generation if preincubated with the V (but not with Xa, prothrombin, or phospholipid),4 the inhibition was overcome with time. At the higher concentrations reported here, thrombin formation was linear with time allowing a straightforward determination of Factor V activity. The results support our conclusion and of Coots et al. (38) that the antibody is directed against V and that Factor V is, at least in part, the platelet Xa binding site.

DISCUSSION

Normal hemostasis requires both platelets and the circulating proteins of the blood coagulation system. Although the synergistic participation of these components has been recognized previously, few detailed mechanisms have been elucidated. Loose association of plasma clotting factors with platelets has been described and considered a special “plasma atmosphere” surrounding platelets (39) which might accelerate fibrin formation (for reviews, see Refs. 40 and 41). Specific intraplatelet coagulation factors are secreted from granules during the release reaction, including fibrinogen (42), probably Factor V (43), and possibly others (44). The ability of platelets to shorten the clotting time of plasma treated with Russell's
viper venom has been considered a measure of the ability of platelets to accelerate thrombin formation from prothrombin (49). This platelet function has previously been termed "platelet Factor 3" and has been presumed to involve phospholipids (4, 46-49), although many investigators have suggested that platelet lipoprotein may play a part and Marcus (49) has noted that phospholipid alone cannot account for the total procoagulant effect of platelets. Based on experiments involving primarily coagulation assays, Walsh has postulated that platelets participate in the formation of other active coagulation factors including Factors XI, and X.

We previously showed that platelets possess specific receptors for thrombin (6) and the current results that thrombin may be formed on the platelet surface as a result of the action of X, bound to its receptor. Under the in vitro conditions we used with low concentrations of X, and platelets, the time required to reach equilibrium thrombin generation and X, binding is relatively long. The small number of receptor sites (~200/platelet) of high affinity (K_a = 3.4 x 10^10 M^-1) could be saturated when 0.1% conversion of plasma X (5 to 10 ng/ml) to X, occurs. The ability of the saturated receptor to form thrombin is great; for example, 10^8 platelets saturated with X, (2 x 10^7 ng) can form thrombin at a rate of 50 units/min at room temperature. Rates much faster than this could occur in vivo where platelet concentrations at sites of injury are greater, the temperature is higher, etc. That we have defined a true receptor is suggested both by the correlation of high affinity binding with thrombin generation rates and the specificity of binding for Factor X. However, unlike most receptors the receptor-bound X, can form thrombin approximately 15-fold faster than an optimal mixture of X,, X, and phospholipids. The relation of the platelet X that participates in X, binding with thrombin generation rates and the specificity of binding is relatively long. The small number of receptor sites may be formed on the platelet surface as a result of the action of Factor X, occurs.'
Properties of the factor Xa binding site on human platelets.
J P Miletich, C M Jackson and P W Majerus


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