A Binding Site Expressed on the Surface of Activated Human Platelets Is Shared by Factor X and Prothrombin†

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ABSTRACT: We have demonstrated the presence of a saturable, reversible, and Ca^{2+}-dependent binding site for ^{125}I-labeled factor X ([^{125}I]factor X) on human platelets (16 000 ± 2 000 sites per platelet, K_d = 320 ± 40 nM, n = 12) activated with either thrombin or the thrombin receptor agonist peptide, SFLLRN-amide, but not with ADP. Bound [^{125}I]factor X could be completely removed by the addition of a Ca^{2+} chelator or an excess of unlabeled factor X. Antibodies that inhibit binding of factor X to the MAC-1 integrin receptor of monocytes and those directed against human factor V, failed to disrupt [^{125}I]factor X binding to platelets. Prothrombin, but neither factor VII, factor IX, protein C, nor protein S, was an effective competitor of [^{125}I]factor X binding with a $K_i \approx K_d$. [^{125}I]Prothrombin also binds to activated (but not unactivated) platelets in a saturable, reversible, and Ca^{2+}-dependent manner (20 500 ± 1 500 sites, K_d = 470 ± 110 nM, n = 3). Annexin V potently inhibited the binding of both [^{125}I]factor X and [^{125}I]prothrombin ($IC_{50} \approx 3$ nM). Factor X, prothrombin, and prothrombin fragment 1 (residues 1−155) were equipotent inhibitors of [^{125}I]prothrombin and [^{125}I]factor X binding, whereas Gla-domain-less factor X was unable to compete with [^{125}I]factor X for platelet binding sites. Thus, it is the Gla-domains of factor X and prothrombin that appear to contain the regions necessary for platelet binding. The results of studies utilizing artificial phospholipid surfaces have led to the hypothesis that the substrates (FX and prothrombin) for the intrinsic pathway FXase and prothrombinase complexes are bound to the phospholipid surface. The factor X/prothrombin binding site we have described on the surface of activated platelets permits the utilization of surface-bound substrates by these complexes when they are assembled on a *physiologic* surface.

The coagulation of blood consists of an intricate cascade of limited, proteolytic events which ultimately lead to the formation of an insoluble fibrin clot. Several enzymatic reactions within this cascade exhibit an effective requirement for the simultaneous presence of multiple cofactors. The activation of blood coagulation factor X (FX)[1] by FIXa_β occurs at physiologically significant rates only in the presence of calcium ions, FVIIIa and either activated platelets or negatively charged phospholipids. Analogously, prothrombin activation by FXa requires calcium ions, FVa and an appropriate surface (platelets or phospholipid). The activity of platelets in promoting coagulation has been hypothesized to result from the condensation of enzyme, substrate, and cofactors on their surface, thus requiring these proteins to interact with each other on the platelet surface and for at least one of them, and perhaps all, to interact with the surface directly.

Previous studies from our laboratory (Ahmad et al., 1989b) have demonstrated the presence of specific, saturable receptors for human FIXa_β on the surface of thrombin-activated platelets [∼600 sites per activated platelet with a dissociation constant (K_d) of 2.5 nM]. Similarly, Nesheim et al. (1988) have shown that rFVIII also binds to platelets in a saturable and specific manner (∼500 sites per activated platelets; $K_d \approx 3$ nM). The affinity of the interaction between FIXa_β and activated platelets is increased ≥5-fold in the presence of both FVIIIa and FX (but not in the absence of either), and occupancy of these receptors by FIXa_β is closely correlated with the rate of FX activation (Ahmad et al., 1989a).

Over the past fifteen years, there has been much learned about the assembly of the prothrombinase complex on both human (Miletich et al., 1977, 1978; Kane et al., 1980; Kane & Majerus, 1982; Trace et al., 1985) and bovine platelets

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[1] Abbreviations: FX, factor X; FIXa_β, activated factor IX; FXa, activated FX; FVIII, factor VIII; FVIIIa, activated FVIII; rFVIII, recombinant-derived FVIII; FV, factor V; FVa, activated FV; FVIIa, activated factor VII; FXI, factor XI; FXIa, activated FXI; ATIII, antithrombin III; BAEC, bovine aortic endothelial cells; BSA, bovine serum albumin; wGFP, albumin density gradient washed gel-filtered platelets; DAPA, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide; DEGR-CK, dansyl-L-glu-gly-L-arg chloromethyl ketone; EDTA, disodium ethylenediaminetetraacetic acid; Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TSV, crude *Oxyuranus scutellatus* (Taipan Snake) venom; FPR-CK, D-phe-L-pro-L-arg chloromethyl ketone; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HEPPS, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid; NPP, normal pooled plasma; PRP, platelet-rich plasma; RVV-X, the FX-activating enzyme isolated from *Vipera russeli* (i.e., Russell's Viper); S-2238, *H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; S-2765, *H*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide dihydrochloride; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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(Dahlback & Stenflo, 1978; Tracy et al., 1979, 1981). Although there are differences in some of the particular results of these studies, it is evident that FV(a) binds directly to platelets (Tracy et al., 1979, 1981; Kane & Majerus, 1982) and then provides a high-affinity binding site for FXa (Miletich et al., 1978; Kane et al., 1980; Tracy et al., 1981). In the presence of excess human FVa, there are between 200 (Miletich et al., 1978; Kane et al., 1980) and 5000 (Tracy et al., 1985) sites for FXa on human platelets with a K_d between 30 pM (Miletich et al., 1978; Kane et al., 1980; Kane & Majerus, 1982) and 200 pM (Tracy et al., 1985). The binding of human FVa to human platelets is not saturated (Kane & Majerus, 1982; Tracy et al., 1985) at concentrations up to ~12 nM where, in the presence of FXa and prothrombin, ~3000 molecules are bound per cell (Kane & Majerus, 1982). Nonetheless, the kinetic sequelae of FVa binding, in terms of its ability to enhance the catalytic efficiency of FXa toward prothrombin, saturates between 200 and 500 pM (Kane & Majerus, 1982; Tracy et al., 1985).

Activated platelets alone promote FX activation by $FIXa_\beta$ primarily through effecting a 2−3 orders of magnitude decrease in the apparent Michaelis constant (K_m). One of the ways by which activated platelets could promote this reaction is by expressing functional binding sites for FX in addition to those for $FIXa_\beta$ and FVIII. By analogy, part of the decrease in the K_m, from 80 μM (Rosing et al., 1980) to 0.5 μM (Tracy et al., 1985), brought about by platelets for the activation of prothrombin by FXa may result from a direct interaction between prothrombin and platelet binding sites. The following studies were undertaken to determine the existence of the postulated FX and prothrombin binding sites on platelets and to elucidate the nature of these zymogen binding interactions, if found.

EXPERIMENTAL PROCEDURES

Materials. Heparin from porcine intestinal mucosa (168 units/mg), biotin-labeled lactoperoxidase (35 units/mg), Sepharose 2B-CL, Kodak X-AR film, EGTA, TSV, ovalbumin, ELISA-grade BSA, and Sephadex G-50 (fine) were purchased from Sigma Chemical Co. (St. Louis, MO). Calcium nitrate and hydrogen peroxide were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Methyl silicon oil (1.0 DC200) and Hi Phenyl silicon oil (125 DC550) were purchased from William F. Nye, Inc. (Fairhaven, MA). Carrier-free $Na^{125}I$ was obtained from Amersham Corp. (Arlington Heights, IL). Avidin coupled to an agarose matrix and the BCA protein assay reagent were obtained from Pierce Chemical Co. (Rockford, IL). TCA and disodium EDTA were obtained from Fisher Scientific (Springfield, NJ). HEPES, HEPPS, FRP-CK, and DEGR-CK were obtained from Calbiochem-Behring Corp. (San Diego, CA). The specific thrombin inhibitor, DAPA (Nesheim et al., 1979), was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Chromogenic substrates, S-2238 and S-2765, were purchased from AB Kabi Diagnostica (Stockholm, Sweden). Normal pooled human plasma and factor-deficient human plasmas were obtained from George King Biochemical (Overland Park, KS). Nucleopore polycarbonate membranes (100 nm pore diameter) were purchased from Costar Corp. (Cambridge, MA). Bovine brain phosphatidylserine and L-α-dioleoylphosphatidylcholine were purchased from Avanti Polar Lipids (Birmingham, AL). All reagents used for SDS−PAGE were purchased from Bio-Rad Laboratories, Inc. (Melville, NY). The thrombin receptor agonist peptide, SFLLRN-amide (Scarborough et al., 1992; Hui et al., 1992), was synthesized using 9-fluorenylmethyloxycarbonyl (FMOC) chemistry on a Applied Biosystems 430A synthesizer and reverse-phase HPLC purified to greater than 99% homogeneity. All other chemicals were reagent grade or better.

Proteins. Human FX was purchased from both Enzyme Research Laboratories, Inc. (South Bend, IN) and Haematologic Technologies, Inc. Other highly purified human coagulation factors FXa, FIX (200 units/mg), $FIXa_\beta$, FVII (2000 units/mg), protein C, protein S (100 units/mg), prothrombin (11 units/mg), RVV-X, and human ATIII (1,250 antithrombin units/mg) were all purchased from Enzyme Research Laboratories, Inc. Human α-thrombin (4165 units/mg) was purchased from Sigma or was kindly provided by Dr. Genesio Murano (CBER, Bethesda, MD) (3008 units/mg). High-purity rFVIII (>4000 units/mg) was the generous gift of Genetics Institute (Cambridge, MA) and Baxter Healthcare Corp. (Duarte, CA). Human prothrombin fragment 1 was liberally supplied by Dr. Sriram Krishnaswamy (Emory University, Atlanta, GA). Gla-domain-less FX ($FX_{DES1-44}$) was the kind gift of Drs. Ray Rezaie and Charles T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). A monoclonal antibody to human FV which is known to inhibit prothrombinase activity was purchased from Haematologic Technologies, Inc. and burro anti-human FV IgG was the generous gift of Drs. Paula Tracy and Kenneth Mann (University of Vermont, Burlington, VM). Antibodies to the CD11b/CD18 or MAC-1 receptor (OKM-1) and to the "activated" form of MAC-1 (7A10) were kindly provided by Dr. Thomas Edgington (Scripps Research Institute, La Jolla, CA). OKM-1 was purified from ascites by sequential octanoic acid and ammonium sulfate precipitation (McKinney & Parkinson, 1987; Ogden & Leung, 1988; Perosa et al., 1990). Human placenta annexin V (Funakoshi et al., 1987) was given to us by Dr. Kazuo Fujikawa (University of Washington, Seattle, WA). All proteins were >95% pure as judged by SDS−PAGE and protein staining by Coomassie Brilliant Blue.

Molecular Weights and Extinction Coefficients. The following published values for molecular weights (Da) and extinction coefficients ($\epsilon_{280}^{0.1\%}$) were used: FX, 58 900, 1.16 (Fujikawa et al., 1972); FXa, 46 000 (Di Scipio et al., 1977a), 1.16 (Di Scipio et al., 1977b); FIX, 55 000 (Osterud et al., 1978), 1.32 (Di Scipio et al., 1977b); $FIXa_\beta$, 45 000 (Di Scipio et al., 1977b), 1.40 (Lindquist et al., 1978); prothrombin, 72 000, 1.42 (Tracy et al., 1992); prothrombin fragment 1, 21 700, 1.19;[2] ATIII, 58 000 (Damus & Rosenberg, 1976), 6.5 (Nordenman et al., 1977); RVV-X, 79 000 (Kisiel et al., 1976), 1.34 (Williams & Esnouf, 1962); thrombin, 36 700, 1.83 (Fenton et al., 1977); the average molecular weight for FVIII was 220 000 (Fay, 1988), and the porcine extinction coefficient 1.2 (Lollar et al., 1988) was used in absence of a value for the human protein. The molecular weight for SFLLRN-amide was calculated from the amino acid content to be 748 Da.

Protein Measurement. Protein concentration was determined by the absorption at 280 nm using published extinction coefficients and correcting for the contribution of Raleigh scattering (Mach & Middaugh, 1993) or by using the BCA

[2] S. Krishnaswamy, personal communication.

protein assay from Pierce. The concentration of SFLLRN-amide was calculated from the absorbence at 257 nm using the published extinction coefficient for phenylalanine, 200 M^{-1} cm^{-1} (Freifelder, 1982).

Phospholipid Vesicles. Phospholipid vesicles (composed of bovine brain phosphatidylserine and L-α-dioleoylphosphatidylcholine in a 1:3 molar ratio) were synthesized in a custom-designed apparatus by high-pressure nitrogen extrusion through Nucleopore polycarbonate membranes essentially as described by Hope et al. (1985; Mayer et al., 1986).

Buffers. Buffer A (0.5 mg of BSA/mL, 126 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.38 mM NaH_2PO_4, 5.6 mM dextrose, 15 mM HEPES, pH 7.4 at 37 °C) was used for most experiments when a physiologic buffer was desired. Buffer B (0.5% ovalbumin, 175 mM NaCl, 50 mM HEPES, pH 8.1 at 37 °C) was used in many enzyme assays because of the increased activity of the serine proteases in this buffer. Stopping buffer (buffer B containing 20 mM EDTA) was used to measure amidolytic activity of activated coagulation factors. Stock protein solutions were dialyzed into and stored in buffer C (135 mM NaCl, 15 mM HEPES, pH 7.4 at 37 °C). ACD (71.4 mM citric acid, 85 mM trisodium citrate, 111 mM dextrose) was used as an anticoagulant and platelet stabilizer for blood collection.

Active-Site Titration of Coagulation Factors. FXa and thrombin were active-site titrated with FMGB·HCl essentially as described (Melhado et al., 1982; Bock et al., 1989). Human ATIII, calibrated with active-site titrated thrombin, was used in the molar determination of $FIXa_\beta$ active-sites.

Briefly, a linear dilution series of calibrated ATIII (0−100 nM) was made into buffer A containing 20 μg of heparin/mL and 5 mM $CaCl_2$. $FIXa_\beta$ was diluted to approximately 100 nM (estimated from protein concentration) in buffer A containing 5 mM $CaCl_2$ and then mixed (1:1 v/v) with each of the ATIII dilutions. After a 20 min incubation at 37 °C, the mixture was further diluted (1:9 v/v) into buffer A.

The remaining $FIXa_\beta$ activity was measured as follows: (i) 20 μL of buffer A containing 1.2 μM FX, 12 units rFVIII/mL, 60 μM phospholipid vesicles, and 15 mM $CaCl_2$ were added to Eppendorf tubes; (ii) 20 μL of a solution containing the residual $FIXa_\beta$ activity (0−10 nM) was then added; (iii) the reaction was started by the addition of 20 μL of 10 units of thrombin/mL in buffer A, and the mixture was placed in a 37 °C water bath; (iv) after a 2 min incubation, 50 μL of the mixture was diluted into 50 μL of ice-cold stopping buffer. The FXa generated was then quantified in the following manner. Aliquots of the solution (75 μL) were placed into wells of a microtiter plate and warmed to 37 °C. To each well was added an equal volume of warm substrate solution (stopping buffer containing 700 μM S-2765 and 2 μM DAPA), and the release of *p*-nitroaniline from the substrate was monitored kinetically at 405 nm in a Molecular Devices ThermoMax microplate reader (Menlo Park, CA) at 37 °C.

Data were analyzed by plotting the final velocity of *p*-nitroaniline generation vs the concentration of ATIII added in the initial incubations. A line was then fitted to the data by nonlinear least-squares regression, and the parameters so obtained (slope and *y*-intercept) were used to calculate the *x*-intercept. When plotted in this manner the *x*-intercept is equal to the concentration of the activated coagulation factor in the initial dilution.

Iodination of FX and Prothrombin. Human FX and prothrombin were radioiodinated via lactoperoxidase with minor modifications of a method used to radiolabel porcine FVIII.[3] Briefly, FX or prothrombin (0.5−5 mg/mL) was exchanged into 5 mM calcium nitrate, 40 mM sodium acetate, pH 6.5. To this were added biotinylated lactoperoxidase (0.8 units/mL, final concentration), carrier-free $Na^{125}I$ (5−60 mCi per mL, final concentration), and H_2O_2 (0.0001% w/v, final concentration) in rapid succession. Incubation was carried out for 3−8 min at ambient temperature with intermittent vortex mixing. At this time, sodium azide and avidin-agarose were added to 0.1% (w/v) and 5% (v packed bed/v), respectively. The mixture was vortex mixed and incubated for 5 min after which a small aliquot was removed for determination of percent incorporation and the remainder was applied to a 1 mL G-50 Sephadex Spin-Column made in a tuberculin syringe fitted with a porous polyethylene disk and equilibrated with buffer C containing 0.1% PEG 8000. Typical yields were as follows: (i) 75−95% incorporation of ^{125}I into the protein; (ii) greater than 99% of the radioactivity eluted from the Spin-Column was trichloroacetic acid precipitable; (iii) approximately 90% of the original protein was recovered; (iv) greater than 90% retention of the coagulation factor's functional activity as measured in the FX-activation assay, by active-site titration after activation with RVV-X or TSV (for FX and prothrombin, respectively) and by standard one-stage clotting assay (Biggs, 1972); (v) specific radioactivity of the labeled protein varied from 2×10^7 CPM/μg (approximately 0.05−0.5 mol of ^{125}I per mol of protein); and (vi) the labeled proteins and its activated form were indistinguishable from the native proteins upon SDS−PAGE and autoradiography (FX and prothrombin were activated for gels with RVV-X and crude Taipan snake venom, respectively). Iodinated proteins were stored at 4 °C and used within 4 days of labeling.

FX Activity Measurement. All activity measurements were performed at 37 °C in buffer B. Samples consisted of normal pooled plasma (NPP), [^{125}I]FX, and the unlabeled FX from which the [^{125}I]FX was prepared. Each sample was diluted into buffer B to less than approximately 10 nM, and then a linear dilution series spanning the 0.1−10 nM range was made from the initial dilution. These dilutions were each processed as follows: (1) To each tube was added 20 μL of a solution containing rFVIII (1 unit/mL) and 2 mM $CaCl_2$ in buffer B. (2) Next, 20 μL of the FX-containing sample (diluted into buffer B) was added. (3) The reaction was started by the addition of 20 μL of buffer B containing $FIXa_\beta$ (100 nM), thrombin (100 nM), phospholipid vesicles (60 μM), and $CaCl_2$ (30 mM). The mixture was vortexed and placed in a 37 °C water bath. (4) After 60 s, 50 μL was removed from the reaction mixture and diluted into 100 μL of ice-cold stopping buffer. (5) FXa activity was measured as the velocity of *p*-nitroaniline release from S-2765 as described above. The averaged, background-subtracted, velocity measurements were plotted versus the inverse of the stock sample's dilutional titer. Slopes were fitted to each series of sample dilutions. By plotting the data in this manner, the ratio of the slopes of any two curves is equal to

[3] J. S. P. Lollar, personal communication.

the ratio of the FX concentration in the samples from which the dilution series were made.

Prothrombin Activity Measurement. Approximately 1 μM prothrombin was incubated in buffer A containing $CaCl_2$ (5 mM), phospholipid vesicles (50 μM), and crude Taipan snake venom (5 μg/mL) for 20 min at 37 °C. The mixture was then diluted 10-fold into buffer A containing 5 mM $CaCl_2$ and active-site titrated as usual. The molar concentration of active-sites so obtained was then used to calculate the specific activity of the unactivated prothrombin.

Platelet Isolation. Human venous blood was collected into ACD (7:1 v/v) through a 16 gauge needle. Twice washed, concentrated platelets were isolated with an albumin density gradient essentially as described by Walsh et al. (1977), resuspended in buffer A to $^1/_3$ to $^1/_5$ the original volume of PRP, and then gel-filtered on a Sepharose 2B-CL column equilibrated with buffer A. We refer to these platelets as albumin density gradient washed, gel-filtered platelets (wGFP). Platelets isolated in this manner were found to contain no detectable levels of prothrombin or FX activity (Walsh et al., 1977), nor were detectable levels of iodinated protein that had been added to the freshly collected blood found in the wGFP. Similarly, platelets prepared in this manner have no detectable contamination with ristocetin cofactor activity (vWF) whereas platelets isolated by gel-filtration alone have a large contamination with this protein. The concentration of wGFP was determined with an electronic particle counter (Coulter Electronics, Hialeah, FL).

Binding Experiments. All binding experiments were performed in straight-sided microfuge tubes having a narrow, 15 μL tip at the bottom. To each tube was added 15 μL of a mixture of silicon oils (Dow-Corning 500 and Dow-Corning 200, mixed 4:1 v/v), and each tube was briefly centrifuged horizontally at 12 000*g* in a Beckman Microfuge E to localize the oil to the tube's tip. Additions were made to the tube so that a total of 50 μL was located above the oil layer. This 50 μL contained [^{125}I]FX or [^{125}I]prothrombin, and, when needed, $CaCl_2$, a platelet activator (thrombin, ADP, or SFLLRN-amide), and/or competitors all at twice their desired final concentration. The tubes were again centrifuged briefly to centralize the liquid to the bottom of the tube. At specified times, 50 μL of the wGFP were added to each tube and the tubes were briefly vortexed to ensure adequate mixing. After a fixed interval of time (generally 30 min unless otherwise specified), the tubes were centrifuged for 5 min to pellet the platelets and any bound proteins through the silicon oil mixture. Following centrifugation, a 10 μL aliquot of the supernatant was removed and ^{125}I content was determined by counting γ-emission in a Wallac 1470 Wizard γ-Counter (Gaithersburg, MD) using an ^{125}I energy window. The tubes were then frozen in dry ice, and afterward the tips containing the platelet pellets were amputated and the radioactivity measured. The radioactivity in the supernatant sample and the pellet of each tube were used to calculate the free [^{125}I]zymogen concentration and the total bound [^{125}I]zymogen, respectively.

Nonspecific binding was measured as the binding observed in the presence of excess unlabeled zymogen, FX or prothrombin, and a plot of the nonspecific binding was entirely linear. The concentration of unlabeled zymogen used to determine nonspecific binding was a 10−50-fold molar excess over the sum of the average K_d and the concentration of [^{125}I]zymogen. "Specific" binding was obtained by subtracting from the total binding measurements the nonspecific component calculated with the slope obtained from the nonspecific data and the [^{125}I]zymogen concentration.

The volume of supernatant fluid that is trapped in the platelet pellet was determined on seven separate occasions by incubation of platelets [$(1-5) \times 10^8$ per mL] with [^{125}I]-BSA. When normalized to the number of platelets, the trapped volume was insensitive to the platelet concentration used in the incubation (at very high platelet concentrations, i.e., $\geq 10^9$ per mL, the normalized trapped volume was less consistent). The trapped volumes observed for activated and unactivated platelets were very similar to each other (7−14 fL per activated platelet and 6−10 fL per unactivated platelet), and they were very similar to the value reported by Greengard and Griffin (1984) using a similar technique. This volume corresponds to 4−9 molecules trapped per activated platelet per nM in the supernatant. Nonspecific binding was judged to be entirely due to this supernatant fluid trapped in the platelet pellet.

The specific binding was best represented as a simple binding phenomenon, and parameters were obtained by a direct nonlinear least-squares fit to the following equation:

$$B = \frac{B_{max}F}{K_d + F}$$

where B is the amount of specifically bound [^{125}I]FX or [^{125}I]-prothrombin per platelet, B_{max} is the amount of specifically bound [^{125}I]FX or [^{125}I]prothrombin per platelet at saturation, F is the concentration of [^{125}I]FX or [^{125}I]prothrombin in the supernatant, and K_d is the dissociation constant for the binding interaction.

Solubilization of Platelet Pellets and Electrophoresis. Binding experiments were carried out as described above with the following modifications: (1) the incubations were conducted in standard 1.5 mL Eppendorf tubes; (2) 100 μL of the silicon oil mixture was placed in the bottom of the tube; and (3) the volume of the incubation was increased to a total of 500 μL while the ratios were kept fixed. After the incubation and centrifugation steps, an aliquot of the supernatant or the platelet pellet was solubilized in 10% acetic acid, 1% SDS, and then heated for 10 min at 100 °C to denature any proteases. Before SDS−PAGE, the samples were lyophilized overnight and then resolubilized in sample buffer, heated at 100 °C for 3 min, and centrifuged at 16 000*g* for 5 min to remove particulate material. SDS−PAGE was carried out essentially as described by Laemmli (1970), and exposure of autoradiography film to fixed and dried gels was conducted at −70 °C in the presence of an Cronex Lightning Plus intensifying screen (DuPont-NEN, Boston, MA).

Numerical Analysis. All curve fits were obtained using the program Kaleidagraph 3.0 running on a Macintosh Quadra 900 personal computer (Apple Computer, Inc., Cupertino, CA). This program utilizes a nonlinear curve-fitting algorithm known as the Levenberg−Marquardt method (Marquardt, 1963).

RESULTS

Binding of Factor X to Activated Platelets. The specific binding of [^{125}I]FX to wGFP activated with SFLLRN-amide is shown in Figure 1. Parameter values obtained from the

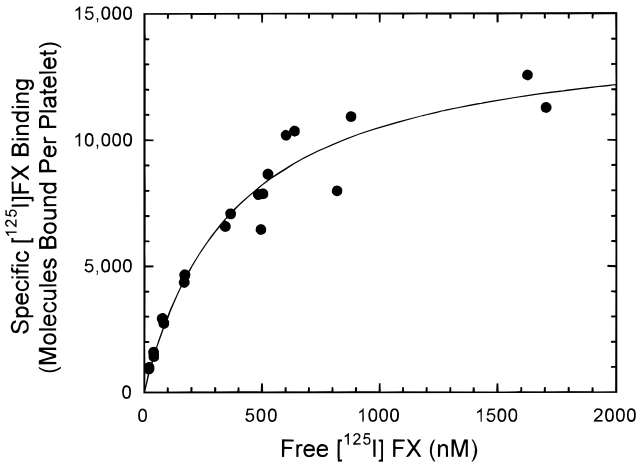

FIGURE 1: Specific binding of [^{125}I]FX to activated platelets. Human platelets (4.1×10^8) were incubated with [^{125}I]FX (20−1750 nM), SFLLRN-amide (25 μM), and $CaCl_2$ (5 mM) at 37 °C for 40 min (Experimental Procedures). The filled circles represent the specific binding determined as the difference observed in the quantity of [^{125}I]FX bound when incubations were carried out in the absence and presence of 25 μM unlabeled FX. The solid line represents the nonlinear least-squares fit of this data to the simple binding equation and yields parameters (K_d = 380 nM, B_{max} = 15 000) that are in close agreement with the average values obtained from eleven similar experiments.

nonlinear least-squares fit to twelve independent experiments (i.e., distinct platelet donors and different preparations of [^{125}I]FX) were averaged yielding 16 000 ± 2 000 binding sites per platelet with a K_d of 320 ± 40 nM (average ± standard error).

Despite our inability to demonstrate a functional deficiency in the radiolabeled FX preparations using several different assay systems (see Experimental Procedures), it was necessary to ensure that the [^{125}I]FX had not suffered any untoward effects with respect to its ability to interact with activated platelets. As shown in Figure 2, [^{125}I]FX was found to bind platelets in a manner that is indistinguishable ($K_d \approx K_i$) from native FX.

Requirements for Factor X Binding to Platelets. Platelets must be activated before they express [^{125}I]FX binding sites (Figure 3A). In the presence of 5 mM $CaCl_2$, platelets exposed to 25 μM SFLLRN-amide expressed 17 000 sites per platelet with a K_d of 260 nM while unactivated platelets, incubated with 1 μM PGE, exhibited only low-level binding which saturated at approximately 400 molecules per platelet. Binding to unactivated platelets may represent a small population of platelets that were activated during the isolation procedure. Similarly, platelets incubated with 10 nM thrombin that had been irreversibly inhibited with FPR-CK expressed no specific binding sites for FX (data not shown). Calcium was found to be necessary for [^{125}I]FX to bind to sites expressed on the surface of activated platelets (Figure 3B). In the presence of 5 mM $CaCl_2$, SFLLRN-amide (25 μM)-activated platelets bound [^{125}I]FX in the typical manner (14 000 sites per platelet with a K_d of 200 nM). However, when the incubation was carried out with 5 mM EDTA (or EGTA, not shown), slightly less [^{125}I]FX was bound per platelet than was determined from the nonspecific binding data measured using excess unlabeled FX; hence, total binding in the presence of EDTA is shown in Figure 3B.

Thrombin and SFLLRN-amide were equally efficacious in their ability to bring about the expression of FX binding

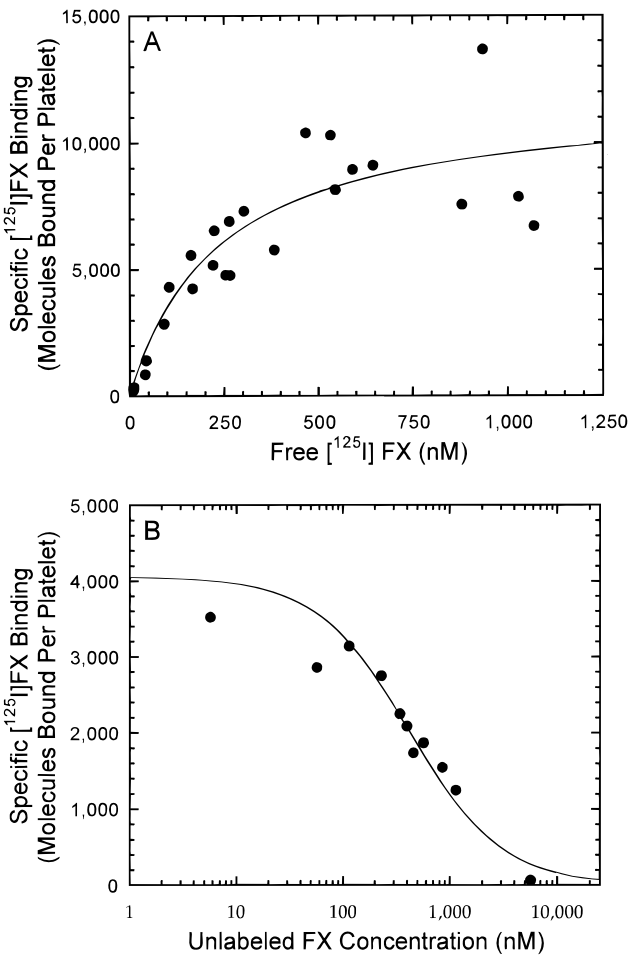

FIGURE 2: Parallel experiments determining the K_d for [^{125}I]FX binding to activated platelets and the K_i for FX competition. (A) Specific binding of [^{125}I]FX to platelets activated by SFLLRN-amide (20 μM) in the presence of 5 mM $CaCl_2$. Incubations were carried out for 30 min at ambient temperature. The solid line represents the nonlinear least-squares fit of this data to the simple binding equation and yields 12 000 binding sites per platelet with a K_d of 240 nM. (B) Competition of unlabeled FX for the binding of 120 nM [^{125}I]FX to activated platelets. The solid line represents a nonlinear least-squares fit of this data to the competitive binding equation (i.e., $B = B_{max}F/(K_d(1 + I/K_i) + F)$) where B_{max} and K_d values from panel A were inserted along with the measured concentration of [^{125}I]FX. The fit yielded a K_i = 270 nM for unlabeled FX.

sites by platelets (data not shown), and, as expected, thrombin was much more potent than the peptide (EC_{50}'s of 1.6 nM and 14 μM, respectively). Curiously, ADP did not induce the expression of FX binding sites on platelets even at concentrations (up to 50 μM) well in excess of those required to induce platelet aggregation.

Kinetics and Reversibility of Factor X Binding to Platelets. At all times, the binding of [^{125}I]FX to platelets could be reversed by the addition of excess unlabeled FX (Figure 4A), EDTA (Figure 4B), or by dilution of the incubation mixture with buffer (buffer A containing SFLLRN-amide and $CaCl_2$) (data not shown). The dissociation of bound [^{125}I]FX from the platelet surface is virtually instantaneous when EDTA is added (10 mM final), and the amount of [^{125}I]FX pelleted with the platelets after EDTA treatment is that which is expected for the volume of trapped supernatant. When excess unlabeled FX was added after 20 min, dissociation of the bound [^{125}I]FX was observed with ≥70% of the specific binding removed in the first 40 s and ≥85% removed

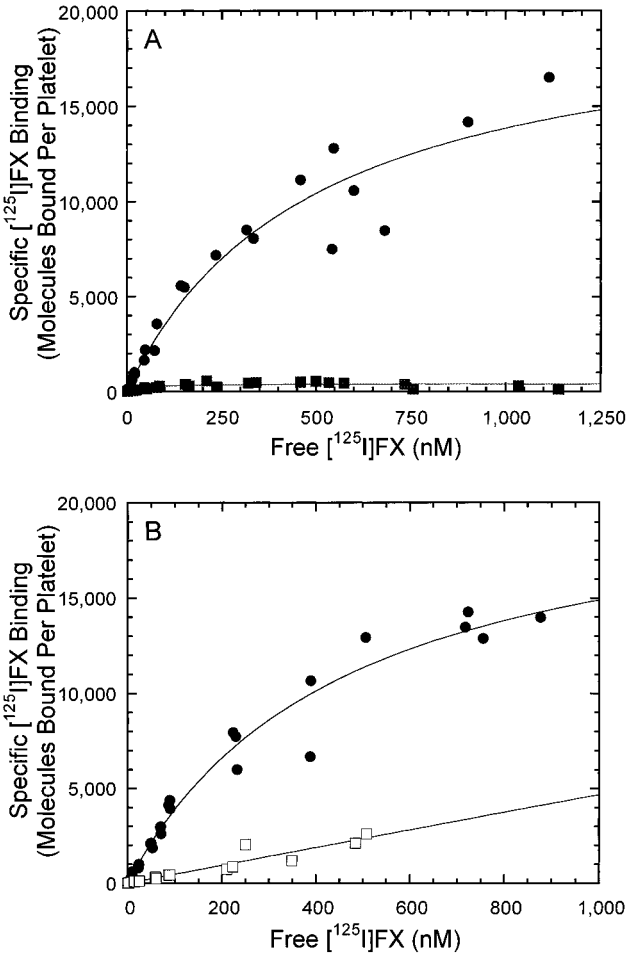

FIGURE 3: FX binding requires platelet activation and calcium ions. (A) [^{125}I]FX binding to platelets which had either been exposed to 25 μM SFLLRN-amide (filled circles) or 1 μM PGE1 (filled squares) in the presence of 5 mM $CaCl_2$. The curves are nonlinear least-squares fits to the simple binding equation (B_{max} was 21 000 and 400 for activated and unactivated platelets, respectively; the K_d was 490 nM). (B) [^{125}I]FX binding to SFLLRN-amide (25 μM)-activated platelets in the presence of either 5 mM $CaCl_2$ (filled circles) or 5 mM EDTA (open squares). The data from platelets in the presence of $CaCl_2$ are measurements of the specific binding (B_{max} = 22 000 and K_d = 460 nM) determined with excess unlabeled FX, whereas the data from platelets in the presence of EDTA are the total amounts bound under these conditions (4.7 molecules of [^{125}I]FX bound per platelet per nM [^{125}I]FX).

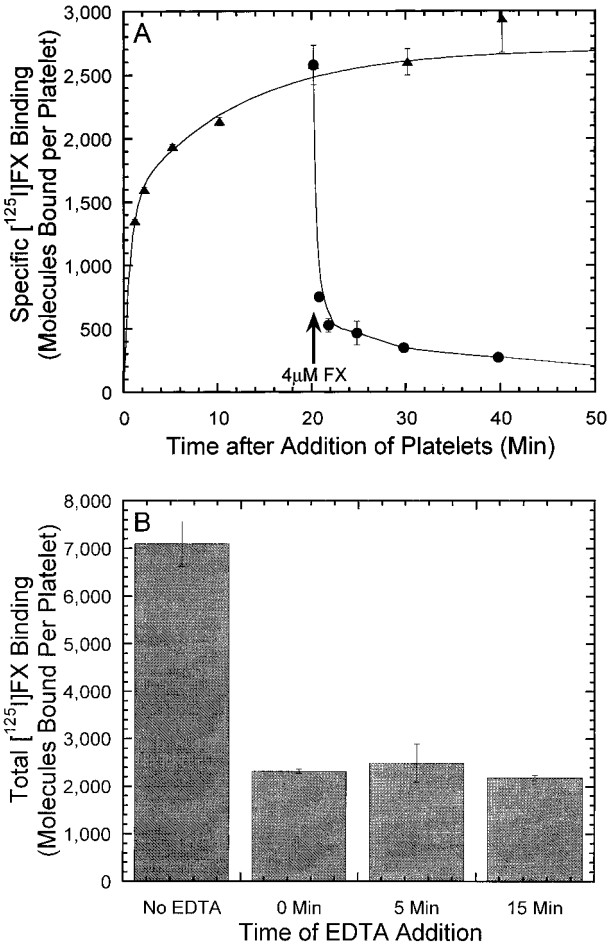

FIGURE 4: [^{125}I]FX binding to activated platelets is reversible at all times. All incubations were carried out at ambient temperature in the presence of 150 nm [^{125}I]FX, 25 μM SFLLRN-amide, and 5 mM $CaCl_2$. Error bars represent the standard error in the mean of two measurements. (A) Specific binding of [^{125}I]FX measured at various times after the addition of platelets (Experimental Procedures) to the incubation mixture (filled triangles). The dissociation of bound [^{125}I]FX by unlabeled FX (4 μM), added after a 20 min pre-incubation of the platelets with [^{125}I]FX (150 nM), is also shown (filled circles). Smooth curves are drawn. (B) [^{125}I]FX bound to activated platelets after a 20 min incubation with 150 nM [^{125}I]-FX. The first bar represents the total binding in the presence of 5 mM $CaCl_2$; the three bars to the right represent the [^{125}I]FX in the pellet at 20 min when EDTA (10 mM final) was added just before, 5 min after, or 15 min after platelet addition.

after 10 min (theoretically, ~89% of the [^{125}I]FX would be displaced at equilibrium). Similar results were obtained when the reaction mixture was diluted 6-fold with buffer after a 20 min preincubation. Nonspecific binding was constant after platelet activation (data not shown), implying that the trapped volume remained constant and that the [^{125}I]-FX was neither internalized nor irreversibly sequestered by the platelets.

Specifically of the FX Binding Site on Platelets. Several vitamin K-dependent coagulation factors were screened for their ability to compete with [^{125}I]FX for binding sites on activated platelets. In the presence of 5 mM $CaCl_2$ and a plasma concentration of [^{125}K]FX (~140 nM), neither FIX (up to 700 nM, 7 times the plasma FIX concentration, a 5-fold molar excess) nor FVII (up to 250 nM, 25 times the plasma concentration of FVII, a 2-fold molar excess) could effectively prevent the binding of [^{125}I]FX to activated platelets (Figure 5). Protein S possessed a moderate ability to compete with [^{125}I]FX, preventing ~40% of the [^{125}I]FX from binding when present at a concentration of 4 μM (20 times the concentration of free protein S in plasma, ~30-fold molar excess over the [^{125}I]FX concentration). Prothrombin, however, was found to be a potent inhibitor of [^{125}I]FX binding, having an IC_{50} (650 nM) approximately equal to that of unlabeled FX (680 nM).

These initial experiments were designed to elucidate the factors which are physiologically relevant competitors for the platelet site to which FX binds. It is clear that neither FIX, FVII, nor protein S has a significant effect on the binding of FX when all are present at their plasma concentrations. However, prothrombin, with a plasma concentration (~1.4 μM) which is approximately 10 times that of FX, will displace ~75% of the bound [^{125}I]FX.

Under specific conditions, all vitamin K-dependent coagulation factors are capable of reducing the extent to which [^{125}I]FX binds to activated platelets; yet their capacity to do so is unequal. In an experiment measuring the ability of

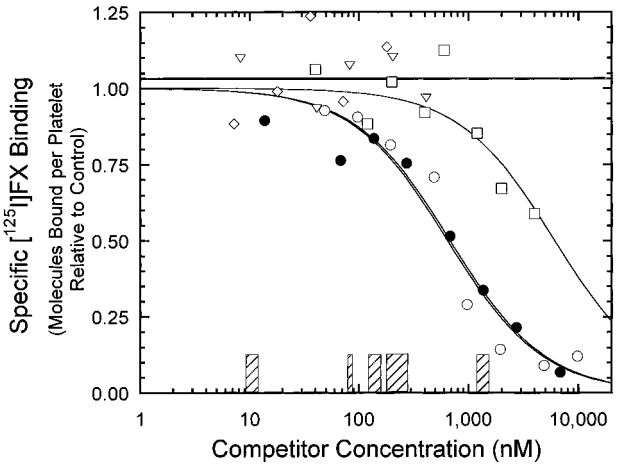

FIGURE 5: Competition for [^{125}I]FX binding to activated platelets by vitamin K-dependent coagulation factors. All incubations were carried out in parallel for 30 min at ambient temperature in the presence of 25 μM SFLLRN-amide, 5 mM $CaCl_2$, 140 nM [^{125}I]-FX, and increasing concentrations of FX (filled circles), prothrombin (open circles), protein S (open squares), FIX (open triangles), or FVII (open diamonds). Boxes at the bottom of the figure represent the plasma concentrations of FVII, FIX, FX, protein S, and prothrombin (left to right, respectively).

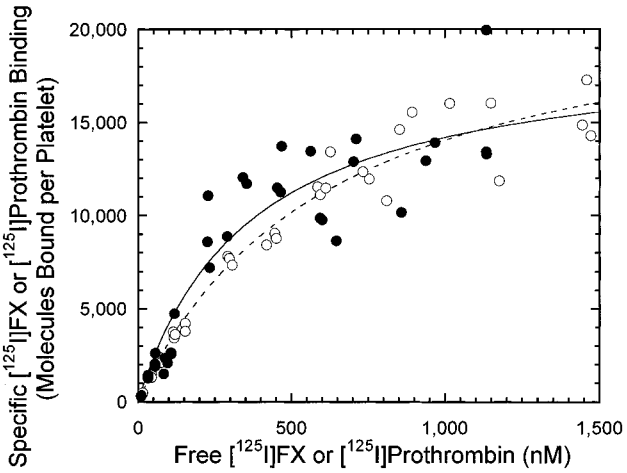

FIGURE 6: [^{125}I]Prothrombin also binds to a site on activated platelets. Direct comparison of specific binding of [^{125}I]prothrombin (filled circles) and [^{125}I]FX (open circles). The solid line represents the best fit of the simple binding equation to the prothrombin data and yields 19 000 sites for [^{125}I]prothrombin with a K_d of 360 nM. Similarly, the dashed line yields 22 000 sites with K_d of 610 nM for [^{125}I]FX. All incubations were carried out for 40 min at ambient temperature in the presence 5 mM $CaCl_2$ and 25 μM SFLLRN-amide.

various factors present at 2.5 μM to compete with [^{125}I]EX (45 nM) for platelet binding sites, the competitors were found to fall into one of two general categories: (1) factors that compete about the same as unlabeled FX (i.e., prothrombin); and (2) those that compete in a manner similar to protein S such as FVII, FIX, and protein C. In the experiment outlined above, FX and prothrombin displaced 82%[4] and 73% of the specifically bound [^{125}I]FX whereas FVII, FIX, protein C, and protein S displaced 33%, 30%, 23%, and 29% of the [^{125}I]FX, respectively.

Prothrombin Can also Bind Directly to a Site on Activated Platelets That Has Characteristics Similar to the Site Expressed for Factor X. The ability of prothrombin to prevent [^{125}I]FX from binding in competition studies suggested that it may be interacting directly with the binding site expressed by activated platelets for FX. To investigate this hypothesis, we radioiodinated prothrombin and conducted a series of binding experiments. The data shown in Figure 6 represents parallel binding measurements for [^{125}I]-prothrombin and [^{125}I]FX. The average parameters obtained from nonlinear least-squares fits to three direct binding experiments yields 21 000 $\pm$ 2 000 sites (mean $\pm$ SEM) available for [^{125}I]prothrombin on activated platelets with a dissociation constant of 470 $\pm$ 110 nM. The binding site for [^{125}I]prothrombin, like that for [^{129}I]FX, requires calcium ions and is not expressed by unactivated or ADP-stimulated platelets (data not shown). Further, prothrombin and FX are equally efficacious as inhibitors of either [^{125}I]FX or [^{125}I]-prothrombin binding to activated platelets (Figure 7A and B, respectively). It appears that the nature of the site expressed by activated platelets for prothrombin is identical to that of the site for FX; henceforth, we will refer to the site as the "zymogen binding site".

Structural Characterization of Factor X and Prothrombin Bound to Platelets. In order to ensure that the bound [^{125}I]-FX or [^{125}I]prothrombin pelleted with the platelet in binding studies was not a modified subset of those in the supernatant, we solubilized the platelet pellets and subjected them to SDS−PAGE and subsequent autoradiography. Autoradiographs of [^{125}I]FX and [^{125}I]prothrombin obtained from both the supernatant and pelleted fractions of binding experiments demonstrate that the proteins have mobilities identical to the native zymogens (Figure 8). When the autoradiography film was grossly overexposed (data not shown), no additional bands were observed in either the supernatant or pellet of the [^{125}I]FX incubation, but a few light bands with apparent molecular weights greater than 50 000 Da were observed in the [^{125}I]prothrombin supernatant and significantly fewer were observed in the pellet.

Nature of the Zymogen Binding Site on Platelets. Human platelets contain a significant quantity of FV within their α-granules (Miletich et al., 1978; Kane et al., 1980; Tracy et al., 1982; Schmaier, 1985), which is released and subsequently activated by thrombin. At the platelet concentrations used in our binding studies, enough FV could be released to bring the nominal concentration into the low nanomolar range (0.5−3 nM). We initially recognized the possibility that released FV may be contributing to the zymogen binding site. This concern was addressed with the use of antibodies to human FV which are known to disrupt the activity of FV in the prothrombinase complex. Both a monoclonal antibody to the light chain of FV and purified burro anti-human FV IgG were used (Table 1). Neither antibody could effectively compete with either [^{125}I]FX or [^{125}I]prothrombin for the binding sites expressed by activated platelets. Thus, it is unlikely that platelet FV provides the binding site for FX or prothrombin.

The binding site for FX on platelets is quite dissimilar from that expressed by activated monocytes (Altieri & Edgington, 1988). Antibodies (OKM1 and 7A10) that completely displace FX from the monocyte surface were found to be without effect in their ability to displace [^{125}I]-FX from the platelet surface (Table 1).

[4] Assuming the average K_d for FX binding and that labeled and unlabeled FX are competing equally for platelet binding sites, 2.5 μM FX should reduce the extent of [^{125}I]FX bound by 87%.

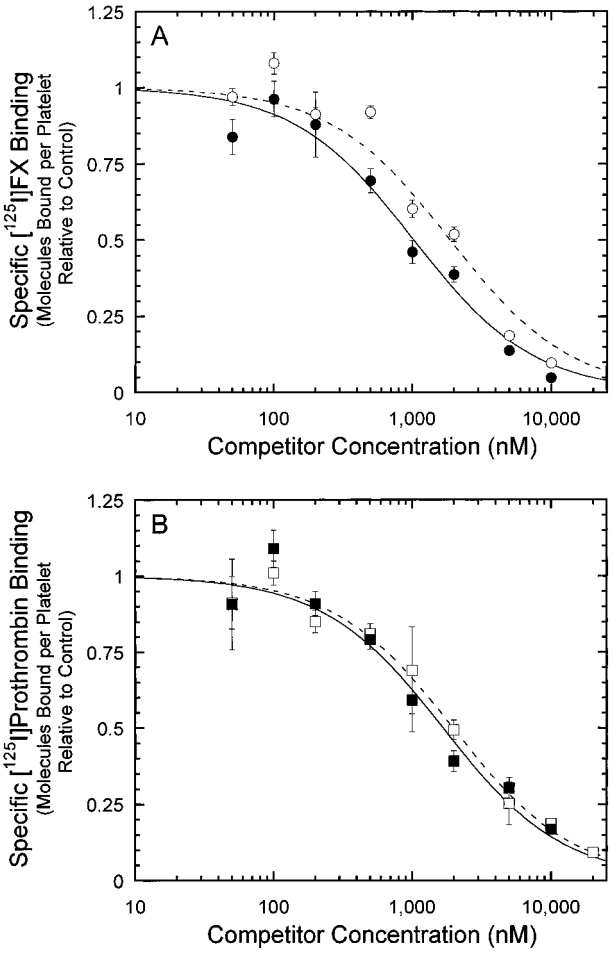

FIGURE 7: FX and prothrombin compete for each others' binding sites on activated platelets. Incubations were carried out in parallel for 40 min at ambient temperature in the presence 5 mM $CaCl_2$ and 25 μM SFLLRN-amide. Binding is expressed relative to the total quantity bound in the absence of competitor. Error bars represent the standard error in the mean of two measurements. (A) Relative amount of [^{125}I]FX bound when incubations were carried out in the presence of 150 nM [^{125}I]FX and either unlabeled FX (filled circles) or prothrombin (open circles). (B) Relative amount of [^{125}I]prothrombin bound when incubations were carried out in the presence of 150 nM [^{125}I]prothrombin and either unlabeled FX (filled squares) or prothrombin (open squares).

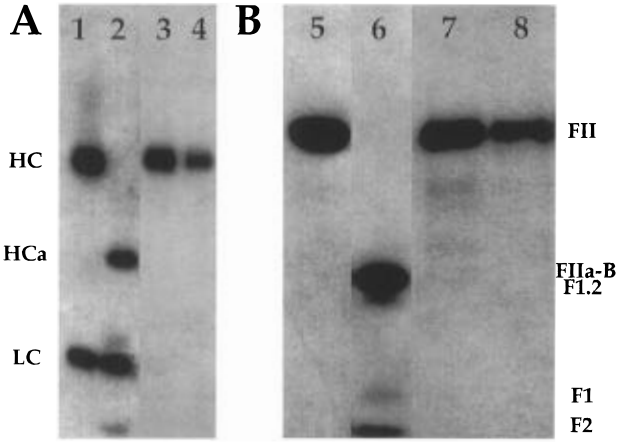

FIGURE 8: [^{125}I]FX and [^{125}I]prothrombin are electrophoretically unmodified when bound to sites on activated platelets. All samples were reduced with 5% β-mercaptoethanol prior to electrophoresis. (A) Autoradiograph of SDS−polyacrylamide gel, 12% acrylamide. Lane 1, [^{125}I]FX; lane 2, ^{125}I]FXa; lane 3, supernatant after a 30 min incubation of [^{125}I]FX (150 nM) with platelets at ambient temperature in the presence of SFLLRN-amide (25 μM) and 5 mM $CaCl_2$; lane 4, Pellet of the incubation described for lane 3. FX is a two-chain molecule consisting of a heavy chain (HC) containing the activation peptide and serine protease domain and a light chain (LC) that is composed of the Gla-containing region and two homologous EGF-like domains; the two chains are connected through a single disulfide linkage. The heavy and light chains have apparent molecular weights of 42 000 and 17 000, respectively. FX is activated by the cleavage of a single peptide bond with the subsequent release of the heavily glycosylated activation peptide from the heavy chain. The heavy chain of FXa (HCa) migrates with an apparent relative molecular weight of 29 000. The mobility of the FXa light chain is identical to that of FX. (B) Autoradiograph of SDS−polyacrylamide gel, 4%−20% acrylamide gradient. Lane 5, [^{125}I]prothrombin; lane 6, [^{125}I]prothrombin activated with TSV; lane 7, supernatant after a 30 min incubation of [^{125}I]prothrombin (150 nM) with platelets at ambient temperature in the presence of SFLLRN-amide (25 μM) and 5 mM $CaCl_2$; lane 8, pellet of the incubation described for lane 5. Prothrombin consists of a single polypeptide chain which runs with an apparent molecular weight of 72 000 (FII). When prothrombin is activated to thrombin by TSV, a number of cleavages are observed. All of the cleavages yield fragments which are readily distinguished from native prothrombin after SDS−PAGE. The bands labeled FIIa-B, F1.2, F1, and F2 are the B-chain of thrombin, prothrombin fragment 1.2, prothrombin fragment 1, and prothrombin fragment 2, respectively.

Table 1: Binding of neither [^{125}I]FX nor [^{125}I]prothrombin Is Inhibited neither by Antibodies That Inhibit FX Binding to Monocytes nor by Those Directed Against Human FV[a]

competing protein	% control binding of [^{125}I]FX	% control binding of [^{125}I]FII
none	100 ± 9	100 ± 7
FX (10 μM)	5.3 ± 0.3	4.9 ± 0.2
prothrombin (10 μM)	9 ± 1	9.8 ± 2
anti-hFV monoclonal (807 nM)	109 ± 9	101 ± 9
anti-hFV polyclonal (0.4 mg/mL)[b]	107 ± 14	92 ± 10
anti-MAC-1, OKM1 (1 μM)[c]	95	ND
anti-MAC-1, 7A10 (1 μM)[c]	102	ND

[a] All incubations were carried out at ambient temperature in the presence of 50 nM [^{125}I]FX or [^{125}I]prothrombin, 5 mM $CaCl_2$, and 25 μM SFLLRN-amide. [b] The IgG fraction of burro anti-hFV serum. [c] OKM1 is a monoclonal antibody directed against MAC-1, and 7A10 is a monoclonal specific for an activated form of MAC-1. [d] ND, not done.

Annexin V has a high affinity for phosphatidylserine-containing phospholipid vesicles and has been shown to bind to human platelets (Thiagarajan & Tait, 1990; Sun et al., 1993). As shown in Figure 9, annexin V is a very potent inhibitor of both FX and prothrombin binding to activated platelets. The observed IC_{50} of annexin V toward FX and prothrombin binding (3.1 and 2.6 nM, respectively) is close to the reported K_d for annexin V binding to activated platelets (7 nM). The inhibition curve is quite steep and is not well described by a simple competitive inhibition model. Since the binding of annexin V to platelets and phospholipid vesicles has not been reported as having an apparent positive cooperativity, its inhibition of FX and prothrombin binding may be the result of a more complex phenomenon than simple competition for binding sites.

Gla-Containing Regions of Factor X and Prothrombin May Mediate Binding to Activated Platelets. We wondered whether the Gla-containing regions of FX and prothrombin which are known to be important for phospholipid binding were also critical to their interaction with platelets. As an initial approach to this question, we measured the ability of prothrombin fragment 1 to compete with both [^{125}I]prothrombin and [^{125}I]FX for the zymogen binding site on activated platelets. Prothrombin fragment 1 was found to

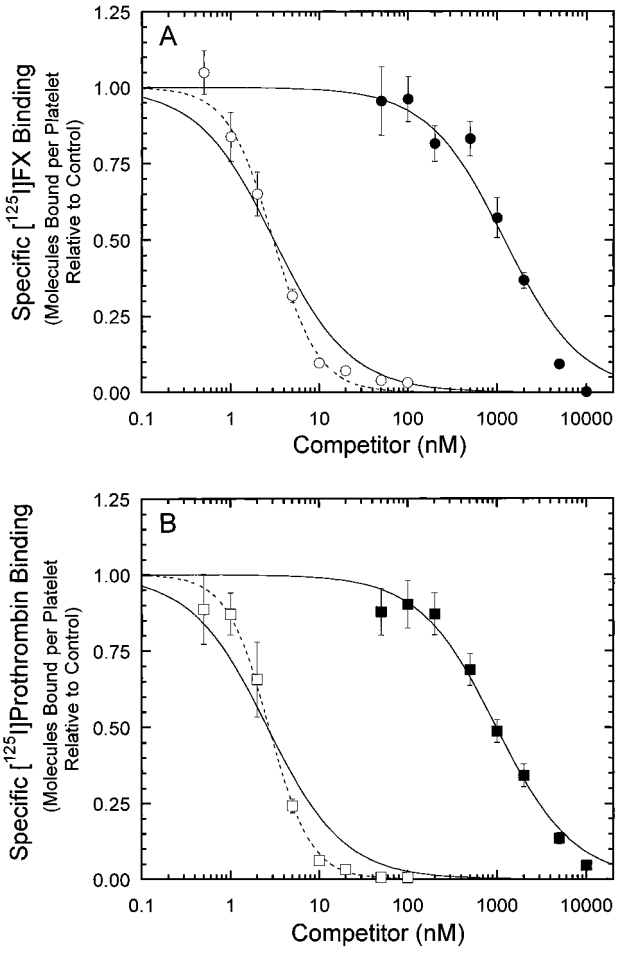

FIGURE 9: Competition for the zymogen binding site by prothrombin fragment 1 and annexin V. All incubations were carried out at ambient temperature in the presence of 5 mM $CaCl_2$, 25 μM SFLLRN-amide and increasing concentrations of either prothrombin fragment 1 (filled symbols) or annexin V (open symbols). The solid lines represent the best nonlinear least-squares fit to an equation describing simple competition. The dashed line represents an empirical equation which provides a smooth curve through the data. Error bars represent the standard error in the mean of two measurements. (A) Competition for the binding of 150 nM [^{125}I]-FX. (B) Competition for the binding of 150 nM [^{125}I]prothrombin.

be an effective inhibitor of both FX and prothrombin binding (Figure 9A and B, respectively). When competition experiments were done in parallel, FX, prothrombin, and prothrombin fragment 1 were found to have roughly equivalent IC_{50}'s in competition for the binding of either [^{125}I]prothrombin or [^{125}I]FX to SFLLRN-amide-activated platelets (data not shown). Further, no statistically significant competition was observed between Gla-domainless FX and [^{125}I]FX for zymogen binding sites. When activated platelets were incubated with 50 nM [^{125}I]FX, 2 μM $FX_{DES1-44}$ displaced only 8% $\pm$ 13% of the specific binding whereas 2 μM FX displaced more than ten times that amount (81% $\pm$ 12%).

DISCUSSION

The activation of FX and prothrombin represents two sequential, surface-dependent reactions which are required for effective hemostasis. The data presented here demonstrate that both FX and prothrombin interact directly and in a saturable manner with the platelet surface. The bulk of evidence supports the hypothesis that the two coagulation factors are binding to a single class of sites.

The similarities between the FX site and the prothrombin site and the interaction of the two proteins with these sites are as follows: (1) There are approximately the same number of sites on the surface of activated platelets for FX, 16 000 $\pm$ 2 000, as for prothrombin, 21 000 $\pm$ 2 000 ($p = 0.25$); (2) the apparent dissociation constants are very similar for FX and prothrombin, 320 $\pm$ 40 nM and 470 $\pm$ 110 nM ($p = 0.21$), respectively; (3) both proteins are able to completely displace the other from the platelet surface; (4) annexin V is able to compete with both coagulation factors for sites on the platelet surface with the same high potency; (5) binding is Ca^{2+}-dependent; (6) the binding site for each is only expressed on the surface of activated platelets; and (7) neither the FX nor the prothrombin site is expressed by platelets stimulated with ADP.

Our data demonstrate that prothrombin fragment 1 can completely and potently displace both FX and prothrombin from the platelet surface. Others have shown that prothrombin fragment 1 can displace FX (Krishnaswamy et al., 1992) and prothrombin from phospholipid surfaces and that the region of prothrombin which endows the protein with its ability to bind to phospholipid vesicles lies within fragment 1. Prothrombin fragment 1 is composed of the first 155 residues of prothrombin and contains two well-defined domains: (1) the γ-carboxyglutamic acid-containing domain (Gla-domain, residues 1−48); and (2) the first of two Kringle domains in prothrombin (residues Cys65−Cys143). The Gla-domain is highly conserved among the vitamin K-dependent coagulation factors, and within this domain all of the determinants required for phospholipid binding are believed to reside (Schwalbe et al., 1989). It is known that neither prothrombin nor FX, which have had their Gla-containing regions removed enzymatically (Skogen et al., 1984; Morita & Jackson, 1986) or which have Gla residue deficiencies due to incomplete γ-carboxylation (Borowski et al., 1985; Malhotra et al., 1985), chemical modification (Sugo et al., 1990), or site-directed mutagenesis (Ratcliffe et al., 1993), will bind to phospholipid vesicles. It is unlikely, however, that either Kringle domain in prothrombin is important for phospholipid binding since recombinant-derived prothrombin mutants lacking either one or both Kringle domains retain their ability to bind phospholipid vesicles (Kotkow et al., 1993). FX and prothrombin fragment 1 are 68% identical in their Gla-containing regions but have little similarity beyond that point (FX has EGF-like domains in place of Kringles). From this limited homology, we infer that it is the Gla-domain which is essential for binding to the zymogen binding site on platelets. Consistent with this interpretation is the inability of $FX_{DES1-44}$ to prevent or displace the binding of [^{125}I]FX to activated platelets.

Calcium ions must be present for both FX and prothrombin to bind to activated platelets. There are two possible mechanisms by which calcium may be required: (1) calcium is necessary for expression of the zymogen binding site by platelets, or (2) calcium is necessary for the interaction between the binding site and either FX or prothrombin. Our data favor the latter mechanism because the addition of a calcium chelator (i.e., EDTA or EGTA) at any time—before, during, or after a prolonged period of platelet activation—quickly removes the bound proteins; exposure of the zymogen binding sites is essentially complete within the first minute of platelet stimulation. Calcium ions are also known to be necessary for factor X and prothrombin to bind to

artificial phospholipid membranes (Nelsestuen et al., 1976, 1978; Nelsestuen & Lim, 1977) and have been described as having "two roles" (Nelsestuen et al., 1976) in this process. The first is to bring about a conformational change in vitamin K-dependent coagulation factors: inducing the relatively unordered Gla-containing region to form a well-structured, compact domain (Soriano-Garcia et al., 1992). The second role of Ca^{2+} is believed to be the formation of "bridges" (Dombrose et al., 1979) between anionic sites in the folded Gla-domain and negatively charged amino phospholipids (e.g., phosphatidylserine). We believe that FX and prothrombin require Ca^{2+} to induce the proteins' Gla-domains to attain a conformation which interacts with the platelet zymogen binding site; an interaction that could involve the formation of calcium "bridges".

Binding sites for FX on a number of different cell types have been described by other authors; however, we are unaware of any reports of cellular binding sites for prothrombin. The binding sites for FX can be loosely categorized as the monocyte type or the endothelial cell type. Altieri and Edgington (1988) have provided convincing evidence of a relatively high-affinity (K_d = 20−40 nM) interaction between FX and the MAC-1 integrin (CD11b/CD18) exposed on ADP-stimulated monocytes and cell lines of monocytic/myeloid differentiation (Altieri et al., 1988). The zymogen binding site on platelets is clearly not MAC-1, nor is it an immunologically similar protein because antibodies found by Altieri and Edgington to completely displace FX from the monocyte surface are without effect on the platelet site (Table 1). Further, MAC-1 specifically recognizes FX among the other vitamin K-dependent coagulation factors including prothrombin whereas the site on platelets does not have this capacity. The regions of FX believed to interact with MAC-1 are found within three noncontiguous heavy-chain loops that surround the active-site (Altieri et al., 1991), a region structurally unrelated to the Gla-domain which we propose is responsible for binding to the platelet site.

A lower affinity FX-binding site is constitutively expressed by cultured bovine aortic endothelial cells (BAEC), and this site seems distinct from the sites for FIX and FIXa_β on these cells (Stern et al., 1984). The affinity of the interaction between bovine FX and BAEC (K_d = 480 nM) is similar to that reported here between human FX and human platelets, and the region of bovine FX that interacts with BAEC has been suggested to be the Gla-domain (Persson et al., 1991), as has been suggested by us for the site on platelets. However, prothrombin has no capacity to compete with FX for the site on BAEC and the site on human platelets is unable to make this discrimination.

Studies directed at the elucidation of interactions between FX or prothrombin and platelets have not been reported. Where binding studies have been conducted by other authors, our results are either consistent with their findings or provide a ready explanation of discrepancies. Miletich et al. (1977) demonstrated that human FX (≤170 nM) will not bind to unactivated human platelets in the absence of calcium nor will it compete with FXa for its binding site on activated platelets. These results are in agreement with those presented here, for we have found that FX binding occurs only after platelet activation and only in the presence of calcium (Figure 3). Further, we have shown that FV(a) is not the binding site for FX (Table 1), whereas others have shown that FVa provides the binding site for FXa. Similarly, Tollefsen et al. (1975) were not able to demonstrate the binding of bovine prothrombin (≤1.2 μM) to unactivated human platelets nor could they measure binding of low concentrations of prothrombin (≤1 nM) to activated platelets. In light of our measurements, one would expect only a small number of specifically bound prothrombin molecules to be observed at these low concentrations. Dahlback and Stenflo (1978) have also reported that low nanomolar concentrations of bovine FX (≤1.7 nM) did not lead to measurable specific binding as determined in the presence of an 100-fold excess of unlabeled FX. In this case, we would expect ≤35% of the specific binding to be displaced by this concentration of cold competitor ($K_i \approx K_d \approx$ 320 nM) and such a small change in the already small number of molecules bound could reasonably have been considered negligible.

It appears that the zymogen binding sites are quite distinct from those for FIX, FIXa_β, and probably FVIIIa. Previous reports from this laboratory have shown that neither FX nor prothrombin (up to 1 μM) is able to displace a significant fraction of bound FIX or FIXa_β from the platelet surface. In fact, when FVIIIa is present, FX helps bring about an increase in the affinity of the interaction between FIXa_β and activated platelets. Further, the number of sites (i.e., 300−600) for FIX, FIXa_β, FVIII, and, presumably, FVIIIa is dramatically lower than the number of zymogen binding sites. In a like manner, the presence of prothrombin has not been found to decrease the binding of FV, FVa (Miletich et al., 1978; Kane et al., 1980; Tracy et al., 1981; Kane & Majerus, 1982), or FXa to unactivated platelets, and FX does not affect FXa binding to activated platelets (Miletich et al., 1977, 1978).

We have provided evidence that FV does not contribute to the zymogen binding site. Using both monoclonal and polyclonal anti-human FV antibodies that are known to disrupt the activity of FVa in the prothrombinase complex, we were unable to demonstrate any alteration in the capacity of activated platelets to bind either FX or prothrombin (see Table 1). The dissociation constant of the interaction between the monoclonal antibody and FV is 3 nM, yet at concentrations over 800 nM this antibody is unable to compete for the zymogen binding site. When one considers that the concentration of platelet-released FV in our assay is sufficient to support the binding of only a few hundred FV molecules per platelet (Kane & Majerus, 1982), it becomes clear that there is an inadequate quantity of bound FV to provide the 15 000−20 000 binding sites shared by FX and prothrombin.

The moderate affinity that both FX and prothrombin show for the platelet surface is comparable to their affinity for phosphatidylserine-containing phospholipid vesicles (Nelsestuen et al., 1978; Wei et al., 1982; Cutsforth et al., 1989). Similarly, the potency with which the other Gla-containing coagulation factors displace [^{125}I]FX from the platelet surface is stratified in a manner consistent with the proteins' affinities for phospholipid vesicles, FX and prothrombin having higher affinity than protein S and the other proteins. After platelets are activated with strong agonists (Bevers et al., 1982, 1983), low levels of hydrolyzable phosphatidylserine are exposed in the outer leaflet of their plasma membrane; however, the quantity of phosphatidylserine exposed is far less than that which is known to be required for FX or prothrombin to bind to vesicles. Nonetheless, the homology between the

zymogen binding site and the phosphatidylserine-containing "site(s)" on phospholipid vesicles is enticing and leaves open the possibility that phosphatidylserine could contribute to the zymogen binding site. To investigate this hypothesis, we measured the binding of FX and prothrombin in the presence of annexin V.

The ability of annexin V to completely and potently prevent both FX and prothrombin from binding to activated platelets is striking. Annexin V binds to platelets directly (Thiagarajan & Tait, 1990; Sun et al., 1993), and there are many similarities between the platelet annexin V binding site and the zymogen binding site. Stimulation with ADP does not lead to the appearance of additional annexin V binding sites on platelets, and the number of annexin V sites presented in response to thrombin (15 000 sites) agrees extremely well with the number of zymogen binding sites exposed. When platelets are stimulated by increasing concentrations of agonists, the exposure of platelet sites for annexin V (Thiagarajan & Tait, 1990) closely parallels the appearance of hydrolyzable phosphatidylserine in the outer membrane leaflet (Bevers et al., 1983). Comparable agonist profiles exist for platelet-dependent acceleration of both FX and prothrombin activation (Bevers et al., 1982; Rosing et al., 1985). Given these similarities, it is attractive to hypothesize that annexin V, FX, and prothrombin compete for the limited number of phosphatidylserine molecules exposed in the outer leaflet of an activated platelet's membrane.

There are, however, inconsistencies in this hypothesis. Whereas we have found none of the zymogen binding sites exposed on the surface of unactivated platelets, roughly $^1/_5$ to $^1/_3$ (3 000−5 000) of the annexin V binding sites are exposed (Thiagarajan & Tait, 1990). We are not confident that different requirements for phosphatidylserine can adequately resolve this discrepancy since it appears that annexin V (Tait et al., 1989) needs more phosphatidylserine in synthetic vesicles than does either FX or prothrombin (Cutsforth et al., 1989). Further, if this difference were solely due to a lesser requirement by annexin V for phosphatidylserine, then we would expect the surface of thrombin-stimulated platelets to have a greater capacity for annexin V than for FX or prothrombin, and this is not the case.

We have found that annexin V prevents both FX and prothrombin from binding in a manner that is not adequately described as simple competition (Figure 9): the inhibition appears to have a cooperative character. In addition to the well-known affinity of annexin V for phosphatidylserine-containing phospholipid vesicles (Tait et al., 1989), annexin V is known to have many and diverse *in vitro* effects [see Walker et al. (1992) for review] including the inhibition of enzymes involved in signal transduction (e.g., protein kinase C and phospholipase C) and the formation of ion channels in artificial membranes. Little is known about the *physiologic* role(s) of this ubiquitous protein although it has been found in many cells including platelets where thrombin-stimulation causes cytosolic annexin V to associate with internal membranes in both a calcium-dependent and calcium-independent manner (Orchard et al., 1993). A very curious characteristic of annexin V is its formation of a triskelion-like matrix (Mosser et al., 1991) on the surface of phospholipid bilayers. These extensive networks of annexin V may be responsible for the observed distortion of normally spherical phospholipid vesicles into polyhedrons through the formation of "planar facets with sharp edges" (Andree et al., 1993). Clearly, any of annexin V's diverse effects could alter the expression of the zymogen binding site, be it of protein or lipid composition. Thus, the mechanism by which annexin V prevents the binding of FX and prothrombin to activated platelets remains elusive despite the superficial attractiveness of a model focused on the phospholipid binding behavior of annexin V.

Although there has been considerable debate as to whether kinetically relevant substrates for the FXase and prothrombinase enzyme complexes assembled on artificial phospholipid membranes are phospholipid bound (van Dieijen et al., 1981; Nesheim et al., 1984, 1992; Mertens et al., 1985; Forman & Nemerson, 1986; Krishnaswamy et al., 1992; Pusey & Nelsestuen, 1993), the binding of neither FX nor prothrombin to a physiologic surface has been previously reported. The pertinence of "bound substrate" hypotheses derived from the results of studies using synthetic surfaces is dependent upon the existence of interactions between FX or prothrombin and platelets, the surface upon which they are believed to be activated *in vivo*. The zymogen binding site described herein may represent such an interaction. There are many potential benefits that the presence of a surface may confer upon an enzymatic reaction including: aiding the enzyme and substrate to attain the relative orientation necessary for the assembly of the Michaelis complex, and directly affecting the enzyme and/or substrate to make them better at their respective roles. Although the data presented in this paper permit "bound-substrate" models of FX and prothrombin activation, the exact mechanism(s) by which phospholipid or platelet surfaces lead(s) to accelerated zymogen activation is outside the focus of this report. We have explicitly dealt with the functional significance of the zymogen binding site in the accompanying paper.

The zymogen binding site on activated platelets has many characteristics that one would expect for an effective substrate binding site. Previous results from our laboratory and from others have demonstrated that zymogen will not displace from the platelet surface any of the complexes that are responsible for their activation. The existence of far more binding sites available to the zymogens, FX, and prothrombin, than to the complexes that convert them to active enzymes is not surprising, for if these sites are important to zymogen activation, then the reaction (enzyme−substrate) is implicitly two-dimensional—driven by the *surface density* of bound substrate molecules—and favored by a high density of substrate binding sites. Nor is it unexpected that, when both FX and prothrombin are present at their plasma concentrations, most of the zymogen binding sites will be occupied by prothrombin since an effective coupling of the FXase and prothrombinase activities would demand a greater supply of prothrombin than FX.

Exposure of binding sites for coagulation factors by platelets represents an additional level of control in blood coagulation. Through the controlled exposure of these binding sites the procoagulant response can be constrained to sites of vascular injury by way of adherent and aggregated platelets. The platelet zymogen binding site may represent yet another component in the regulation and localization of hemostasis as well as thrombosis.

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