Coordinate Binding Studies of the Substrate (Factor X) with the Cofactor (Factor VIII) in the Assembly of the Factor X Activating Complex on the Activated Platelet Surface†

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ABSTRACT: The assembly of the factor X activating complex on the platelet surface requires the occupancy of three receptors: (1) enzyme factor IXa, (2) cofactor factor VIII(a), and (3) substrate factor X. To further evaluate this three-receptor model, simultaneous binding isotherms of ^{125}I-factor X and ^{131}I-factor VIII(a) to activated platelets were determined as a function of time and also as a function of the concentrations of both ligands in the presence of active site-inhibited factor IXa (45 nM) and 5 mM $CaCl_2$. In the presence of active site-inhibited factor IXa and factor VIIIa there are two independent factor X binding sites: (1) low affinity, high capacity (∼9000 sites/platelet; K_d ∼380 nM) and (2) low capacity, high affinity (1700 sites/platelet; K_d ∼30 nM). A single specific and selective factor X binding site was expressed (1200 sites/platelet; K_d ∼9 nM) when the shared factor X/factor II site was blocked by excess factor II (4 μM). In the presence of active site-inhibited factor IXa (4 nM) and factor II (4 μM), factor X binds to 3-fold more platelet sites than procofactor VIII with relatively low affinity (K_d ∼250 nM). The activation of procofactor VIII to factor VIIIa increases the affinity of binding to platelets of both factor VIIIa (∼4-fold to K_d ∼0.8−1.5 nM) and factor X (∼25−50-fold to K_d ∼5−9 nM). In the presence of excess zymogen factor IX, which blocks the shared factor IX/factor IXa binding site, the substrate, factor X, and the active cofactor, factor VIIIa, form a 1:1 stoichiometric complex. These coordinate binding studies support the conclusion that factor X initially binds to a high-capacity, low-affinity platelet binding site shared with prothrombin, which then presents factor X to a specific high-affinity site consisting of factor VIIIa bound to a high-affinity, low-capacity receptor on activated platelets.

The interaction between blood platelets and coagulation factors is essential for normal coagulation and hemostasis. In previous studies it has been shown that the assembly of the factor X (FX)[1] activating complex on the surface of activated platelets requires the occupancy of binding sites for each of the following ligands: (1) the enzyme, FIXa (*1*), (2) the cofactor, FVIII(a) (*2*, *3*), and (3) the substrate, FX (*4*). Receptor[2] occupancy of the components of the FX activating complex is closely correlated with rates of FX activation on the platelet surface resulting in >20 million-fold acceleration of catalytic efficiency (*3*−*5*). We have shown that the presence of the cofactor (FVIIIa) and the substrate (FX) increases 5-fold the affinity of FIXa for the surface of activated platelets (*1*). We have demonstrated that both the procofactor (FVIII) and the active cofactor (FVIIIa) bind platelets with enhanced affinity in the presence of active site-inhibited FIXa (EGR-FIXa) and FX and that FVIIIa binding to activated platelets in the presence of FIXa and FX is closely coupled with rates of FX activation (*3*). In addition to high-affinity specific receptors on activated platelets for FIXa and FVIIIa, activated platelets express a shared FX/prothrombin (FII) receptor which has the characteristics of a low-affinity (dissociation constant, K_d ∼320 nM), high-capacity (n ∼15000 sites/platelet) binding site (*4*), occupancy of which is closely correlated with rates of FX activation (*6*). Additional studies from our laboratory suggest that the zymogen (FIX) and the enzyme (FIXa) interact with a shared binding site (K_d ∼3 nM; n ∼250 sites/platelet) mediated by residues G_4−Q_{11} within the γ-carboxyglutamic acid (Gla) domain, occupancy of which may be required but is not sufficient for the functional assembly of the FX activating complex (*7*). In addition, FIXa binds specifically to a site not occupied by FIX (n ∼250 sites/platelet) which is mediated by the second epidermal growth factor domain of FIXa (*8*−*11*). Occupancy of this site is closely correlated with enhanced rates of FX activation (*8*−*11*).

The results of these studies described above strongly support the view that a ternary complex of FIXa, FVIIIa,

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[1] Abbreviations: FX, factor X; FIX, factor IX; FVIII, factor VIII; FII, prothrombin; Gla, γ-carboxyglutamic acid; EGR-FIXa, active site-inhibited Glu-Gly-Arg-factor IXa.

[2] The term "receptor", as used in this paper, refers to a cell surface membrane binding site, occupancy of which has biologically important consequences, including either transmembrane signaling or acceleration of extracellular enzymatic reactions.

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and FX is assembled on the activated platelet surface and that the enzyme (FIXa) catalyzes the activation of bound rather than solution-phase substrate molecules. The formation and maintenance of this ternary complex are dependent on the stability of the cofactor (FVIII). It is generally believed that the primary mechanism for decay of the FX activating complex under physiological conditions is the dissociation of the A2 subunit (*12*−*14*). Fay et al. (*15*) demonstrated that the A2 subunit of FVIIIa markedly increases the catalytic activity (i.e., k_{cat} of FIXa-catalyzed FX activation). The presence of an excess of the FVIII A2 subunit enhances the affinity of active cofactor FVIIIa binding to activated platelets in the presence of EGR-FIXa and FX (*3*), and a progress curve analysis of FX activation on the platelet surface suggests that the instability of thrombin-activated FVIII is an important control mechanism in intrinsic pathway F-X activation (*6*). Thus, FX activation is a platelet receptor-mediated process tightly coupled to receptor occupancy by FIXa, FVIIIa, and FX.

The purpose of the present studies was to examine the hypothesis that the assembly of the FX activating complex involves the coordinated interactions of each of the three components, the enzyme (FIXa), the cofactor (FVIIIa), and the substrate (FX), with binding sites exposed on the surface of the activated platelets and with binding sites that mediate the interaction of each of the protein components with one another. The predictions arising from this hypothesis are that the presence of each of the protein components should affect the affinity and stoichiometry of binding to the platelet surface of the other two and that the binding energy reflecting the assembly of each component into the complex should maintain thermodynamic equilibrium, reflecting each protein−protein and each protein−platelet interaction. In the present paper we have examined the coordinated interaction of FX and FVIII(a) with the activated platelet surface using dual-labeled proteins in equilibrium binding studies.

EXPERIMENTAL PROCEDURES

Materials. *p*-Aminobenzamidine was obtained from Sigma Chemical Co. (St. Louis, MO). D-Phenylalanylprolylarginyl chloromethyl ketone (PPACK) was purchased from Calbiochem-Behring Corp. (San Diego, CA). Carrier-free $Na^{125}I$ and $Na^{131}I$ were obtained from Amersham Corp. (Arlington Heights, IL). Thrombin receptor agonist peptide SFLLRN-amide was synthesized as previously described (*4*) using 9-fluorenylmethyloxycarbonyl (FMOC) chemistry on an Applied Biosystems 430A synthesizer and reverse-phase HPLC purified from greater than 99% homogeneity by the Protein Chemistry Laboratory (Dr. John Lambris) of the University of Pennsylvania (Philadelphia, PA). All other reagents and chemicals used were the same as previously reported (*3*) and were obtained from Sigma Chemical Co., Aldrich Chemical Co. (Milwaukee, WI), or Calbiochem-Behring Corp. and were of the highest grade commercially available.

Proteins. Human FIX, FIXa, EGR-FIXa, FX, and FII were purchased from Enzyme Research Laboratories, Inc. (South Bend, IN), and Haematologic Technologies, Inc. (Essex Junction, VT). Other highly purified human coagulation factors (RVV-X and antithrombin III) were from Enzyme Research Laboratories, Inc. Human α-thrombin (4165 units/mg) was purchased from Sigma. Highly purified recombinant FVIII (rFVIII, >4000 units/mg) was the generous gift of the Genetics Institute (Cambridge, MA) and Baxter Health Care Corp. (Duarte, CA). All proteins were >95% pure as judged by SDS−polyacrylamide gel electrophoresis (*16*) and protein staining by Coomassie Brilliant Blue.

Radiolabeling and Structural Characterization of Platelet-Bound Components of the FX Activating Complex. We have developed a highly reproducible method utilizing the Bolton−Hunter reagent (*17*) to radiolabel FVIII to high specific radioactivity while retaining virtually 100% of its specific biological activity (*3*). In this procedure, 20−50 μg of FVIII was precipitated with 25% PEG 8000, washed, and then resuspended in 50 μL of 0.5 M NaCl, 20 mM HEPES, pH 8.0, 5 mM $CaCl_2$, and 0.1% Tween 80 just before use. The protein was then added to a siliconized glass vial containing 0.25−0.5 mCi of iodinated Bolton−Hunter reagent, which had been previously dried onto the surface. The mixture was incubated for 45 min at 0 °C and then quenched by the addition of 5 μL of 0.2 M glycine and 0.1 M borate, pH 8.5, followed by further incubation for 45 min at 0 °C. Usually 85−95% of the original protein was recovered after labeling. The ^{131}I-FVIII obtained had a specific radioactivity of 400−1200 cpm/ng (0.015−0.09 mol of ^{131}I/mol of FVIII). Finally, the functional activity of the labeled FVIII was compared to cold FVIII as described previously (*3*). Human FX was radiolabeled via lactose peroxidase (*4*). The functional activities of the proteins were determined as described previously (*1*, *4*). The activation of FX by FIXa was also determined at 37 °C in the presence of thrombin-stimulated platelets, FVIIIa, and $CaCl_2$ as described previously (*1*, *4*). To characterize the platelet-bound substrate (FX), procofactor (FVIII), and the active cofactor (FVIIIa) structurally, platelets were incubated with either thrombin (0.1 unit/mL) or thrombin-receptor agonist peptide, SFLLRN-amide (25 μM), and $CaCl_2$ (5 mM) for 20 min at 37 °C in the presence of either radiolabeled substrate (^{125}I-FX) or cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) and centrifuged through 20% sucrose to separate the bound from free ligand as described under Experimental Procedures. Platelet pellets were solubilized in SDS and were analyzed as described previously (*1*, *3*, *4*). When we examined the autoradiograms of bound ligands, the substrate (^{125}I-FX) on nonreduced 13% polyacrylamide gel migrated as a single band at M_r 58000 (Figure 1, lane 1). In contrast, the autoradiogram of platelet-bound ^{131}I-FVIII molecules was run on a 4−20% gradient gel in SDS. The bound radioligand migrated under reduced conditions as two major bands, i.e., HC, molecular mass 90−200 kDa, and LC, molecular mass 80 kDa, respectively (Figure 1, lane 2). The active cofactor (FVIIIa) migrated under reduced conditions as several bands of molecular mass ∼90, 72, 50, and 43 kDa representing the A1-A2 domain, A3 plus C1 and C2, A1, and A2, respectively (Figure 1, lane 3). All bound ligands (FX, FVIII, and FVIIIa) were indistinguishable from free ligands (data not shown). The data provided in Figure 1 show no evidence for the formation of high molecular mass covalent complexes or for proteolytic degradation of any radiolabeled ligand by platelets, thus confirming that the bound radioactivity consists entirely of either the substrate (^{125}I-FX) or the cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) and not a radiolabeled contaminant. The fact that the bound ligands

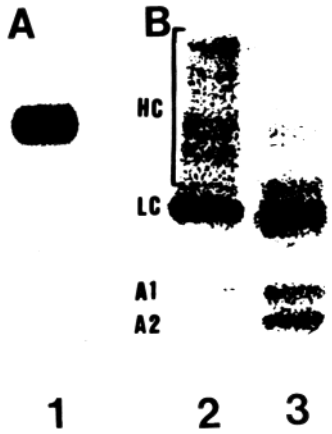

FIGURE 1: Autoradiogram of platelet-bound radiolabeled FX and FVIII molecules. Gel-filtered platelets (3.8×10^8/mL) were incubated for 10 min at 37 °C with either the substrate (^{125}I-FX) or cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) (~5 nM), SFLLRN-amide (25 μM), and $CaCl_2$ (5 mM). After centrifugation through 20% sucrose, the platelet pellet was solubilized in 20% SDS containing 1 mM diisopropyl fluorophosphate, 1 mM benzamidine, and 1 mM EDTA and applied to appropriate SDS gels. Autoradiograms are shown (lane 1) of a 13% polyacrylamide gel electrophoretogram in sodium dodecyl sulfate of ^{125}I-labeled FX (nonreduced, lane 1) and migrated as a single chain 58 kDa protein. Lanes 2 and 3 represent an autoradiogram of 4%−20% gradient gel (reduced with 5% β-mercaptoethanol) in sodium dodecyl sulfate of ^{131}I-labeled FVIII (lane 2) and ^{131}I-labeled FVIIIa (lane 3). Lane 2 represents the heavy chain (HC), M_r ~90−200 kDa, and light chain (LC), M_r 80 kDa, and lane 3 represents the A3 + C1 + C2 domain, M_r 72 kDa, A1 domain, M_r 50 kDa, and A2 domain, M_r 43 kDa.

are structurally intact and indistinguishable from free ligands provides strong evidence that the functional FX activating complex consists of bound substrate, FX (*4*), bound enzyme, FIXa (*1*), together with bound cofactor, FVIIIa (*3*), on the surface of platelets and forms a ternary complex.

Platelet Isolation and Binding Studies. Albumin density gradient washed gel-filtered platelets were isolated from human venous blood by a modification of the method of Walsh et al. (*18*), as recently reported by us (*3*, *4*). In a typical binding experiment, washed gel-filtered platelets (3.5×10^8/mL) in Ca^{2+}-free HEPES Tyrode's buffer, pH 7.4, were incubated at 37 °C in a 1.5 mL Eppendorf plastic centrifuge tube with mixtures of unlabeled and radiolabeled FX, FVIII, or FVIIIa, $CaCl_2$, platelet agonists (thrombin or thrombin-receptor peptide, SFLLRN-amide), and other proteins for coordinate binding studies. After incubation, aliquots (100 μL) were removed and centrifuged through a mixture of silicone oils as previously described (*1*, *3*−*5*). All radioactivity measurements were made in a 1470 Wallac Wizard gamma counter. Double isotope counting conditions were established so that the single isotope count in dual-labeled samples can be determined by the method described in the instruction manual for the Wallac gamma counter. The data were analyzed, and the number of binding sites and K_d were calculated from the means of four determinations using platelets from four different donors, each done in triplicate using a Macintosh G4 computer (Apple Computer, Cupertino, CA) and the Ligand Program as modified by G. A. McPherson (Elsevier Science Publishers BV, Amsterdam, The Netherlands, 1985).

Conversion of the K_d values to free energy of binding ($\Delta G°$) was calculated using the equation $\Delta G° = -RT \ln K_d$, where R is the gas constant (1.987 cal mol^{-1} K^{-1}), T is the absolute temperature (310 K), and K_d is the dissociation constant for protein−protein or protein−platelet interactions.

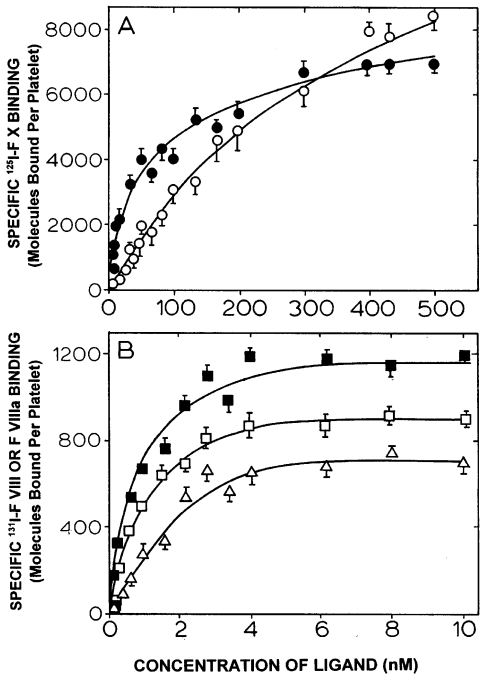

FIGURE 2: Specific binding of substrate (^{125}I-FX) and cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) to activated human platelets in the absence or presence of enzyme (EGR-FIXa). Washed gel-filtered platelets (wGFP) (3.5×10^8/mL) were incubated at 37 °C with thrombin receptor peptide, SFLLRN-amide (25 μM), $CaCl_2$ (5 mM), and either ^{125}I-FX (from 0.5 to 5000 nM) (panel A) or ^{131}I-FVIII or ^{131}I-FVIIIa (from 0.1 to 10 nM) (panel B) in the absence or presence of EGR-FIXa (10 nM) and FVIIIa (10 nM) in panel A and in the absence or presence of EGR-FIXa (10 nM) and FX (1.5 μM) in panel B. The binding was determined as detailed under Experimental Procedures. The results shown represent the specific binding of FX (○, ●), FVIII (△), and FVIIIa (□, ■) in the absence (open symbols) or in the presence of FVIIIa (●) or FX (■) and EGR-FIXa. Nonspecific binding was determined in the presence of excess unlabeled enzyme or cofactor and was subtracted from total binding to obtain specific binding. The plotted results are the means ± SE of triplicate observations from four separate experiments using platelets from four different donors.

RESULTS

Specific Binding of Substrate (^{125}I-FX) and Cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) to Activated Human Platelets in the Absence or Presence of Enzyme (EGR-FIXa). Initially, we examined the specific binding of substrate ^{125}I-FX and cofactor ^{131}I-FVIII or ^{131}I-FVIIIa to activated human platelets in the absence or the presence of EGR-FIXa, after subtracting the nonspecific binding, which was measured in the presence of a 100-fold molar excess of unlabeled ligands. In the absence of EGR-FIXa and FVIIIa (Figure 2A, open circles), analysis of specific ^{125}I-FX isotherms yielded a value of 15000 ± 1800 sites/platelet with a K_d of 500 ± 112 nM. In the presence of EGR-FIXa (10 nM) and FVIIIa (10 nM), Scatchard analysis (not shown) of specific ^{125}I-FX binding isotherms (Figure 2A, closed circles) was consistent with the presence of two distinct binding sites: (1) low affinity, high capacity (~9000 sites/platelet; K_d ~380 nM) and (2) low capacity, high affinity (~1700 sites/platelet; K_d ~30 nM). In contrast, the binding of both the procofactor

Table 1: Binding Constants for the Coordinated Interaction of Substrate (Factor X) with Procofactor (Factor VIII) or Activated Cofactor (Factor VIIIa)[a]

binding ligand	fixed components	$K_{d,app}$ (nM)		no. of sites per platelet	
		FX	FVIII or FVIIIa	FX	FVIII or FVIIIa
FX FVIII	EGR-FIXa (10 nM) FII (4 μM)	250 ± 80	4.0 ± 1.9	1400 ± 210	495 ± 66
FX FVIIIa	EGR-FIXa (10 nM) FII (4 μM)	9 ± 2.2	1.5 ± 1.1	1200 ± 175	750 ± 101
FX FVIIIa	EGR-FIXa (10 nM) FII (4 μM), FIX (250 nM)	5 ± 1.5	0.8 ± 0.08	1000 ± 150	990 ± 120

[a] Binding parameters shown are the means (±SE) for four separate experiments, each done with platelets obtained from different normal subjects.

(^{131}I-FVIII) and the active cofactor (^{131}I-FVIIIa) approached saturation at ~4 nM (Figure 2B). In the absence of the enzyme (EGR-FIXa) and the substrate (FX), the maximum amount of specific binding observed for ^{131}I-FVIII and ^{131}I-FVIIIa was 3.9 and 6.5 pmol/10^{10} platelets, respectively, representing 480 ± 60 sites/platelet (K_d 3.9 ± 0.30 nM) for procofactor ^{131}I-FVIII (Figure 2B, open triangles) and 780 ± 150 sites/platelet (K_d 1.6 ± 0.15 nM) for active cofactor ^{131}I-FVIIIa (Figure 2B, open squares). The presence of the enzyme (45 nM EGR-FIXa) and the substrate (1.5 μM FX) increased both the number (from 480 ± 60 to 700 ± 80 sites/platelet) and affinity of binding sites (K_d from 3.9 ± 0.30 to 1.6 ± 0.17 nM) on activated platelets for procofactor (^{131}I-FVIII) (data not shown). Similarly, the presence of both enzyme and substrate increased both the number (from 800 ± 155 to 1250 ± 210 sites/platelet) and affinity of binding sites (K_d 1.6 ± 0.17 to 0.7 ± 04 nM) for the activated cofactor, ^{131}I-FVIIIa (Figure 2B, closed squares). Thus, compared with the procofactor (FVIII), the active cofactor (FVIIIa) interacts with an additional 300−500 sites/platelet with enhanced affinity, and the presence of both the enzyme (EGR−FIXa) and the substrate (FX) increases both the number (by ~200−450 sites/platelet) and affinity of binding sites on activated platelets for both FVIII and FVIIIa.

Since FX and FII bind to a shared site on activated platelets (*4*), we examined the binding of FX to activated platelets in the presence of a saturating concentration of FII (4 μM). In the absence of FIXa and FVIIIa (Figure 3A, open circles), no saturable, specific binding is observed since saturating concentrations of prothrombin compete with FX for binding to this low-affinity, high-capacity FX/FII binding site. However, in the presence of EGR-FIXa (10 nM) and FVIIIa (10 nM) a lower capacity binding site appears on the surface of platelets which is selective for FX over prothrombin and binds FX much more tightly than does the shared site (Figure 3A, closed circles). The binding isotherm (Figure 3A, closed triangles) for the difference between the FX binding in the absence of EGR-FIXa and FVIIIa (open circles) and in the presence of EGR-FIXa and FVIIIa (closed circles) demonstrates the presence of a lower capacity (1200 sites/platelet), higher affinity (K_d ~9 nM) binding site for FX. It is also important to note that the affinity of the selective FX binding site (i.e., in the presence of 4 μM FII) is 25-fold higher (K_d ~9 nM) in the presence of the active cofactor (FVIIIa) than in the presence of the procofactor (K_d 250 nM), as summarized in Table 1. Moreover, the presence of FII (4 μM) does not affect the binding of the procofactor (^{131}I-FVIII) or active cofactor (^{131}I-FVIIIa) in the presence of EGR-FIXa and FX since the stoichiometry and the affinity of cofactor

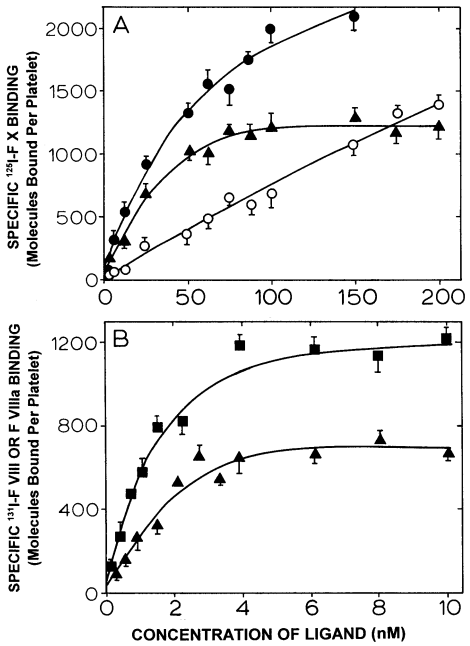

FIGURE 3: Specific binding of substrate (^{125}I-FX) and cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) to activated human platelets in the presence of enzyme (EGR-FIXa) and a saturating concentration of prothrombin (FII). Results shown in panel A represent specific binding of the substrate ^{125}I-FX to activated platelets in the presence of a saturating concentration of FII (4 μM) with (●) or without (○) the addition of EGR-FIXa (10 nM) and FVIIIa (10 nM). The additional binding sites that result from the presence of the enzyme (EGR-IXa) and cofactor (FVIIIa) are shown by closed triangles. Panel B represents the specific binding of the cofactors ^{131}I-FVIII (▲) and ^{131}I-FVIIIa (■) to activated platelets in the presence of a saturating concentration of FII (4 μM), EGR-FIXa (10 nM), and FX (1.5 μM). The plotted results are the means ± SE of triplicate observations from four separate experiments using platelets from four different donors.

interaction with activated platelets in the presence of prothrombin (Figure 3B) were indistinguishable from those observed in the absence of prothrombin (Figure 2B).

Specific Binding of Substrate (^{125}I-FX) and Active Cofactor (^{131}I-FVIIIa) to Activated Human Platelets in the Presence of Enzyme (EGR-FIXa), Excess Zymogen (FIX), and Prothrombin (FII). To determine the affinity and stoichiometry of simultaneous substrate and cofactor binding to activated platelets, binding studies of both the substrate (^{125}I-FX) and the active cofactor (^{131}I-FVIIIa) were carried out in the presence of both excess zymogen (FIX, 250 nM) and excess prothrombin (FII, 4 μM). The rationale for using excess zymogen is that the zymogen (FIX) and the enzyme (FIXa) bind to a shared binding site (~250 sites/platelet; K_d ~3 nM) that is mediated by residues G_4−Q_{11} within the Gla domain

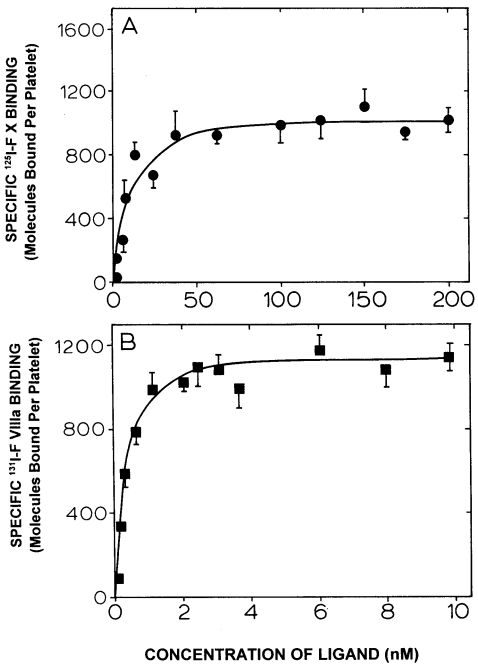

FIGURE 4: Specific binding of substrate (^{125}I-FX) and cofactor (^{131}I-FVIIIa) to activated platelets in the presence of enzyme (EGR-IXa), excess zymogen FIX (250 nM), and excess FII (4 μM). Results shown in panel A represent specific binding of substrate ^{125}I-FX (●) and in panel B cofactor ^{131}I-FVIIIa (■) to activated platelets in the presence of a saturating concentration of zymogen (FIX, 250 nM) and FII (4 μM) and with the addition of EGR-FIXa (10 nM). The FX titrations in panel A were carried out in the presence of FVIIIa (10 nM) whereas the FVIIIa titrations in panel B were carried out in the presence of FX (1.5 μM). The plotted results are the means ± SE of triplicate observations from four separate experiments using platelets from four different donors.

(*7*). Recent data suggest that the Gla-mediated FIX binding site, although possibly required, is not sufficient for the functional assembly of the FX activating complex (*7*, *11*). Rather, a distinct set of FIXa binding sites (~250 sites/platelet; K_d ~0.5 nM), probably mediated via the second epidermal growth factor domain, is involved in the functional assembly (*8*−*11*). We hypothesized that by blocking the shared FIX/FIXa binding site we would be able to define the contribution to substrate and cofactor binding of platelet membrane-bound FIXa molecules that can interact only with the specific FIXa binding site. Specific and selective FX binding to activated platelets was assured by carrying out the coordinate binding studies in the presence of excess FII (4 μM). As shown in Figure 4A (closed circles), equilibrium binding studies of ^{125}I-FX in the presence of saturating concentrations of both FII (4 μM) and FIX (250 nM) and in the presence of EGR-FIXa (10 nM) and FVIIIa (10 nM) demonstrated a new class of low-capacity (1000 ± 150 sites/platelet), high-affinity (K_d ~5 nM) FX binding sites. Blocking the shared sites (FX/FII and FIX/FIXa) (Figure 4B, closed squares) also resulted in the appearance of a specific high-affinity FVIIIa binding site (K_d ~0.5 nM; ~990 ± 120 sites/platelet). Thus, the presence of both excess FII (4 μM) and excess FIX (250 nM) along with the enzyme EGR-FIXa confers specificity and high affinity (K_d ~5 nM; ~1000 sites/platelet) upon a class of FX binding sites which are superimposed upon a low-affinity and high-capacity shared site. Under the same experimental conditions both the substrate (^{125}I-FX) and the active cofactor (^{131}I-FVIIIa) interacted with an identical number of sites, thus forming an equimolar stoichiometric complex (Figure 4, Table 1).

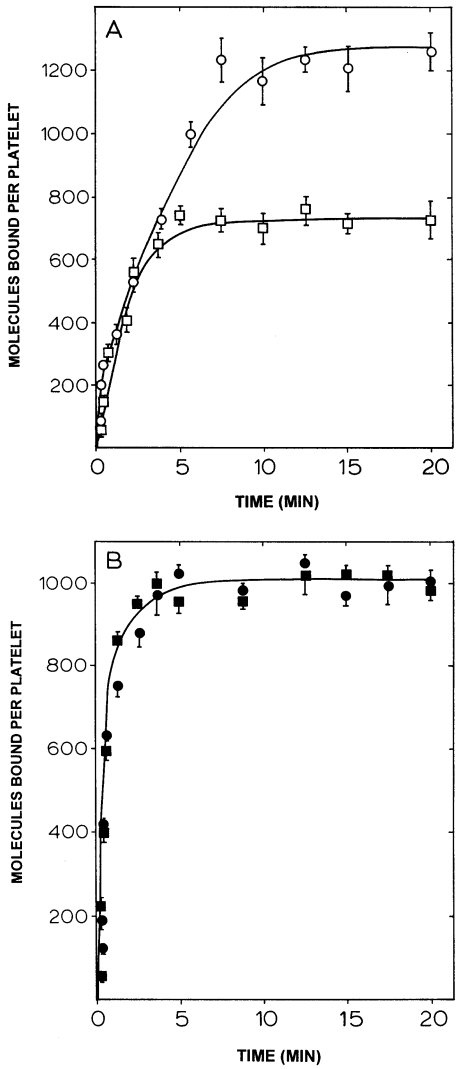

FIGURE 5: Simultaneous binding measurements of substrate (^{125}I-FX) and cofactor (^{131}I-FVIIIa) to activated platelets as a function of time in the presence of enzyme (EGR-FIXa) and excess FII (4 μM) and in the absence (panel A) or in the presence (panel B) of excess (250 nM) FIX. The time course of specific binding of both ^{125}I-FX (○, ●) and ^{131}I-FVIIIa (□, ■) to activated human platelets was made by making binding measurements at 0.5, 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15, and 20 min after the addition of both ligands (1.5 μM FX and 10 nM FVIIIa) to the reaction mixtures. Nonspecific binding of the ligands was determined in parallel reaction mixtures containing a 100-fold molar excess of unlabeled FX or FVIIIa. Specific binding of both ligands was determined by subtracting the nonspecific binding from total binding. The plotted results are the means ± SE of triplicate observations from four separate experiments using platelets from four different donors.

Figure 5A shows the time course of specific substrate (^{125}I-FX) binding (open circles) and the active cofactor (^{131}I-FVIIIa) binding (open squares) to activated human platelets in the presence of the enzyme (EGR-FIXa, 10 nM) and excess FII (4 μM). Nonspecific binding was determined from parallel reaction mixtures containing a 100-fold excess of unlabeled ligand. The data shown in Figure 5A indicate that both the substrate (^{125}I-FX) and active cofactor (^{131}I-FVIIIa) bind to activated platelets at similar rates. The initial binding of each ligand is rapid, with equilibrium being achieved at ~6−10 min. At equilibrium (~15 min), the substrate (^{125}I-FX) interacted with ~1200 sites/platelet compared to the

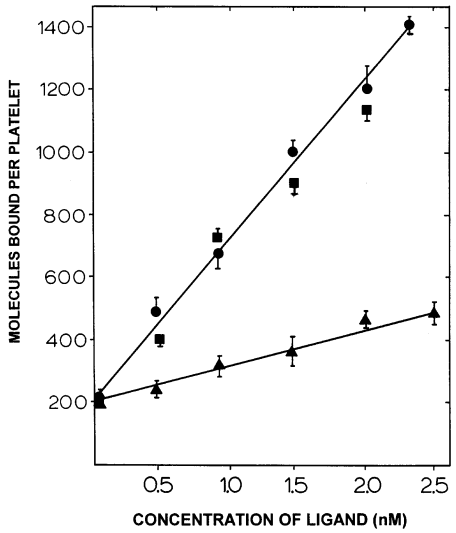

FIGURE 6: Simultaneous binding measurements of substrate (^{125}I-FX) and cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) to activated human platelets in the presence of enzyme (EGR-FIXa, 10 nM), excess FIX (250 nM), and FII (4 μM) as a function of FVIII or FVIIIa concentration. Binding measurements were made following a 20 min incubation. This figure shows specific binding of ^{125}I-FX (●), ^{131}I-FVIII (▲), and ^{131}I-FVIIIa (■). The plotted results are the means ± SE of triplicate observations from four separate experiments using platelets from four different donors.

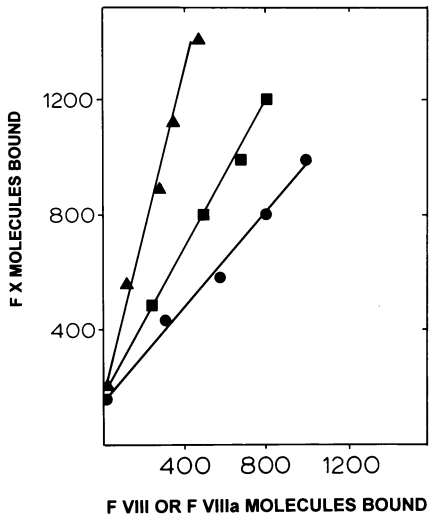

FIGURE 7: Determination of the ratio of FX to FVIII or FVIIIa bound per platelet following a 20 min incubation in the presence of FII (4 μM) as a function of FVIII or FVIIIa concentration. Linear regression analysis of the data produced a slope equal to ~3.05 molecules of FVIII per FX molecule bound to platelets (▲) and ~1.7 FVIIIa molecules per FX molecule bound to platelets (■). In the presence of saturating concentrations of both the zymogen (FIX, 250 nM) and FII (4 μM), data analysis produced a slope equal to 0.99 molecule of FX bound to each molecule of FVIIIa on activated platelets (●).

active cofactor (^{131}I-FVIIIa), which interacted with only 750 sites/platelet. We then carried out simultaneous binding measurements of the substrate (^{125}I-FX) and active cofactor (^{131}I-FVIIIa) to activated human platelets in the presence of enzyme (EGR-FIXa, 10 nM) at a saturating concentration of both the zymogen (FIX, 250 nM) and prothrombin (FII, 4 μM). The results (Figure 5B) demonstrate that the addition of saturating concentrations of FIX zymogen has the unexpected and interesting effects (compared with Figure 5A) of markedly increasing the rate of FVIIIa and FX binding and also of increasing the amount of FVIIIa bound without any effect on the amount of FX bound. Thus, in the presence of FIX (in addition to FIXa and FII), the binding of FVIIIa and FX to activated platelets was simultaneous, with equimolar stoichiometry maintained throughout the time course of the experiment (Figure 5B).

Simultaneous Binding Measurements of Substrate (^{125}I-FX) and Cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) to Activated Human Platelets in the Presence of Enzyme (EGR-FIXa), Excess Zymogen (FIX), and Prothrombin (FII) as a Function of Procofactor (FVIII) or Active Cofactor (FVIIIa) Concentration. Next we determined the amount of substrate (^{125}I-FX) and cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) bound after a 20 min incubation as a function of cofactor concentration with the concentrations of both the enzyme (EGR-FIXa) and the substrate (^{125}I-FX) held constant at 10 nM each. In the presence of saturating concentrations of prothrombin (FII, 4 μM) and zymogen (FIX, 250 nM) as the concentration of cofactor (FVIII or FVIIIa) was increased, the binding of the active cofactor (FVIIIa) and substrate (FX) was coordinate and equimolar, indicating that the binding of substrate (FX) is directly related to the binding of active cofactor (FVIIIa) (Figure 6). In contrast to the coordinate binding of FX and FVIIIa, the procofactor (FVIII) interacted with only ~25−30% of the number of sites occupied by the active cofactor (FVIIIa).

Determination of the Ratio of Substrate (FX) to Procofactor (FVIII) or Active Cofactor (FVIIIa) Bound per Platelet as a Function of Procofactor (FVIII) or Active Cofactor (FVIIIa) Concentration. In Figure 7, coordinate binding of ^{125}I-FX and either ^{131}I-FVIII or ^{131}I-FVIIIa was determined and expressed as the number of substrate (FX) molecules bound per platelet versus the number of molecules of bound procofactor or active cofactor. The experiment was carried out in the presence of excess FII (4 μM) and in either the absence or presence of saturating FIX (250 nM). The slope of the line provides an estimate of the stoichiometric relationship between the number of substrate (FX) and cofactor (FVIII or FVIIIa) molecules bound to the activated platelet membrane. We determined by linear regression analysis (r ~0.950) that the ratio of substrate (FX) molecules to procofactor (FVIII) molecules bound per platelet is ~3.05:1 in the presence of excess prothrombin to block the shared FX/FII binding site (Figure 7, closed triangles). In contrast, the ratio of substrate (FX) molecules to active cofactor (FVIIIa) molecules in the presence of excess prothrombin was 1.7:1 (r ~0.960), whereas when both saturating FII (4 μM) and saturating FIX (250 nM) were present, the stoichiometry between the substrate (FX) and the active cofactor (FVIIIa) was 1:1 (r ~0.990), indicating that, under relatively physiological conditions, for each molecule of active cofactor (FVIIIa) that was bound to the platelet surface, a single molecule of substrate was bound.

DISCUSSION

The intrinsic pathway FX activating complex consists of the vitamin K-dependent glycoproteins FIXa and FX (*19*, *20*) together with the heterotrimeric active cofactor (FVIIIa) in the presence of calcium ions and phospholipids (*21*−*23*). The binding to a platelet receptor complex of FIXa (*1*), FVIIIa (*3*), and FX (*4*) is essential for physiologically

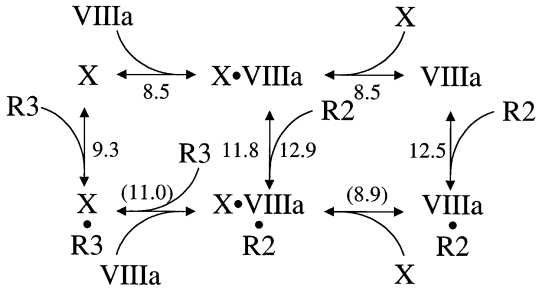

FIGURE 8: Thermodynamic boxes representing interactions of FVIIIa and FX. Shown in the upper portion of the figure are the interactions of FVIIIa and FX in solution as determined by Lapan and Fay (*24*). The vertical arrows represent reversible interactions of FX with its platelet (FX/FII shared site) receptor (designated R3), of FVIIIa with its platelet receptor (designated R2), and of the FVIIIa/FX complex with R2. Not shown in the figure for simplicity is the interaction of the enzyme, FIXa, with its receptor, designated R1. The numbers adjacent to the arrows represent the fee energy of binding in kilocalories per mole, calculated from measured values of equilibrium dissociation constants as described under Experimental Procedures. The values in parentheses are calculated from measured values within the same box.

relevant FX activation *in vivo*. The combined effect of receptor occupancy by the components of the FX activating complex on the activated platelet surface is in an overall increase in catalytic efficiency (k_{cat}/K_m) of more than 2×10^7-fold (*3*−*5*). We now report simultaneous binding measurements using dual labeled ligands to probe the coordinate interaction between the substrate (^{125}I-FX) and the cofactor (^{131}I-FVIII or ^{131}I-FVIIIa) in the presence of enzyme (EGR-FIXa) for the purpose of determining their relative stoichiometry and affinity. The hypothesis underlying the present studies is that interactions of the three protein components (FIXa, FVIIIa, and FX) of the enzyme−cofactor−substrate complex on the surface of activated platelets should reflect a composite of the interactions of each component with the platelet surface and the interactions of each of the protein components with one another. A prediction arising from this hypothesis is that the free energy of binding of each component to the activated platelet surface should be determined by both protein−surface and protein−protein interactions. This concept is presented in Figure 8, which depicts the free energies of protein−receptor and protein−protein interactions in thermodynamic boxes which incorporate the results of the present studies and those from previously published work on the interaction of FVIIIa and FX in solution and on the activated platelet membrane.

FX and FII bind to a shared high-capacity (16000 ± 2000 sites/platelet), low-affinity ($K_d \sim 320$ nM) site on platelets activated with either thrombin ($EC_{50} = 0.15$ unit/mL) or the thrombin-receptor activated peptide, SFLLRN-amide ($EC_{50} = 15$ μM), but not with ADP at concentrations up to 100 μM) (*4*). Occupancy of this FX binding site is closely coupled to rates of FX activation (*6*). This biologically relevant interaction is displayed in the thermodynamic box in Figure 8 as the binding of FX to a receptor on activated platelets designated R3 with the binding energy of 9.3 kcal/mol, reflecting a $K_d \sim 320$ nM.

In the present study (Figure 2), we show that the presence of EGR-FIXa (10 nM) and FVIIIa (10 nM) leads to the appearance of two independent binding sites, one with low affinity and high capacity (~9000 sites/platelet; $K_d \sim 380$ nM) and the other with lower capacity and higher affinity (1700 sites/platelet; $K_d \sim 30$ nM). When we carried out substrate (FX) binding studies to activated platelets in the presence of EGR-FIXa (10 nM) and FVIIIa (10 nM) and a saturating concentration of FII (4 μM), we observed an absolutely selective and specific FX binding site (1200 sites/platelet; $K_d \sim 9$ nM) (Figure 3A, Table 1). Thus, specificity is conferred for FX and not FII in the presence of other components of the FX activating complex, specifically FVIIIa which binds to its own receptor, designated as R2 in Figure 8. Thus, FVIIIa binds to 750 ± 146 sites/platelet with a $K_d = 1.5 \pm 0.11$ nM in the absence of other components (*3*), i.e., with a binding energy of 12.5 kcal/mol. In solution, it has been demonstrated that the catalytic domain of FX binds to the A1 subunit of FVIIIa, with a $K_d \sim 1-3$ μM comprising the totality of the known binding energy between the two proteins or 8.5 kcal/mol (*24*), as depicted in Figure 8. Therefore, the present studies indicate that FX can bind by two alternative routes to the site comprised by FVIIIa bound to its receptor, R2. FX can either bind to R3 (the shared FX/FII binding site), thereafter dissociating from R3 to bind to R2-bound FVIIIa, or, alternatively, the FX/FVIIIa complex formed by three-dimensional diffusion in solution can bind to the FVIIIa (R2) binding site.

In the present study, we carried out coordinate binding studies of the substrate (^{125}I-FX) with cofactor (^{131}I-FVIIIa) in the presence of the active site-inhibited enzyme (EGR-FIXa), prothrombin (FII), and excess zymogen (FIX). The rationale for adding a saturating concentration of zymogen (FIX) is to block the shared FIX/FIXa binding site (*7*), so that the coordinate binding of the substrate (FX) with active cofactor (FVIIIa) will reflect the true affinity and stoichiometry of these two components in the FX activating complex under physiological conditions. As shown in Figures 4 and 5 and in Table 1, when shared sites (FX/FII and FIX/FIXa) were blocked, both the substrate (^{125}I-FX) and the active cofactor (^{131}I-FVIIIa) interacted with identical numbers of platelet membrane receptors. Thus, all of the binding parameters describing the interaction of FX and FVIIIa with activated platelets, either obtained by equilibrium binding measurements in this study or inferred from kinetic studies (*6*, *25*), indicate coordinated equimolar stoichiometry (1000 sites/platelet for both FVIIIa and FX) with high affinities ($K_d \sim 5-10$ nM for FX; $K_d \sim 0.8$ nM for FVIIIa).

The thermodynamic boxes depicted in Figure 8 show the free energies of binding for each known protein−receptor interaction of FVIII and FX and for the solution-phase interaction of FVIII and FX, all of which are subject to direct measurement. In contrast, the free energy of interaction of platelet-bound FX with platelet-bound FVIIIa is not directly measurable. However, they can be deduced as shown in Figure 8 as the numbers within parentheses. The inference to be drawn from these calculations (which are based on the assumption that thermodynamic equilibrium must be maintained) is that the binding of R3-bound FX to R2-bound FVIIIa is considerably tighter (i.e., ~11 kcal/mol) than FX binding to FVIIIa in solution (i.e., ~8.5 kcal/mol). This influence, combined with the fact that the stoichiometry of FVIIIa/FX interaction on the surface is equimolar, strongly suggests that one of the major biological functions of FVIIIa bound to its platelet receptor (R2) is to serve as the receptor for FX and to present it to the platelet-bound enzyme, FIXa,

for efficient catalysis. Thus, it appears that FX initially binds to its high-capacity, low-affinity shared FX/FII binding site, from which it dissociates to bind by lateral diffusion to R2-bound FVIIIa.

A comparison of the assembly of the FX activating complex with the analogous assembly of the prothrombinase complex on platelet membranes is instructive. Despite some major differences [e.g., the presence of platelet FV comprising 18−25% of the FV in blood (*26*), which interferes with the determination of exogenous ligand receptor concentration], it is evident that FV(a) binds directly to platelets (*21*−*23*) and provides a high-affinity binding site for FXa (*22*, *27*, *28*). In the presence of excess human FVa there are between 200 (*19*, *27*) and 5000 (*29*) sites for FXa on human platelets with a K_d between 30 pM (*23*, *27*, *28*) and 200 pM (*29*). The binding of FVa to human platelets is not saturated at concentrations up to ∼12 nM (*21*, *29*) where, in the presence of FXa and FII, ∼3000 molecules are bound per cell (*21*). Nevertheless, the kinetic sequel of FVa binding, in terms of its ability to enhance the catalytic efficiency of FXa toward prothrombin, saturates between 200 and 500 pM (*21*, *29*). An elegant study of the coordinate binding of FVa and FXa to platelet membranes (*22*) has shown that the functional prothrombinase complex consists of a calcium-dependent, stoichiometric (1:1) complex of the cofactor (FVa) and the enzyme (FXa). In the prothrombinase complex prothrombin contributes to the assembly of the FVa−FXa complex on a phosphatidylserine-containing phospholipid membrane (*30*). Therefore, prothrombin concentration near the surface, controlled by the prothrombinase activity and mass transfer, is an important regulator of prothrombinase surface density (*30*). Whether FVa and FXa confer specificity upon prothrombin binding to platelets as do FIXa and FVIIIa to FX binding in the FX activating complex will be an interesting subject of study.

In conclusion, as shown in Figure 8, both protein−receptor interactions of the components of the FX activating complex described herein and protein−protein interactions are required to optimally regulate FX activation on the surface of activated platelets. Our recent studies aimed at defining the molecular domains within FVIII/FVIIIa that interact with other components of the FX activating complex on the surface of activated platelets (*31*−*34*) provide strong support for the existence of three separate, unique, and specific but contiguous receptors occupied independently by three-dimensional diffusion that mediate the assembly of the enzyme−cofactor−substrate complex by lateral, two-dimensional diffusion.

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