Factor X Bound to the Surface of Activated Human Platelets Is Preferentially Activated by Platelet-Bound Factor IXa†

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ABSTRACT: Factor X is a zymogen in the blood coagulation system which is activated by the serine protease, factor IXa, in a reaction that is promoted by the presence of stimulated platelets. We have shown previously that platelets possess a binding site for factor IXa, the occupancy of which is correlated with the rate of factor X activation (Ahmad et al., 1989b,c). Similarly, we have described a different binding site on the surface of activated platelets to which the substrate for this reaction, factor X, can bind (see the accompanying paper). This "zymogen binding site" is of moderate affinity and is relatively nonspecific; apparently shared to some degree by factor X and other vitamin K-dependent proteins, most notably prothrombin. We have found that prothrombin fragment 1 not only is able to displace factor X from this platelet binding site but also possesses the ability to inhibit the platelet-dependent activation of factor X. We have developed two mathematical models for the activation of factor X by platelet-bound factor IXa. The first model assumes that factor X is activated in a manner that is totally unrelated to the presumptive zymogen binding site, whereas the second model requires factor X to first bind to this site before it may interact with platelet-bound factor IXa and become activated. Within the context of each of these models, we have evaluated three mechanisms by which prothrombin fragment 1 may inhibit factor X activation. The data presented herein are most consistent with the precept that platelet-bound factor X is activated by platelet-bound factor IXa ($k_{cat} \approx 0.0011\ s^{-1}$) in an explicitly two-dimensional reaction ($K_{m,2D} \approx 230$ molecules per platelet). Prothrombin fragment 1 is believed to disrupt this reaction by competing with factor X for the zymogen binding site ($K_i \approx 470$ nM) and, to a lesser degree, by displacing factor IXa from its binding site ($K_i \approx 7\ \mu$M). These findings suggest that platelet-bound zymogen factor X represents a kinetically important pool of substrate that is preferentially activated on the surface of activated platelets.

Within the blood coagulation system, there exist several enzymatic reactions which proceed at significant rates only in the presence of specific cellular membrane surfaces. Potential advantages to be realized by such reactions are many; some apparent others more enigmatic. For instance, the ability to confine reactions within the locus of appropriate cell surfaces is of obvious utility in blood coagulation, for it is location that discriminates hemostasis from pathological thrombus formation. Further, regulated exposure of an "appropriate" cell surface brings about regulation of the surface-dependent reaction itself and the importance of controlled coagulation is manifest. Less readily understood, however, is the basis for the membrane dependency demonstrated by these reactions: the rate at which they proceed is up to 7 orders of magnitude faster when the membrane is present than when it is not.

There are three general means by which the presence of a surface could enhance an enzymatic reaction. These modalities are formally presented below as suppositions with a brief and inexhaustive set of examples for each.

Supposition 1: The Surface Affects Enzyme Molecules. A surface may attract enzyme molecules which, when bound to the surface, exhibit improved catalytic capabilities. This improvement may result from the direct influence a surface has on enzymes bound to it, such as its stabilization of an advantageous enzyme conformation, or the improvement may result from an indirect effect; for instance, a surface could facilitate the delivery of substrate molecules to surface-bound enzymes. In theory (Berg & Purcell, 1977), by simply providing a barrier to the diffusional movement of substrate molecules, a surface can increase the flux of substrate to a surface target (e.g., bound enzyme).

Supposition 2: The Surface Affects Substrate Molecules. Alternatively, substrate molecules, but *not* enzyme molecules, could be adsorbed to a surface where they are converted to product more readily than when in solution. For example, if surface-bound substrate molecules underwent a conformational change that transformed them into "super substrates," more susceptible to activating proteases than their unbound counterparts, then the inclusion of such a surface in a reaction mixture would increase the rate at which the reaction proceeds.

Supposition 3: The Surface Affects Both Enzymes and Substrates. Finally, the *colocalization* of enzyme and substrate molecules to the same surface could increase the reaction velocity. The potential kinetic advantages enumerated above still pertain yet other mechanisms are possible

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as well. It has been shown (Adam & Delbruck, 1968; Berg, 1985; Berg & von Hippel, 1985) that a freely diffusing molecule (e.g., substrate) which can be adsorbed to a macromolecular surface may reach surface-bound targets (e.g., bound enzymes) more efficiently if it conducts a two-step search for them (i.e., by reduction of dimensionality): the diffusing molecule first finds the surface from three dimensions and then finds the target in the two dimensions of the surface. Simplistically, it has been posited that the colocalization of enzyme and substrate molecules to a surface increases their "local concentration," hence, the frequency of enzyme−substrate collisions, and, resultantly, accelerates the velocity of their reaction (van Dieijen et al., 1981; Nesheim et al., 1984). Of course, for a reaction to occur, it is not sufficient for two molecules to collide. They must collide with a specific orientation, and the severe limitations imposed upon bimolecular reactions by such orientational constraints has been demonstrated theoretically (Shoup et al., 1981; Berg, 1985). The polarized binding of enzyme and substrate molecules to a surface could help alleviate these effects by limiting the number of possible orientations attainable by the reactants, thereby increasing the proportion of "effective collisions" and the overall reaction velocity.

It is obvious that all surface-mediated reactions rely upon an interaction between the surface and either the enzyme, the substrate, or both. Nonetheless, the specific mechanism by which cellular membrane surfaces promote such reactions remains obscure.

Factor VIIa (FVIIa),[1] the protease believed to initiate the coagulation "cascade", effectively activates FX and FIX only after it has formed a complex with the integral membrane protein, tissue factor (Osterud & Rapaport, 1977; Ruf et al., 1991), which is a protein that can be expressed on cells of the subendothelium. We have shown previously that FIXa binds to sites expressed on the surface of activated human platelets and that this interaction is enhanced by the presence of the protein cofactor, FVIIIa (Ahmad et al., 1989c). Similarly, FXa binds to platelets and appears to do so primarily through platelet-associated FVa (Miletich et al., 1978; Kane et al., 1980), a protein with a high degree of homology to FVIIIa. The pluripotent enzyme thrombin, which is generated by the platelet-bound FXa/FVa complex (prothrombinase), catalyzes the activation of the anticoagulant, protein C, only after it binds to thrombomodulin (Owen & Esmon, 1981) on the surface of endothelial cells. Activated protein C may then bind to procoagulant surfaces where it proteolytically inactivates FVIII(a) and FV(a) (Walker et al., 1979; Fay et al., 1991b). Thus, enzymes catalyzing cell-surface dependent reactions in the blood coagulation system bind with high affinity to selected cell surfaces either directly or via protein cofactors which are associated with the surface. Therefore, these cell surface-dependent reactions cannot be promoted by the expression of surface binding sites for the substrates *alone*; therefore, supposition 2 can be rejected, and the mechanism(s) by which cell surfaces exert their effects must fall under the rubric of supposition 1 *or* supposition 3. Discriminating these two remaining possibilities requires an understanding of whether the substrate is bound to the surface or free in solution.

The utilization of cell surfaces by the substrates for these reactions is less well studied. When the character of the "true" substrates for membrane-bound enzyme complexes in the blood coagulation system have been investigated on synthetic surfaces (i.e., phospholipid vesicles), the results have led to divergent interpretations. Some authors have proposed that the true substrates for prothrombinase and FXase are bound to the vesicle surface; hence, "bound substrate" models consistent with supposition 3 (van Dieijen et al., 1981; Nesheim et al., 1984, 1992; Mertens et al., 1985; Ruf et al., 1991; Krishnaswamy et al., 1992). Others (Forman & Nemerson, 1986; Pusey & Nelsestuen, 1993), however, have presented data in support of models wherein the substrates for these reactions reach the membrane-bound enzymes directly from the bulk solution (i.e., "free substrate" models consistent with supposition 1). Thus, essential features of the interaction between membrane bound enzyme complexes in the blood coagulation system and their substrates remain controversial.

The goal of this study was to assess the role of the human platelet surface in the activation of FX. We have described a binding site on activated human platelets that we believe has many characteristics of a good substrate binding site. We have used our knowledge of this site as well as that of the platelet binding site for FIXa to design and conduct experiments that elucidate whether the FX that is activated by platelet-bound FIXa is free in solution or bound to a platelet binding site. We have compared the results of these studies to simulated data obtained from the mathematical implementation of both "bound substrate" and "free substrate" models of the activation of FX by platelet-bound FIXa.

EXPERIMENTAL PROCEDURES

Materials. Proteins, reagents, and platelet preparation were as described in the accompanying paper.

Factor-X Activation Kinetics. To wells of a microtiter plate, maintained at 37 °C in a dry-block heater, was added 60 μL of wGFP or buffer A alone (for determination of platelet-independent FX activation). To each well was added 10 μL of buffer A containing FIXa followed by the addition of 10 μL of either buffer A or buffer A containing prothrombin fragment 1. Next, 10 μL of a solution containing SFLLRN-amide and $CaCl_2$ was added to activate the platelets and to allow both FIXa and prothrombin fragment 1 to bind to their respective platelet binding sites. Five minutes thereafter, 10 μL of a solution containing FX was added to start the reaction. The final concentrations in the reactions were as follows: $\sim 3.0 \times 10^8$ of platelets/mL; 25 μM SFLLRN-amide; 5 mM $CaCl_2$; 0.5−16 nM FIXa; 0−20 μM prothrombin fragment 1; and 0.5−200 nM FX. Twenty minutes after the addition of FX (the reaction proceeded in a linear fashion throughout this period), 10 μL of EGTA (100 mM) was added to each well, the plate was chilled on ice, and then the reaction solutions were centrifuged for 10 min at 1600*g* with the temperature held at 2 °C. After the centrifugation, 75 μL of the supernatant was transferred to wells of a second microtiter plate and warmed to 37 °C. To each well was added an equal volume of warm stopping buffer containing S-2765 (700 μM), and the cleavage of substrate was monitored for 60 min at 405 nm in a ThermoMax (Molecular Devices, Menlo Park, CA) microplate reader maintained at 37 °C. The velocities were corrected for platelet-independent FX activation by subtrac-

[1] For abbreviations used, see preceding paper in this issue.

tion of the velocity obtained when the reaction was carried out in the absence of platelets (always less than 10% of the total velocity). Prothrombin fragment 1 had no effect on the activation of FX by FIXa in the absence of platelets nor was any effect observed when, in the presence of platelets, an equivalent volume of a prothrombin fragment 1 ultrafiltrate (using a 3000 MWCO ultrafiltration membrane) was added instead of prothrombin fragment 1 itself (data not shown).

Factor X Activation Kinetics in Presence of FVIIIa. Additions were made to a 1.5 mL Eppendorf tube so that it contained wGFP, $CaCl_2$, FIXa, FVIII, and, in competition experiments, prothrombin fragment 1. The tube was maintained at 37 °C in a dry block heater. To activate the platelets and the FVIII, 20 μL of buffer A containing SFLLRN-amide and thrombin was added, and the mixture was incubated for 1 min. At this point, 20 μL of buffer A containing FX was added, and, after an additional 1 min incubation period, the reaction was quenched by the addition of 100 μL of ice-cold stopping buffer. The final reagent concentrations during the activation of FX were as follows: (i) 1×10^8 platelets/mL; (ii) 5 mM $CaCl_2$; (iii) 100 pM FIXa; (iv) 20 nM FVIII; (v) 0.8 nM thrombin; (vi) 25 μM SFLLRN-amide; (vii) 0−20 μM prothrombin fragment 1; and (viii) 6.25−250 nM FX. These conditions ensure that the FIXa concentration is limiting, and, despite the spontaneous decay of FVIIIa activity (Lollar & Parker, 1989; Fay et al., 1991a), the reaction is linear for more than 3 min. The quantity of FXa generated was determined by diluting the quenched reaction mixture 3-fold into stopping buffer containing 1 mM S-2765, measuring the rate of pNA formation, and comparing this rate to a standard curve generated with active-site titrated FXa.

Development of Models. We have developed two general models of the activation of FX by FIXa in the presence of platelets (see Figure 1). The models differ only in the nature of the "true" substrate for the platelet-bound enzyme. Specifically, model I requires that the substrate reach the enzyme, platelet-bound FIXa, directly from the bulk solution without first binding to the platelet surface; thus, model I is a "free-substrate" model. Model II differs in that the substrate, FX, is obliged to initially bind to an independent platelet binding site before it is permitted to reach platelet-bound factor IXa and become activated—it is a "bound substrate" model.

Both models were developed to represent the activation of FX in the absence of the cofactor, FVIIIa. Although this condition is unphysiological, it entails distinct and compelling advantages which greatly simplify the elaboration of mathematical models. One obvious simplification results from the lesser number of species (FX, FIXa, and platelets) that need be considered when FVIIIa is not included. The dearth of information concerning the manner with which FVIIIa interacts with activated platelets makes quantitative models including this unstable cofactor highly speculative. Further, we have found that thrombin-activated FVIII provides a high-affinity binding site for FX on the surface of activated platelets (Scandura & Walsh, 1995), but our understanding of this interaction is not sufficiently sophisticated for its inclusion in a mathematical model of FVIIIa-dependent FX activation to be other than conjecture. Foremost, we developed models for FX activation in the absence of FVIIIa, a condition for which the turnover number is extremely small ($k_{cat} \approx 10^{-3}$ s^{-1}), since in this state it is reasonable to assume

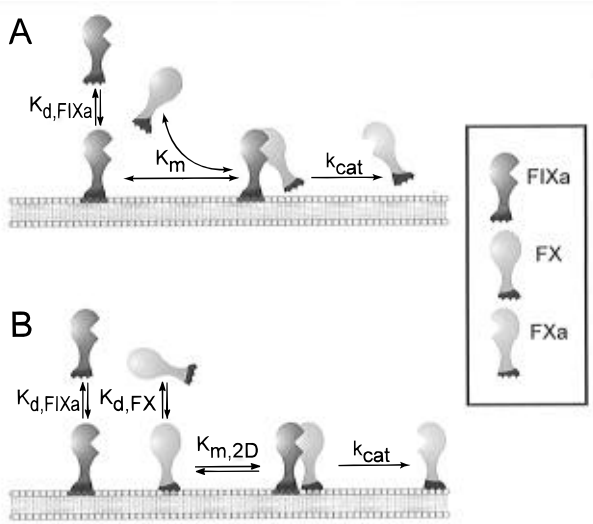

FIGURE 1: Models for surface-dependent activation of factor X. (A) Model I: solution-phase substrate. (B) Model II: surface-bound substrate.

that the system is in rapid equilibrium; thus, dissociation constants measured independently in equilibrium binding experiments could be used explicitly in the evaluation of the models.

Because we were concerned with the platelet-dependent activation of FX, the enzyme species of interest was FIXa bound to the surface of activated platelets. The performance of this species was isolated experimentally by subtracting from the total amount of FXa formed that which was generated in the absence of platelets (i.e., the superimposed contribution of unbound FIXa to the activation of FX).

Model I: "Free Substrate". The molar concentration of platelet-bound FIXa, [$FIXa^B$], at any known, total concentration of FIXa, [$FIXa^T$], can be calculated with eq 1 and the following parameters: (1) the number of platelets per mL, P; (2) the number of binding sites per platelet to which FIXa is able to bind, n_{FIXa}; (3) the dissociation constant, $K_{d,FIXa}$, of this interaction; and (4), Avogardro's number, N_A.

$$[FIXa^B] = \frac{\frac{1000}{N_A} n_{FIXa} P [FIXa^T]}{K_{d,FIXa} + [FIXa^T]} \quad (1)$$

Platelet-bound FIXa is treated as a species that can be titrated with FX, and the initial velocity of FX activation may be calculated with eq 2. This relationship, predicted by model

$$V_i = \frac{k_{cat}[FIXa^B][FX]}{K_m + [FX]} \quad (2)$$

I, resembles a simple Michaelis−Menten mechanism in that the K_m is a dissociation constant reflecting the affinity of the enzyme, platelet-bound FIXa, for the substrate, solution phase FX. However, unlike the Michaelis−Menten equation, wherein the velocity at saturating substrate concentrations (i.e., the V_{max}) increases in direct proportion to the total concentration of enzyme, the V_{max} in eq 2 is a saturable function of the total enzyme concentration and reflects the quantity of FIXa bound to the activated platelet surface, [$FIXa^B$].

Model II: "Bound Substrate". In model II, both the enzyme, FIXa, and the substrate, FX, have independent binding sites on the surface of activated platelets, and, in constrast to model I, only the platelet-bound forms of these

proteins interact with one another to produce FXa. The equilibrium governing the formation of surface-bound enzyme−substrate complexes (i.e., Michaelis complexes) is driven by the surface *density* of the reactants, that is, by the numbers of FIXa and FX molecules bound per unit surface area. We represent surface densities in units of molecules per activated platelet surface area (i.e., molecules per platelet). Accordingly, the surface density of bound FIXa or bound FX can be calculated with eq 3a or 3b, respectively.

$$[\mathrm{FIXa^B}] = \frac{n_{\mathrm{FIXa}}[\mathrm{FIXa^T}]}{K_{\mathrm{d,FIXa}} + [\mathrm{FIX_a{}^T}]} \quad (3A)$$

$$[\mathrm{FX^B}] = \frac{n_{\mathrm{FX}}[\mathrm{FX^T}]}{K_{\mathrm{d,FX}} + [\mathrm{FX^T}]} \quad (3B)$$

The parameters in these equations are the same as those described under eq 1 for FIXa or are the analogous parameters for FX.

Equation 4 represents the molar velocity of FX activation given the surface densities of enzyme, [FIXa^B], and substrate, [FX^B]. The term within the curly brackets describes the

$$V_i = \left(\frac{1000P}{N_A}\right)k_{cat}\left\{[\mathrm{FX^B}] + [\mathrm{FIXa^B}] + K_{m,2D} - \sqrt{([\mathrm{FX^B}] + [\mathrm{FIXa^B}] + K_{m,2D})^2 - 4[\mathrm{FX^B}][\mathrm{FIXa^B}]}\Big/2\right\} \quad (4)$$

formation of the Michaelis complex from surface-bound enzyme and substrate. We have used the more general quadratic form of the binding equation in this calculation because we have no *a priori* knowledge of the magnitude of the 2-dimensional dissociation constant, $K_{m,2D}$. k_{cat} is the constant describing the rate at which product is formed from the Michaelis complex and the first term of eq 4 is a factor used to convert the velocity from units of FXa molecules formed per second per platelet to molar FXa formed per second.

As for model I, the V_{max} predicted by model II (eq 4) demonstrates a saturable dependence upon the total FIXa concentration (due to FIXa binding); however, unlike model I, the substrate concentration leading to half-maximal velocity, the EC_{50}, will not be constant. Instead, this concentration is a complex function of the density of bound FIXa molecules, [FIXa^B], the number of FX binding sites per platelet, n_{FX}, the dissociation constant for FX binding to these sites, K_{dFX}, and the surface-dependent affinity of the interaction between the bound substrate and bound enzyme, $K_{m,2D}$. The EC_{50} can be calculated as shown in eq 5.

$$\mathrm{EC_{50}} = \frac{S_{50}K_{d,FX}}{n_{FX} - S_{50}} \quad (5A)$$

where

$$S_{50} = \{[\mathrm{FIXa^B}]n_{FX} + 2[(\mathrm{FIXa^B}] + K_{m,2\text{-}D})^2 - {n_{FX}}^2 - 3n_{FX}K_{m,2\text{-}D} + (n_{FX} - 2K_{m,2\text{-}D} - 2[\mathrm{FIXa^B}])\sqrt{[\mathrm{FIXa^B}]^2 + 2[\mathrm{FIXa^B}](K_{m,2\text{-}D} - n_{FX}) + (K_{m,2\text{-}D} + n_{FX})^2}\}/8([\mathrm{FIXa^B}] - K_{m,2\text{-}D}) - 4n_{FX} \quad (5B)$$

Models for Prothrombin Fragment 1 Inhibition. In the context of either model I or II, prothrombin fragment 1 has been modeled to inhibit the activation of FX by one of three mechanisms (shown Schematically in Figure 2). In mechanism A, prothrombin fragment 1 acts by competing with the enzyme, FIXa, for its platelet binding site. The mode of inhibition of mechanism B, results from the ability of prothrombin fragment 1 to compete with FX for a shared platelet binding site. Mechanism C permits both mechanisms A and B to operate simultaneously. That is, prothrombin fragment 1 retains its ability to compete for the enzyme binding sites while also preventing the interaction of the substrate with distinct surface binding sites. Clearly, mechanisms B and C are only relevant for model II, and, since we know that prothrombin fragment 1 can compete for the FX binding site (see the accompanying paper), mechanism A only exists in model I; we refer to these three mechanisms as IA, IIB, and IIC to reiterate this point.

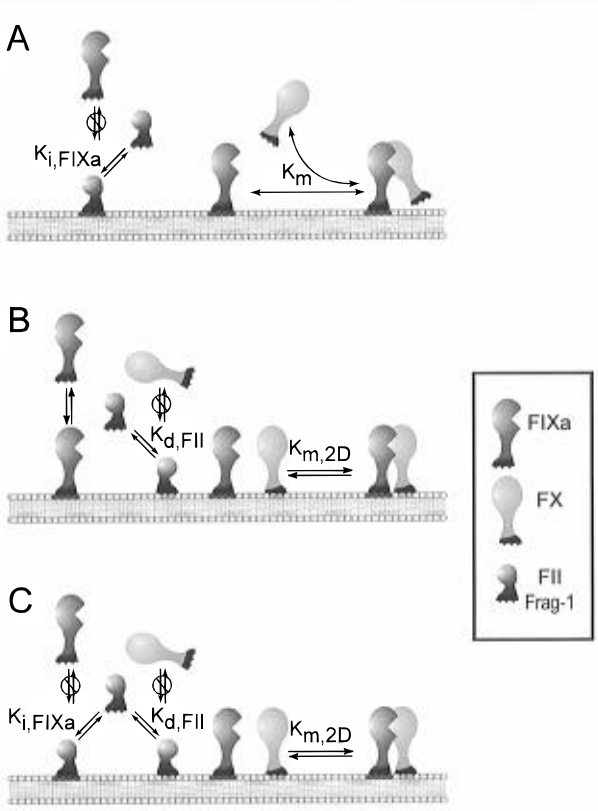

FIGURE 2: Forms of prothrombin fragment 1 inhibition. Prothrombin fragment 1 competes ( ) with FIXa for its platelet binding site, (B) with FX for its platelet binding site, and (C) with both FIXa and FX for their respective platelet binding sites.

According to these mechanisms, the effect that prothrombin fragment 1 has on the velocity of FXa generation is solely due to its ability to displace the enzyme, the substrate or both from the platelet surface. As such, the extent of inhibition predicted for a known concentration of prothrombin fragment 1, I, by each of these mechanisms can be calculated as follows: (mechanism IA) by inserting $K_{d,FIXa}(1 + I/K_{i,FIXa})$ for the $K_{d,FIXa}$ term in eq 1; (mechanism IIB) by inserting $K_{d,FX}(1 + I/K_{i,FX})$ for the $K_{d,FX}$ term in eq 3B; or (mechanism IIC) by making both of these insertions into eqs 3A and 3B. The manner with which the observed EC_{50} is predicted to be affected by the presence of prothrombin fragment 1 and the effect that the concentration of FX has on the IC_{50} observed for prothrombin fragment 1 were solved iteratively (Press et al., 1988).

Data Analysis. All data (i.e., data collected at several substrate concentrations and several enzyme or inhibitor concentrations) from a kinetic experiment conducted in either

Table 1: Parameters Fixed During Curve Fitting

parameter	value
n_{FIXa}	600
$K_{d,FIXa}$	5.5 nM[a]
n_{FX}	15 000
$K_{d,FX}$	320 nM
$K_{i,FX}$	470 nM[b]

[a] This is the average value estimated from the V_{max} vs [FIXa] curve fits as described in the test; the standard deviation was 1.6 nM. [b] This value is the average K_d measured for prothrombin binding to activated platelets.

the absence (activation kinetics) or presence (inhibition kinetics) of prothrombin fragment 1 were fit simultaneously to either model I or model II (activation kinetics) or to inhibition forms IA, IIB, or IIC (inhibition kinetics). A least-squares measure for goodness-of-fit was used, and the individual titration curves within a data set were normalized so that they were equally weighted during the fitting process (Barshop et al., 1983). In this manner, global minimization of *all* data points was obtained, a condition that we believe ensured the most general results. Each titration curve by itself is well described by classical Michaelis−Menten (or simple competitive inhibition) kinetics, and it is only when the data from several such curves are taken *en masse* that aberrations from this simple behavior can be seen.

During the global curve fitting, the only parameters that were varied were the K_m (3D or 2D) and k_{cat} or, in the presence of prothrombin fragment 1, $K_{i,FIXa}$. The other parameters were fixed to those shown in Table 1 and the known, measured concentrations of reactants (i.e., FIXa, FX, and the platelet concentration). A Visual Basic module was written for Microsoft Excel v5 to carry out all calculations and curve fits and was run on either a PowerMacintosh 7100 or 8100 computer.

Although the general phenomena described by models I and II are extensible to the more physiological situation where FVIIIa is included, the extremely rapid turnover number ($\sim$10 s^{-1}) of that reaction likely invalidates the rapid equilibrium assumptions inherent to these models. In as much as that may be true, we have only rigorously analyzed the data for the activation of FX by platelet-bound FIXa alone. The data obtained from the activation of FX in the presence of FVIIIa were fit to the Michaelis−Menten equation to estimate the apparent values of K_m and V_{max}. These values were used to obtain an apparent K_i for prothrombin fragment 1, assuming a competitive inhibition model.

RESULTS

Activation of Factor X by Platelet-Bound Factor IXa. Representative data from one of three independent measurements of the activation of FX by platelet-bound FIXa are shown in Figure 3A. The nine data points collected at each of the eight FIXa concentrations used were fitted to the Michaelis−Menten equation to derive estimates of the velocity at saturation, V_{max}, and the apparent K_m, or rather the EC_{50} (see filled circles in Figure 3B and C, respectively). The eight independently estimated V_{max} values were plotted against the total FIXa concentration, and this curve was fit to a simple binding model to obtain an estimate of $K_{d,FIXa}$. This value for $K_{d,FIXa}$ was held constant during subsequent curve fits to this data set.

The entire data set (eighty points) was fit simultaneously to either model I or model II. The only parameters permitted to vary during the minimization were k_{cat} and K_m (3D or 2D). The other parameters were fixed to the values given in Table 1, and the estimated $K_{d,FIXa}$, for the data set in Figure 3, was 1.6 nM. The best fit to either model I or II yielded values for the Michaelis constant of 16 nM (K_m) and 490 molecules per platelet ($K_{m,2D}$), respectively. The k_{cat} was estimated to be 5.1×10^{-4} s^{-1} by both models. Simulated curves calculated with eq 4 and the parameters fit to model II are represented by the solid lines in Figure 3A. The high degree of correlation between the simulation and the experimental data can be seen.

Shown in panels B and C of Figure 3 (filled circles) are the estimates of V_{max} and EC_{50} which were obtained from the fits of each curve in panel A to the Michaelis−Menten equation. Also shown in these panels is the manner with which model I (broken lines) and model II (solid lines) predict that V_{max} and EC_{50} vary with the total FIXa concentration. According to model I, V_{max} is simply the product of k_{cat} and [$FIXa^B$] ($V_{max} = k_{cat}$[$FIXa^B$]). For model II, it is calculated by substituting n_{FX} for [FX^B] in eq 4. Both models predict an hyperbolic dependency of V_{max} upon the FIXa concentration, and the best fit parameters for both models yield results that are in good agreement with the eight independently estimated V_{max} values. The models differ dramatically in how increasing FIXa concentrations are expected to affect the EC_{50}. Model I requires the EC_{50} to be independent of the total FIXa concentration, whereas it is predicted by model II to vary according to eq 5. Although neither model is in complete concordance with the eight EC_{50} estimates, model II yields values that reproduce the hyperbolic trend observed in the data; model I does not.

Prothrombin Fragment 1 Inhibition of Factor X Activation by Platelet-Bound Factor IXa. Shown in panel A of Figures 4 and 5 is one of three similar data sets generated by activating FX with FIXa (2 nM) for 20 min at 37 °C in the presence of $CaCl_2$ (5 mM), SFLLRN-amide-activated platelets (3×10^8 mL^{-1}), and prothrombin fragment 1 (0−10 μM). The entire data set, consisting of measurements made at eight different FX concentrations and eight prothrombin fragment 1 concentrations, was fit to model IA, model IIB, or model IIC. The only parameters permitted to vary during the minimization process were k_{cat}, K_m (3D or 2D), and $K_{i,FIXa}$. All other parameters were fixed to the values in Table 1. The solid lines in Figures 4 and 5 were calculated with the parameters giving the best fit to model IIC ($k_{cat} = 1.1 \times 10^{-3}$ s^{-1}, $K_{m,2D} = 120$ molecules per platelet, and $K_{i,FIXa} = 5000$ nM). For models IA and IIB, the best fit k_{cat}'s were 1.3×10^{-3} and 1.1×10^{-3} and the best fit K_m's were 11 nM (K_m) and 358 molecules per platelet ($K_{m,2D}$), respectively. The $K_{i,FIXa}$ determined by the fit to model IA was 1900 nM.

Each of the FX titration curves shown in Figure 4A was fit to the Michaelis−Menten equation to derive an estimate of V_{max} and the EC_{50} at each of the eight prothrombin fragment 1 concentrations used; the values obtained from these fits are plotted in panels B and C of Figure 4 (filled circles). It is clear that prothrombin fragment 1 reduces V_{max} in a dose-dependent manner. Accordingly, the two modes of inhibition (i.e., IA and IIC) which permit prothrombin fragment 1 to compete with FIXa for its binding site on activated platelets show a better correlation (see the broken line for IA and the solid line for IIC in Figure 4B) to the

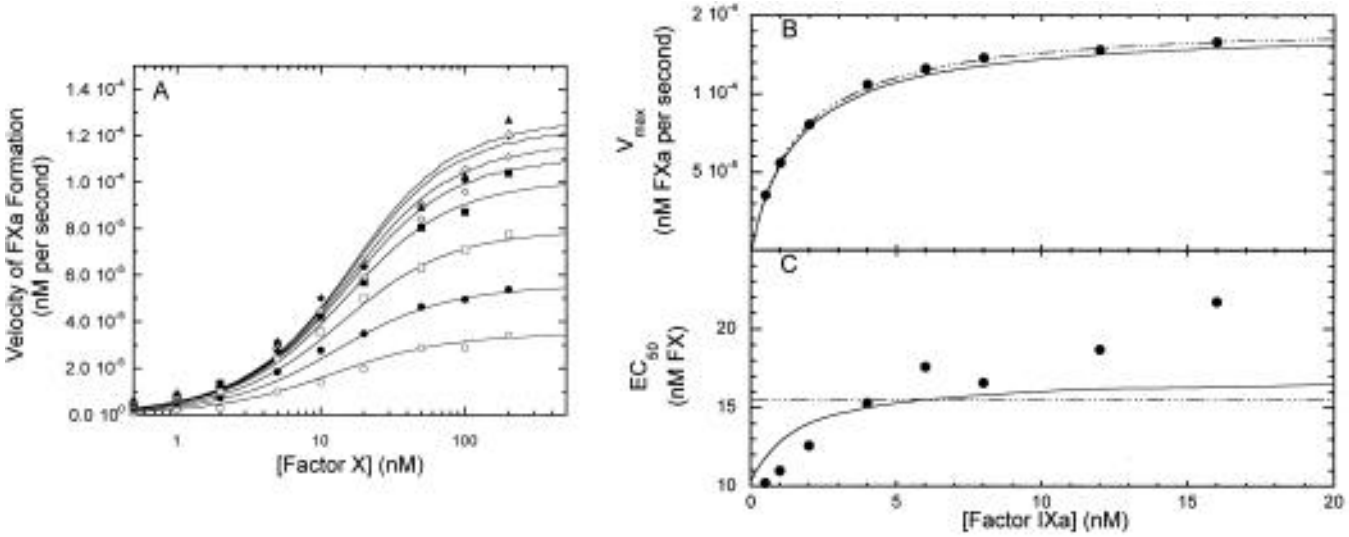

FIGURE 3: Activation of factor X by platelet-bound factor IXa. Factor X was activated for 20 min at 37 °C by factor IXa in the presence of 2.9×10^8 platelets per mL, 25 μM SFLLRN-amide, and 5 mM $CaCl_2$. (A) Velocity of factor X activation by (○) 0.5, (●) 1, (□) 2, (■) 4, (◇) 6, (◆) 8, (△) 12, or (▲) 16 nM factor IXa. Solid lines represent simulated data using parameters obtained when all curves were fit simultaneously to model II as described in the text. (B) V_{max} vs factor IXa concentration. (●) V_{max}'s estimated by fitting each curve independently to the Michaelis−Menten equation. (Broken line) V_{max} predicted with the parameters obtained by simultaneously fitting all of the data in A to model I as described in the text. (Solid line) V_{max} predicted from the best fit of the data in A to model II as described for A. (C) EC_{50} for factor X vs factor IXa concentration. (●) EC_{50}'s estimated by fitting each curve independently to the Michaelis−Menten equation. (Broken line) EC_{50} predicted with the parameters obtained by simultaneously fitting all of the data in A to model I as described in the text. (Solid line) EC_{50} predicted from the best fit of the data in A to model II as described for A.

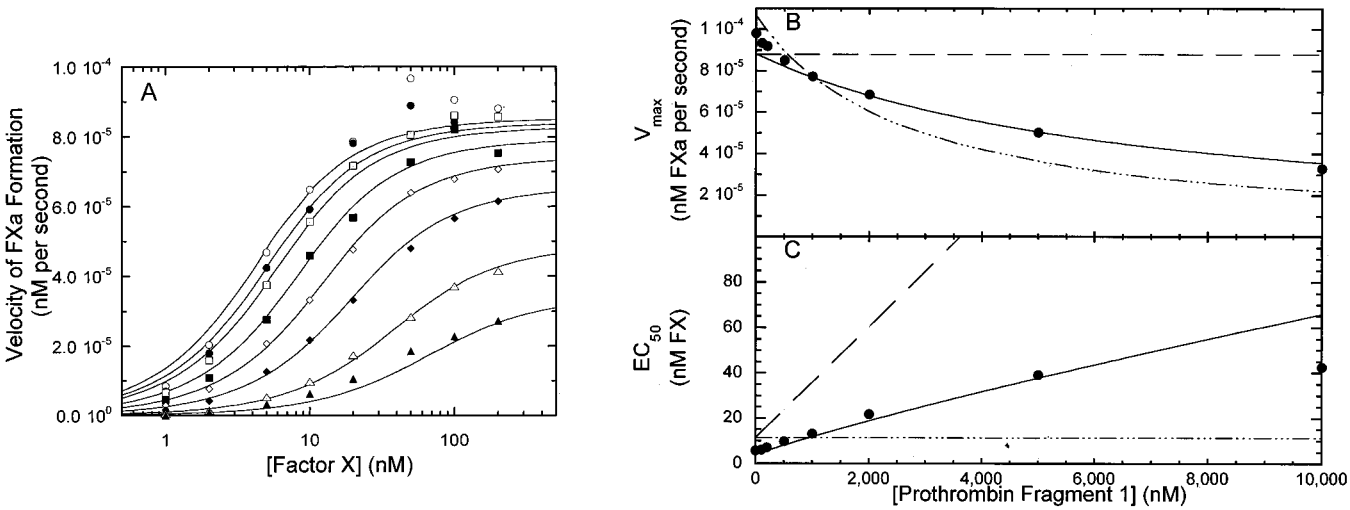

FIGURE 4: Activation of factor X by platelet-bound factor IXa in the presence of prothrombin fragment 1. Factor X was activated for 20 min at 37 °C by factor IXa (2 nM total factor IXa) in the presence of 3.0×10^8 platelets per mL, 25 μM SFLLRN-amide, and 5 mM $CaCl_2$. (A) Velocity of factor X activation by platelet-bound factor IXa in the presence of (○) 0, (●) 100, (□) 200, (■) 500, (◇) 1000, (◆), 2000, (△) 5000, or (▲) 10000 nM prothrombin fragment 1. Solid lines represent simulated data using parameters obtained when all curves were fit simultaneously to model IIC as described in the text. (B) V_{max} prothrombin fragment 1 concentration. (●) V_{max}'s estimated by fitting each curve independently to the Michaelis−Menten equation. V_{max} values calculated with the parameters obtained by simultaneously fitting all of the data in A to model IA (broken line), to model IIB (dashed line), or to model IIC (solid line) as described in the text. (C) EC_{50} for factor X vs prothrombin fragment 1 concentration. (●) EC_{50}'s estimated by fitting each curve independently to the Michaelis−Menten equation. EC_{50} predicted from the parameters obtained by simultaneously fitting all of the data in A to model IA (broken line), to model IIB (dashed line), or to model IIC (solid line) as described in the text.

extrapolated V_{max} values than does model IIB which disallows any effect of prothrombin fragment 1 on V_{max} (see the dashed line for IIB in Figure 4B). In a like manner, the increase in the interpolated EC_{50} observed at increasing prothrombin fragment 1 concentrations is anticipated by the two models (IIB and IIC) which allow prothrombin fragment 1 to occupy the platelet site to which FX must bind to be activated (see the dashed line for IIB and the solid line for IIC in Figure 4C). Model IA does not permit prothrombin fragment 1 to affect the EC_{50} (see the broken line for IA in Figure 4C).

The potency with which prothrombin fragment 1 inhibits FX activation decreases as the concentration of the substrate is increased (Figure 5), a characteristic of competitive inhibition. The curves representing the inhibition of FX activation by prothrombin fragment 1 at each of eight FX concentrations were independently fit to a general equation, $V = \text{constant}/(IC_{50} + I)$ (I is the concentration of prothrombin fragment 1), to interpolate values for the IC_{50}'s. These values are represented by the filled circles in panel B. Again, the manner in which models IA, IIB, and IIC predict the IC_{50} to

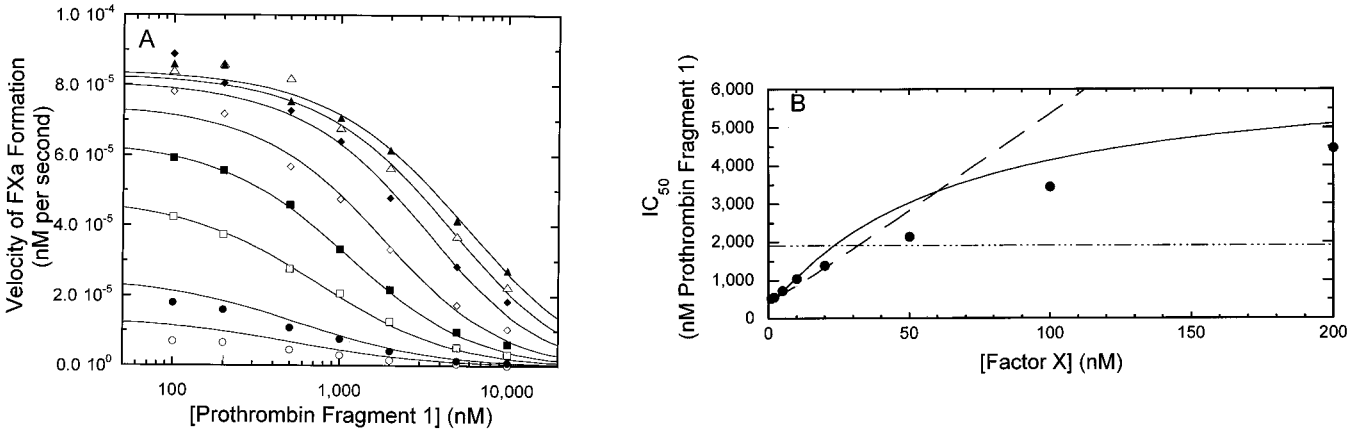

FIGURE 5: Inhibition of Factor X activation by prothrombin fragment 1. Data identical to that in Figure 4. (A) Inhibition by prothrombin fragment 1 of the activation of (○) 1, (●) 2, (□) 5, (■) 10, (◇) 20, (◆) 50, (△) 100, or (▲) 200 nM factor X by platelet-bound factor IXa. (B) IC_{50} for prothrombin fragment 1 vs factor X concentration. (●) IC_{50}'s estimated by fitting each curve independently to a general equation as described in the text. IC_{50} values calculated with the parameters obtained by simultaneously fitting all of the data in A to model IA (broken line), to model IIB (dashed line), or to model IIC (solid line) as described in the text.

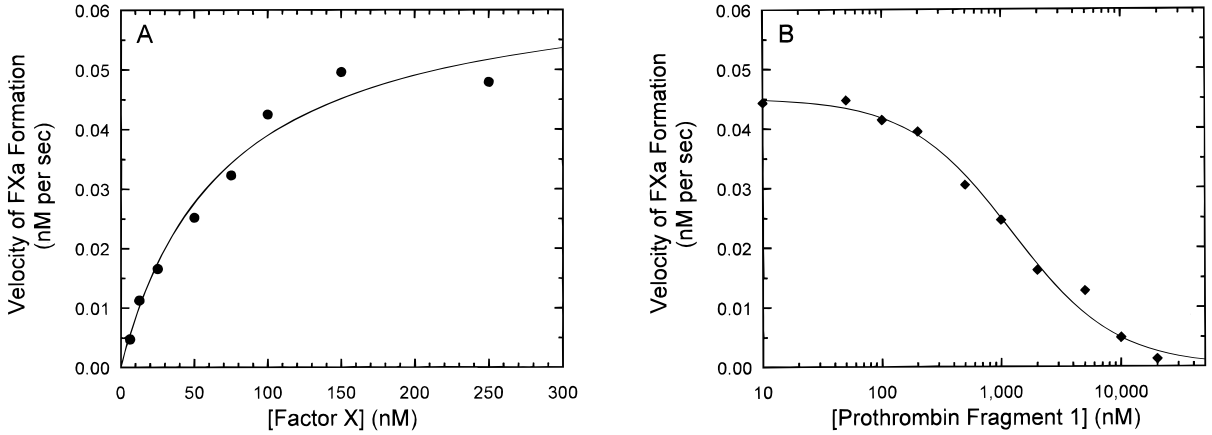

FIGURE 6: Activation of factor X by factor IXa in the presence of factor VIIIa. Factor X was activated by factor IXa (100 pM total) for 1 min at 37 °C in the presence of 20 nM thrombin-activated FVIII, 1×10^8 platelets per mL, 25 μM SFLLRN-amide, and 5 mM $CaCl_2$. (A) (●) Initial velocity of factor X activation. (solid line) Nonlinear least-squares fit of the velocity data to the Michaelis−Menten equation ($V_{max} = 0.066$ nM s^{-1}, $K_{m,app} = 70$ nM). (B) Inhibition of factor X activation by prothrombin fragment 1. (◆) The initial velocity of factor X (150 nM) activation was measured at increasing concentrations of prothrombin fragment 1. (Solid line) Nonlinear least-squares fit of the velocity data to a competitive inhibition model where the V_{max} and $K_{m,app}$ were obtained as described for A and the K_i obtained in the fit was 390 nM (the IC_{50} was 1200 nM).

be affected by the FX concentration is also shown in panel B (see the broken line for IA, the dashed line for IIB, and the solid line for IIC).

Activation of Factor X by Platelet-Bound Factor IXa in the Presence of Factor VIIIa and Its Inhibition by Prothrombin Fragment 1. FX was activated by FIXa (100 pM) in the presence of its protein cofactor FVIIIa (20 nM), and the ability of prothrombin fragment 1 (0−20 μM) to inhibit this reaction was studied. The V_{max} observed for this reaction was 0.066 nM s^{-1}, and the apparent K_m was 70 nM (see solid line in Figure 6A). At the same concentration of enzyme, cofactor, and platelets (1×10^8 mL^{-1}), and when the FX concentration was 150 nM, prothrombin fragment 1 inhibited the activation of FX with an IC_{50} of 1200 nM (see Figure 6B); if a simple competitive inhibition model is assumed, then the K_i is 390 nM. These data were not analyzed according to the models developed for the activation of FX in the absence of FVIII because the very much larger k_{cat} (calculated to be more than 2 s^{-1} for these data)[2] likely invalidates the rapid equilibrium assumption inherent to these models. Nonetheless, these data are consistent with the data obtained for the activation of FX by FIXa in the absence of FVIIIa and with the hypothesis that it is primarily platelet-bound FX which is activated by the intrinsic FXase complex.

DISCUSSION

Our discovery of a site on activated platelets to which FX can bind prompted our efforts to elucidate the physiological significance of this site. Two models of the activation of FX by platelet-bound FIXa have been presented and subjected to extensive analysis. Both of these models—model I, a "free substrate" model, and model II, a "bound substrate" model—contain parameters that are independently measurable and have been studied by our group in the past. We have found that prothrombin fragment 1 is an effective inhibitor of FX activation on the surface of platelets (Figures 4−6). Three plausible mechanisms by which prothrombin fragment 1 exerts its inhibitory effect were presented and utilized to aid in the discrimination of the bound substrate and free substrate models. These models make specific predictions about the form taken by the kinetics of FX activation both

[2] Calculated using the parameters for FIXa binding to activated platelets in the presence of FX and FVIIIa, those being, $K_{d,FIXa} = 0.25$ nM, $n_{FIXa} = 600$.

in the presence and absence of an inhibitor such as prothrombin fragment 1. We have studied the platelet-dependent activation of FX by FIXa under conditions wherein the models are most dissimilar and have constrained the comparison between experimental data and values predicted by the models with parameters that are independently measurable; these parameters are given in Table 1, and they were fixed during all curve fits. We have identified model IIC, a bound substrate model, as that which is most generally consistent with the observed behavior of this system.

It has been shown previously that the rate of FX activation is well correlated with the occupancy of a specific binding site on the surface of activated platelets by FIXa (Ahmad et al., 1989a,b; Rawala-Sheikh et al., 1990). Similarly, the more extensive studies presented herein (Figure 3), show that the maximum velocity of FX activation (i.e., V_{max}) is a saturable function of the total FIXa concentration and is well modeled as a consequence of FIXa binding to the platelet surface with an average dissociation constant (i.e., $K_{d,FIXa}$) of 5.5 nM (range from 1.6 to 8.5 nM), a value which is in close agreement with that obtained in equilibrium binding studies (Ahmad et al., 1989c). The concentration of FX necessary to yield a half-maximal velocity of FXa generation (i.e., the EC_{50}) was also shown to increase, in a saturable manner, in proportion with the concentration of FIXa used in the reaction. As shown in Figure 3C, this dependency is predicted by model II but is not permitted by a "free substrate" model such as model I. Although the observation of a nonconstant EC_{50} was sufficient to eliminate model I from further consideration, we utilized the ability of prothrombin fragment 1 to displace FX from the platelet surface to more clearly discriminate models I and II.

Though prothrombin fragment 1 possesses no ability to affect the activation of FX by FIXa when platelets are not present, Figure 4 clearly demonstrates that it causes a dose-dependent increase in the EC_{50} for FX and a concomitant reduction in the V_{max}. Presumably, the diminution of V_{max} results from a low-affinity interaction between prothrombin fragment 1 and the platelet FIXa-binding site, an effect included in models IA and IIC but not permitted by model IIB. Contrarily, the effect of prothrombin fragment 1 on the EC_{50} is incompatible with model IA. The phenomenon is, however, consistent with bound substrate models such as IIB and IIC which permit prothrombin fragment 1 to compete with FX for substrate binding sites on the platelet surface. The predominantly competitive nature of the inhibition by prothrombin fragment 1 is well demonstrated by Figure 5, where increasing concentrations of the substrate, FX, cause the observed IC_{50} for prothrombin fragment 1 to increase as well. Although each mechanism presented was in qualitative agreement with some of the data presented, it was only mechanism IIC, a form of model II, that was in accordance with all of it. Further, under all circumstances, model IIC was best able to reconcile the sometimes complex behavior observed experimentally.

Most of the parameters were held constant during the curve fit minimizations and we believe this had two major benefits: (1) it placed fairly rigid constraints upon the forms that the models could take; and (2) it ensured that model II, which includes more parameters than does model I, did not yield better approximations simply because it possessed a greater number of degrees of freedom (number of variable parameters). Overall fits to each model were improved (data not shown) by allowing the fixed parameters (i.e., n_{FIXa}, n_{FX}, $K_{d,FX}$, $K_{i,FX}$) to be varied during the nonlinear regression; some experimental variability in each of these parameters has been measured directly. However, without independent, parallel measurements of these parameters and the FX activation kinetics, the inclusion of such flexibility is more cosmetic than informative. On average, the "true" k_{cat}, $K_{m,2D}$, and $K_{i,FIXa}$ should fall within the values that we have obtained with fits to a total of six different data sets; three sets, like those in Figure 3, carried out in the absence of prothrombin fragment 1 and at differential concentrations of FIXa and three sets similar to that presented in Figures 4 and 5 wherein measurements were made at a single concentration of FIXa and at increasing concentrations of FX and prothrombin fragment 1. Table 2 contains the average values of the variable parameters obtained by independent curve fits to model IIC.

Table 2: Average Parameters Obtained from Curve Fits[a]

parameter	value
k_{cat} [b]	$(1.0 \pm 0.40) \times 10^{-3}$
$K_{m,2D}$ [c]	230 ± 100
$K_{i,FIXa}$ [d]	7.2 ± 3.2

[a] These values were obtained by averaging the parameters obtained by fitting individual data sets to model II ($n = 3$) or to inhibition form IIC ($n = 3$). The represent the mean value ± the standard error in the mean. [b] In s^{-1}. [c] In molecules per platelet. [d] In μM.

The average k_{cat}, 1×10^{-3} s^{-1}, is in excellent agreement with that determined by Duffy et al. (1992) using synthetic phospholipid vesicles and is similar to that for the activation of FX by FIXa alone (i.e., in the absence of a surface and FVIIIa) (Rawala-Sheikh et al., 1990; Duffy & Lollar, 1992). The average two-dimensional K_m, $K_{m,2D}$, that we have measured is tight, requiring only a few hundred molecules of FX bound to the surface of activated platelets to form a Michaelis complex with half of the bound FIXa molecules. If $K_{m,2D}$ were much larger, then, according to model II, even extremely high concentrations of FX would not be sufficient to cause all of the bound FIXa molecules to be occupied with substrate. Similarly, if the number of FX binding sites were not in vast excess of those for the enzyme, then there could be many platelet-bound FIXa molecules left unoccupied even at saturation. Under physiological conditions, where up to 1.4 μM prothrombin (or prothrombin fragment 1 or prothrombin fragment 1.2) is present and able to compete with FX (≈140 nM in plasma) for binding sites, the tight $K_{m,2D}$ and the large excess of substrate binding sites permit the activation of FX to proceed at near-normal rates (see open triangles in Figure 5A); only a small fraction of the total number of binding sites need be occupied by FX to nearly completely saturate the bound FIXa. This is an effective utilization of a surface binding site for substrate molecules.

The low EC_{50}'s observed for FX activation in the absence of the cofactor FVIII are at variance with those observed by us (Rawala-Sheikh et al., 1990) and by others in the past. We believe that there are three reasons for these differences. (1) In the past, we have made these measurements with gel-filtered platelets which we now know to have a significant contamination with von Willebrand factor (measured as ristocetin cofactor) and concomitantly, with FVIII. The tremendous effect of FVIIIa on the turnover number (an increase of greater than 3 orders of magnitude) permits even a very small contamination with FVIII to significantly

contribute to and change the character of the observed kinetic data. The experiments reported in this manuscript use platelets that have been washed twice into BSA-density medium prior to gel-filtration and have been shown to be devoid of ristocetin cofactor activity. In support of this interpretation is the observation that the k_{cat} reported previously is roughly 100 times greater than the value reported in this paper and that reported for the activation of FX by FIXa alone or in the presence of purified phospholipid vesicles (Rawala-Sheikh et al., 1990; Duffy & Lollar, 1992). (2) The contribution to the total rate of FX activation by the greater than 97% of the FIXa that is not platelet-bound and that has a K_m in the mid-micromolar range has not been sufficiently accounted for in the past. (3) When phospholipid vesicles have been used to study FX activation, mid- to high-micromolar concentrations of total phospholipid have been used. Assuming the phospholipid to be in the form of smaller unilamellar vesicles (SUV's) with an average diameter of 100 nm, this concentration of phospholipid gives rise to tens to hundreds of nanomolar *discrete* vesicles (Huang & Mason, 1978), conditions where the concentration of vesicles was in vast excess of the total concentration of enzyme used. In this situation, most substrate molecules are bound to vesicles that have no enzyme present on their surface. Not only does this circumstance invalidate the assumption of an isotropic system inherent in the interpretation of data according to classical enzyme kinetic theory, but also, qualitatively, it will cause the EC_{50} to be higher. A decrease in the observed EC_{50} with decreasing total phospholipid concentrations has been generally observed (van Dieijen et al., 1981; Rawala-Sheikh et al., 1990); however, we are not aware of any study in which the lower limit of the EC_{50} has been ascertained, and it is this limiting value that should be compared to the EC_{50}'s obtained with platelets.

The ability of prothrombin fragment 1 to inhibit FIXa binding to platelets is fairly impotent with the average $K_{i,FIXa}$ in excess of 7 μM (see Table 2), which is well above the plasma concentration of prothrombin and, accordingly, well above the maximum concentration of prothrombin fragment 1 attainable physiologically. Even at very high concentrations of prothrombin fragment 1, the dominant mode of inhibition is competitive; this is seen most clearly in Figures 4C and 5B.

As shown in Figure 6, prothrombin fragment 1 also inhibits the activation of FX in the presence of FVIIIa. Since FVIIIa tightens the binding of FIXa more than 5-fold (Ahmad et al., 1989c, 1990), prothrombin fragment 1 should not displace FIXa (0.1 nM) from this tighter site with an IC_{50} of less than 10 μM. The IC_{50} determined in the experiment presented in Figure 6 was approximately 1200 nM; hence, even in the presence of FVIIIa, prothrombin fragment 1 inhibits FX activation primarily by displacing FX from the zymogen binding site. When a purely competitive character of this inhibition was assumed, the K_i calculated from the data in Figure 6 was 390 nM, a value very close to the K_d for prothrombin binding to the zymogen binding site on platelets.

Interestingly, the EC_{50} measured for FX activation in the presence of FVIIIa (for the conditions in Figure 6 it was 70 nM) is in excess of that which we have observed in the absence of FVIII at any FIXa concentration. We have found this to be generally true. There are two plausible explanations for this observation: (1) the presence of FVIIIa decreases the inherent affinity (i.e., increases $K_{m,2D}$) of surface-bound FIXa for FX; or (2) the reaction becomes transport limited and therefore requires a greater diffusive flux of FX to the surface when FVIIIa is present. In light of the tremendous increase in k_{cat} brought about by FVIIIa, we favor the second interpretation. We predict that if extremely low concentrations of phospholipid were used as the "surface," a similarly increased EC_{50} would be observed for the activation of FX when FVIIIa is included.

The favored model presented in this paper has many similarities to and some important differences with the Clotspeed model of prothrombinase developed by Nesheim et al. (1984, 1992). Clotspeed uses the construct of an "interfacial shell" surrounding phospholipid vesicles. Within this shell, it is hypothesized that the binding of enzyme and substrate molecules to the vesicle surface brings their local concentrations to values far exceeding those in bulk solution. In this way, the concentrated reactants overcome the intrinsically low affinity (*intrinsic* $K_m \geq 100$ μM) of the enzyme (in this case FXa) for the substrate (in this case prothrombin). Implicit in both Clotspeed and our own model II is a two-dimensional enzyme−substrate interaction. We have chosen to deal explicitly with this dimensionalization whereas the use of an interfacial shell permits the Clotspeed model to be described in more familiar, though less strictly valid, molar units. The two approaches are largely equivalent.

Assuming an activated platelet to be a *spherical* particle with a volume of roughly 7 fL (and a surface area of $\sim 2 \times 10^{-7}$ cm^2), an interfacial shell extending 10−30 nm from its surface contains a volume of roughly 0.2−0.6 fL. If there are 15 000 substrate binding sites within this shell (a surface density of $\sim 8 \times 10^{10}$ sites cm^{-2}), then the *maximum* "local" concentration attainable is 42−120 μM, and a $K_{m,2D}$ of 230 molecules per platelet (a surface density of $\sim 1 \times 10^9$ molecules $\times$ cm^{-2}) implies that the local concentration required to attain a half-maximal reaction velocity is on the order of 0.6−2.0 μM. Of course platelets are not spheres and an interfacial shell surrounding them need not be uniform (e.g., it may exist as globoid patches); thus, one could posit the existence of a "shell volume" 50−200 times lower than what we have calculated, and, accordingly, our $K_{m,2D}$ could be made to give a Clotspeed-like *intrinsic* K_m that is in the high micromolar range,[3] and equal to that measured for the activation of FX by FIXa alone (Rawala-Sheikh et al., 1990).

Despite the essential similarities between model II and the Clotspeed model, there are differences in the manner with which the models have been applied. The data we have analyzed in terms of model II were generated under conditions where the inherent assumption of rapid equilibrium kinetics is likely to pertain. The Clotspeed model was applied to interpret kinetic data obtained with the full prothrombinase complex which has an extremely high catalytic efficiency and makes the establishment of an equilibrium unlikely. Recent studies (Giesen et al., 1991; McGee et al., 1992; Willems et al., 1993; Billy et al., 1995; Speijer et al., 1995) support the notion that, at moderate prothrombin concentrations, prothrombinase (and FXase) is limited by the rate of substrate delivery (i.e., the chemical reaction proceeds at a rate that outstrips the conveyance of substrate to the enzyme). Ultimately, it is diffusion that physically limits the rate at which substrate *can* be delivered

[3] Adjustment of the shell volume while fixing an *intrinsic* K_m, as was done in Clotspeed, is mathematically identical to varying $K_{m,2D}$.

to an enzyme or surface-bound enzyme complex. A diffusionally limited, surface-bound enzyme depletes the region surrounding it of substrate faster than new substrate can diffuse into the region. Thus, the "local" substrate concentration, which is determined by the rate of substrate influx—driven by the gradient between the bulk and local concentrations and the substrate's diffusion constant—and the rate of substrate efflux (i.e., the rate at which the chemical reaction proceeds), will *always* be lower than that far away from the surface (i.e., the bulk concentration). Therefore, the reaction rate must reach half-saturation at a local substrate concentration that is *lower* than the bulk concentration. If prothrombinase is transport limited, the "K_m" on the surface must truly be *lower* than the K_m measured in the absence of a surface, and one of the central tenets of the Clotspeed model, namely, that the *intrinsic* K_m is equal both inside and outside the "interfacial shell" surrounding the surface, is specious. We prefer the use of $K_{m,2D}$ since, when a model is developed with this construct, mass transport limitations do not invalidate the precepts of the model.

The near 4 orders of magnitude drop observed in the EC_{50} for FX activation observed when platelets are included in the reaction mixture reflects the greater effectiveness of the association between FX and FIXa on the platelet surface compared to their association in bulk solution. If not by concentrating the reactants upon their surface, how then do platelets bring about this dramatic reduction in the EC_{50}? One could hypothesize that FX and FIXa are perturbed by their binding to the platelet surface in such a way that they become a better substrate and a better enzyme for each other. Indeed, there is considerable evidence to suggest that the catalytic enhancement enjoyed by cell-surface-dependent reactions in the coagulation system is, at least partly, the result of direct effects by surface-bound cofactors on the enzymes. This phenomenon is clearly demonstrated by the dramatic change in substrate specificity observed when thrombomodulin binds thrombin; in the absence of thrombomodulin, thrombin has little affinity for protein C, instead preferring procoagulant substrates such as fibrinogen (Esmon & Esmon, 1984; Jakubowski, 1986). Active-site resident fluorophores have revealed that the enzymes thrombin, FVIIa, FXa, and FIXa all undergo conformational changes when they interact with their respective cofactors on the surface of artificial phospholipid vesicles (Husten et al., 1987; Lu et al., 1989; Duffy et al., 1992; Mutucumarana et al., 1992). Nonetheless, when these enzymes bind to vesicles in the absence of their cofactors, similar alterations in their active-site geometry have not been consistently observed; FXa appears to undergo such a transition (Husten et al., 1987), yet FIXa, the enzyme we have studied, does not (Duffy et al., 1992). Study of the substrates for surface-dependent reactions in the coagulation system is largely lacking. To our knowledge, only the prothrombinase reaction intermediate, meizothrombin, has been studied and shown to undergo a conformational change upon its binding to phospholipid membranes (Armstrong et al., 1990). It is *possible* that heretofore unrecognized conformational rearrangements cause platelet-bound FIXa to specifically recognize platelet-bound FX with high affinity, yet such structural perturbations need not be evoked.

In theory, the flux of freely diffusing molecules (e.g., FX) to a small target (e.g., FIXa) can be increased if the target is located on a macromolecular surface (e.g., the plasma membrane of an activated platelet). This effect is a consequence of the restricted freedom of movement which is imposed upon the diffusible molecule when it is in close proximity to even an *inert* surface (i.e., one that does not bind the diffusing molecule) (Berg & Purcell, 1977). Since the inert surface behaves as an extended barrier, a molecule that has collided with it is likely to collide with it several times again before finally moving away. Accordingly, the likelihood of the molecule's finding a small target is increased if the target is located on a membrane. However, if the platelet surface acted in this manner, as an inert barrier to the free movement of FX molecules which allows this substrate to find platelet-bound FIXa molecules more efficiently, then the binding of prothrombin fragment 1 to the platelet surface would simply extend this "inert" surface and prothrombin fragment 1 could not possess the character of a competitive inhibitor of FX activation. It appears unlikely that platelets are purely inert in their interaction with FX.

If a surface is not inert and a "nonspecific" interaction between a diffusible molecule and the surface exists, then the diffusible molecule may conduct a coupled, two-step search for membrane-localized targets (such as platelet-bound FIXa). The character of this coupled search will depend on the affinity of the surface for the diffusible molecule. If the affinity is high, then the molecule's residence time on the surface will be long and the coupled search will be dominated by diffusion of the molecule in the two dimensions of the surface. The two-dimensional diffusion of a surface-bound molecule may result from the diffusion of its binding site within the membrane (McCloskey & Poo, 1986), or, if the binding sites are sufficiently delocalized, the molecule may simply "slide" across the surface (Berg & von Hippel, 1985). In either case, the size of the target is effectively enlarged by a factor determined from the molecule's two-dimensional diffusion constant and the "residence time" of the molecule on the surface. If the affinity of the membrane for the diffusible molecule is small, the molecule will "fall off" the surface quickly and the coupled search will be more strongly influenced by diffusion in bulk solution. Nonetheless, the molecule's spatial and perhaps rotational correlation with the surface will be retained upon its dissociation (Berg, 1985), and, when the molecule hits the surface again, it will likely do so in a region adjacent to the site from which it had dissociated: in effect, the molecule may "skitter" across the surface.

The zymogen binding site on activated platelets permits FX to find and be activated by platelet-bound FIXa via a coupled, two-step search. Yet this capacity does not prohibit FX from reaching platelet-bound FIXa by colliding with it directly; that is, by way of a "one-step" search. It is self-evident that the predominant means by which a substrate reaches a surface-bound enzyme is the most efficient means. That prothrombin fragment 1 inhibits platelet-dependent FX activation demonstrates that the two-step search is efficient. That it can abolish FX activation almost entirely suggests that virtually none of the FX that reaches platelet-bound FIXa does so via a one-step search. Thus, the binding of FX to the platelet surface seems a prerequisite to its forming a Michaelis complex with FIXa.

The binding of both FX and FIXa to platelet surfaces may be polarized in such a way as to align the active-site of the enzyme with the cleavage site in the zymogen. Both FX and FIXa are molecules roughly shaped as elongated (prolate) ellipsoids of revolution (Lim et al., 1977). Both are known to bind to platelets (Rawala-Sheikh et al., 1992; see also

accompanying paper) and phospholipids (Schwalbe et al., 1989) primarily via an interaction between the Gla-domain of the proteins and the surface. It is believed that when the molecules are bound to phospholipid vesicles, both FX (Lim et al., 1977) and FIXa (Mutucumarana et al., 1992) are oriented with their major (long) axes perpendicular to the vesicle surface. Further, the active-site of FIXa has been shown to be located "far above" (Mutucumarana et al., 1992) the vesicle surface, a finding that is consistent with the hypothesis that the active-site and the Gla-domain of FIXa exist at opposite poles along the major axis of the molecule. It is arguable that the site in the zymogen, FX, cleaved by FIXa must also be located "far above" the surface at a distance commensurate with the active-site of surface-bound FIXa. The polarized binding of both FX and FIXa reduces their orientational freedom from three axes of rotation to one, which is that parallel to their major axes, the axis about which they rotate most quickly. Not only is it suggested that the two molecules are aligned on the surface of activated platelets (or phospholipid vesicles), but they are aligned in a fashion that permits them to most quickly achieve the orientation needed for the formation of a Michaelis complex. Thus, FX molecules which are colocalized with FIXa molecules on a membrane surface are more advantageously situated than the more plebeian molecules of FX in bulk solution.

We have presented data consistent with the hypothesis that the preferred substrate for platelet-bound FIXa is platelet-bound FX. Further, a simple model for this reaction has been developed. This model implies that a binding site expressed on the surface of activated platelets which possesses a moderate affinity for zymogen substrates in the blood coagulation system can be effectively utilized by multiple substrates simultaneously. We have postulated that this "zymogen binding site" and the distinct binding site for the enzyme, FIXa, act in concert to both localize the activation of FX to the site of platelet activation and to effectively orient the molecules so that their interaction is made more efficient.

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