Prothrombinase Complex Assembly

CONTRIBUTIONS OF PROTEIN-PROTEIN AND PROTEIN-MEMBRANE INTERACTIONS TOWARD COMPLEX FORMATION*

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The prothrombinase complex of the blood coagulation cascade is composed of the serine protease, factor Xa, associated with the protein cofactor, factor Va, on a membrane surface in the presence of calcium ions (2-9). The assembled complex catalyzes the proteolytic activation of prothrombin to the serine protease, x-thrombin (3-7). Although factor Xa alone is capable of catalyzing prothrombin activation, the incorporation of factor Xa into the prothrombinase complex has a profound influence on the catalytic process (4-6). The rate of prothrombin activation is increased by approximately 10⁵-fold when factor Xa is incorporated into prothrombinase at physiologically relevant concentrations of reaction components (6). This remarkable rate enhancement has been attributed to the co-condensation of the enzyme complex and substrate on the membrane surface and to the influence of the cofactor on the properties of factor Xa (8). Hence, membrane-binding events and complex assembly are fundamental to the process of prothrombin activation in vivo.

Although the surfaces of a variety of blood cell types are considered to provide the relevant membranes required for prothrombinase assembly and activity in vivo (5, 9-11), the existing understanding of the properties of this complex is primarily based on kinetic and binding constants obtained from studies using purified proteins and unilamellar phospholipid vesicles of known size and composition. Kinetic studies of the hydrolysis of synthetic substrates by factor Xa provided initial evidence for complex formation in the absence of the substrate, prothrombin (12). Several subsequent studies (13, 14) have clearly demonstrated that neither prothrombin nor the expression of the catalytic activity of factor Xa is necessary for the assembly of factor Xa and factor V on the membrane surface. As a consequence, equilibrium binding studies have been conducted using factor Xa modified with the active site-directed inhibitor dansyl glutamylglycinyl arginyl chloromethyl ketone (DEGR-Xa) (13, 14). The dansyl moiety is a useful reporter group for the interaction between DEGR-Xa and other constituents of the prothrombinase complex. Changes in fluorescence polarization are observed when DEGR-Xa binds to phospholipid vesicles or is incorporated into the prothrombinase complex (13-15). Changes in fluorescence intensity of this reporter group are observed only when DEGR-Xa is incorporated into prothrombinase and are not observed upon formation of the DEGR-Xa-membrane complex (16). Intensity changes in the fluorophore are therefore a useful and specific signal for complex formation, independent of the pathway of assembly (16).

The accumulated information regarding the properties of prothrombinase indicates that this enzyme complex is composed of 1 mol of factor Xa complexed with 1 mol of factor Va on membrane surfaces containing negatively charged phospholipids (5). Both factor Xa and the substrate prothrombin bind to these membrane surfaces in a calcium-dependent manner (Kd ≈ 10⁶ M and Kd ≈ 10⁷ M, respectively) by virtue of γ-carboxyglutamyl acid residues present in their respective combining sites; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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1 The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DAPA, dansylarginine-N-(3-ethyl-1,5-pentanediyl) amide; DEGR-Xa, factor Xa modified with dansyl glutamylglycinyl arginyl chloromethyl ketone; FPR-Xa, factor Xa modified with diphenylalanineprolylarginyl chloromethyl ketone; PCPS, unilamellar vesicles composed of 75% (w/w) L-α-phosphatidylcholine and 25% (w/w) L-α-phosphatidylserine; VaLc, isolated light chain (M, = 74,000) of factor Va; RMSD, root mean squared deviation; L, lipid-combining sites; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
NH₂ termini (17, 18). Factor Va can also associate reversibly and tightly \((K_d \approx 10^{-12} \text{ M})\) with membranes containing acidic phospholipids through an interaction that is independent of exogenously added calcium ions (19). Complex formation results in an altered kinetic pathway for the activation of prothrombin (20), and some evidence exists for a perturbation in the active site environment of factor Xa upon its incorporation in prothrombinase (15, 16, 21). In addition, distance measurements by fluorescence energy transfer between the fluorophore at the active site of DEGR-Xa and fluorophores in the phospholipid bilayer have indicated that the protease is reoriented or displaced with respect to the membrane surface in the presence of factor Va (21). These data raise the possibility that factor Xa is no longer associated with the phospholipid surface once it is incorporated into prothrombinase. Thus, a variety of protein protein, protein calcium ion, and protein-phospholipid interactions is involved in the formation and stabilization of the enzyme complex and the subsequent expression of its catalytic activity.

Stopped-flow kinetic studies of prothrombinase assembly led to the formulation of a model for the process of complex formation (16). These studies indicated that the rate-limiting combination of factor Va and factor Xa with separate combining sites on the vesicle surface represented initial steps that were required for complex formation. The formation of the protein-phospholipid binary complexes was found to be mutually exclusive, suggesting that factor Xa and factor Va bind to identical or overlapping sites on the membrane surface. All subsequent reaction(s) between membrane-bound proteins were not rate limiting under the conditions studied and were postulated to represent rapid reaction steps involving translational or rotational diffusion of the protein-phospholipid binary complexes on the vesicle surface to form prothrombinase (16).

Although kinetic and equilibrium binding data exist regarding the initial reactions between factor Va or factor Xa and membrane sites (13, 16, 19), little information is available regarding the reactions between membrane-bound components. The only information regarding this step, obtained from kinetic studies of prothrombin activation, has been criticized for not considering the contributions of prothrombin binding to complex formation (22). Therefore, using the kinetic model derived from stopped-flow studies, I have investigated the equilibrium parameters for the interaction between membrane-bound factor Xa and factor Va while considering the linkage between the individual lipid-binding events and complex formation. In addition, direct binding studies of complex formation have been compared with kinetic studies to investigate the contributions of the substrate to the stabilization of the prothrombinase complex.

**EXPERIMENTAL PROCEDURES**

**Materials**

L-α-Phosphatidylcholine (bovine brain), L-α-phosphatidylethanolamine (hen egg), and Hepes were obtained from Sigma. The fluorescent inhibitors dansyl glutamycyanylglycylarginyl chloromethyl ketone and dansylarginine-N-(3-ethyl-1,5- undecanoylamidino) were synthesized by the procedures described previously (23, 24). D-Phenylalanylglycylarginyl chloromethyl ketone was obtained from Calbiochem. Unilamellar vesicles composed of 25% (w/w) L-α-phosphatidylserine and 75% (w/w) L-α-phosphatidylcholine (PCPS) were prepared by the procedures described by Higgins and Mann (25) and were used in binding studies within 2 days of preparation. The concentrations of PCPS are expressed as the concentration of monomeric phospholipid present in the reaction mixture.

**Proteins**

Factor Va was purified from bovine blood obtained from the slaughterhouse by procedures described previously (19). Factor X and prothrombin were isolated from barium citrate-adsorbed bovine plasma as described (28) except that Q-Sepharose (Pharmacia Fine Chemicals Biotechnology Inc.) was used as the resin for the ion exchange step instead of QAE-Sepharose. Factor Xa was activated to factor Xa using the purified activator from Russell’s viper venom (27) and subsequently purified using benzamidine-Sepharose (Pharmacia LKB) (28). The individual subunits of factor Va were purified as described previously (28). Factor Xa was modified with dansyl glutamylglycine-arginyl chloromethyl ketone or D-phenylalanylglycylarginyl chloromethyl ketone as described by Nesheim et al. (13). Following this modification, factor Xa was separated from unreacted inhibitor by chromatography using Sephadex G-25 (Pharmacia-LKB) as described previously (16). The purity of all protein preparations was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and after disulfide bond reduction by the method of Laemmli (29). The molecular weights and extinction coefficients \(\varepsilon^{\text{max}}\) used to determine concentrations were: bovine factor Va, 150,000,1.74 (19); factor Va light chain \((\text{Va}_{\text{LC}})\), 74,000,2.20 (19, 25); bovine factor X, 56,500,1.24; bovine factor Xa, 45,000,1.24 (30, 31); bovine prothrombin, 72,000,1.47 (32).

**Fluorescence Titrations of Complex Formation by Intensity Measurements**

Fluorescence titrations were performed using an SLM8000 fluorescence spectrophotometer (SLM Instruments, Urbana, IL) with hardware and software modifications as described previously (16, 19). For these experiments, the excitation wavelength was 280 nm (band pass = 2 nm), and broadband emission was monitored using a long pass filter (RV-500) in the emission path to minimize scattered light artifacts. All buffer solutions were filtered using 0.45-μm filters, and protein solutions were centrifuged (Eppendorf microcentrifuge) for 10 min to remove particulates prior to use. The sample compartment was maintained at 23 ± 0.1°C with a circulating water bath. The reaction mixture (2.0 ml) in 1 x 1-cm stirred quartz cuvettes contained either 6.0 or 15 mM DEGR-Xa and the indicated fixed concentrations of PCPS in 20 mM Hepes, 0.15 M NaCl, 2.0 mM CaCl₂, pH 7.4. Microliter additions of a concentrated stock solution of factor Va were made, and fluorescence intensity was recorded approximately 2 min after each addition. Intensity measurements were made by averaging 30 readings over 10 s with an electronic time constant of 100 ms. Three reaction mixtures were used to correct for changes in fluorescence due to scattered light. Reaction A was DEGR-Xa, PCPS, and variable concentrations of factor Va; reaction B was DEGR-Xa and PCPS with additions of buffer instead of factor Va; and reaction C was FPR-Xa (factor Xa modified with the nonfluorescent inhibitor D-phenylalanylglycylarginyl chloromethyl ketone), PCPS, and variable concentrations of factor Va. The experimental signal was obtained from reaction A, the fluorescence baseline was obtained from reaction B, and the contribution due to scatter was obtained from reaction C. Fluorescence enhancement was calculated according to the expression

\[
F = \frac{F_A - F_C}{F_B - F_C} \quad (1)
\]

where \(F_A\), \(F_B\), and \(F_C\) represent the fluorescence intensities recorded for reaction mixtures A, B, and C described above. \(F_C\) represents the intensity recorded for reaction C in the absence of factor Va. The ratio \(F/F_C\) then yields the extent of fluorescence enhancement relative to DEGR-Xa or the DEGR-Xa-PCPS binary complex after correction for scattering contributions. All scatter corrections were small (≤10%) compared with the experimental signal, and inner filter corrections were not necessary as the absorbance of the sample at the exciting wavelength \(\lambda_{\text{exc}}\) never exceeded 0.03. In all cases, the fluorescence intensities (corrected for scatter) were identical for mixtures containing DEGR-Xa, DEGR-Xa plus Va, or DEGR-Xa plus PCPS, and enhanced fluorescence was maintained in the presence of DEGR-Xa, Va, and PCPS. Control experiments indicated that at any concentration of factor Xa, the intensity value was stable for at least 4 h, and the achievement of a stable limiting fluorescence intensity was periodically verified by repeating the measurements after approximately 10 min.

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Fluorescence Titration of Complex Formation by Polarization Measurements

Fluorescence polarization measurements were conducted by operating the fluorescence spectrophotometer in T-format (33), as described previously (19). The excitation wavelength was 330 nm (band pass = 8 nm), and the emission monochromator was centered at 545 nm (band pass = 16 nm). Long pass filters (Schott KV-500) were placed in the emission beams to minimize the contributions due to scattered light. Samples contained fixed concentrations of DEGR-Xa and PCPS with variable concentrations of factor Xa or fixed concentrations of DEGR-Xa and factor Va with variable concentrations of PCPS. Fluorescence polarization was measured by manually rotating the excitation polarizer and integrating fluorescence for 10 s at each position. The individual intensities at the two polarizer orientations (I₁ and I₂) were also measured to calculate the fluorescence intensity of the sample (34). The reported values represent the mean of 8–10 separate measurements with a precision of 0.002.

Fluorescence Studies of Prothrombin Activation

Prothrombin activation was continuously monitored using the fluorescent α-thrombin inhibitor DAPA as described previously (7, 24). Fluorescence measurements were obtained using a Perkin-Elmer MFP44-R fluorescence spectrophotometer or the SLM8000 instrument, as described above. The application of either instrument for monitoring this process continuously has been described previously (7, 20, 35). Reaction mixtures (2.0 ml) maintained at 25 °C contained the indicated concentrations of prothrombin and DAPA and 35 μM PCPS in 20 mM Hepes, 0.15 M NaCl, 2.0 mM CaCl₂, pH 7.4. In separate experiments, the concentrations of prothrombin and DAPA used were 0.53 and 1.07 μM; 1.2 and 3.0 μM; 1.6 and 3.0 μM; 5.0 and 10.7 μM. Reactions were initiated by the addition of microliter amounts of factor Va and factor Xa to the indicated concentrations, and the progress of the reaction was recorded continuously by virtue of the enhanced fluorescence of the thrombin-DAPA complex (24). Reaction rates were determined from the initial steady-state portion of the progress curves.

Stopped-flow Fluorescence Measurements

Stopped-flow fluorescence studies of prothrombinase complex formation were performed using instrumentation and methods described previously (16). Complex assembly was initiated by reacting the VA-PCPS binary complex with DEGR-Xa. The reaction was monitored at 25 °C using an excitation wavelength of 280 nm and measuring broad emission with a long pass filter (KV-500) in the emission path. Excitation slits were selected to minimize photobleaching during the collection period.

Data Analysis

Fluorescence Intensity Measurements—Fluorescence titrations of complex formation obtained at two fixed concentrations of DEGR-Xa, fixed and saturating concentrations of PCPS, and variable concentrations of factor Va were analyzed according to Scheme 1 (see "Results"). Intensity values (F/F₀) obtained as a function of factor Va were fit to the quadratic equation described previously (19), except that the limits of the fluorescence data were substituted for the anisotropy values in Equation 1 of Ref. 19. Hence, nonlinear least squares regression analysis (36) was used to extract the Kᵦ, stoichiometry, F/F₀ in the absence of added Va, and F/F₀ at saturating concentrations of factor Va (Fₛ/Fₛ) from the fitted curves. Satisfactory fits were evaluated by the independence of the fitted parameters on initial estimates, the root mean squared deviation (RMSD), the standard errors (95% confidence limits) of the determined parameters, the random distribution of residuals to the fitted lines, and the Durbin/Watson statistic (37).

At nonsaturating concentrations of PCPS, the expected dependence of fluorescence intensity on the concentration of factor Va was determined according to Scheme 1 and the attending equations (see "Results"). These simulations were performed by obtaining numerical solutions to Equations 2–7 by iterative techniques using the equation-solving program MathCad (MathSoft Inc., Cambridge, MA).

Fluorescence Polarization and Activity Measurements Rates of prothrombin activation obtained at varying concentrations of factor Va at different concentrations of prothrombin and fixed concentrations of factor Xa, and PCPS were analyzed by equations described previously (20). Fitted values were obtained for the Kᵦ and stoichiometry for the lipid-dependent Xa-Va interaction and the limits of the rate data. In several experiments in which titration curves were not obtained at two fixed concentrations of factor Xa, the stoichiometry was fixed at 1 (1 mol of Va/mol of Xa at saturation) to extract the other fitted parameters.

Fluorescence polarization values from titration experiments at saturating concentrations of PCPS and variable concentrations of factor Va were converted to anisotropy values using the appropriate equations (38) and used to fit the data according to Equation 1 of Ref. 19. In competition experiments with unlabeled factor Xa, DEGR-Xa and the competition were assumed to compete with identity for the interaction with factor Va. In addition, the maximum amplitude of the signal for the incorporation of DEGR-Xa into prothrombinase was fixed from control measurements. Using these assumptions, competition curves obtained using a fixed concentration of DEGR-Xa, factor Va, and PCPS with increasing concentrations of FPR-Xa were fitted according to Equations 4–7 of Ref. 19 to extract fitted values for the Kᵦ and limiting polarization in the presence of infinite concentrations of competitor.

Titration curves from polarization measurements at fixed concentrations of DEGR-Xa and factor Va with variable concentrations of PCPS were analyzed in two waves. The PCPS requirement for complex formation (mol of PCPS/mol of prothrombinase at saturation) was estimated from the intersection point of a linear extrapolation of data at low PCPS and at saturating PCPS. The stoichiometry was then calculated by dividing the PCPS concentrations at the intersection point by the fixed concentration of DEGR-Xa. This method of estimation is valid if the fixed concentration of reactant is significantly above the dissociation constant for the measured interaction.

Estimates for the dissociation constant for the DEGR-Xa-PCPS interaction in the presence of a saturating concentration of factor Va were obtained by repeated numerical solution of the five simultaneous equations (Equations 2–7) described under “Results” at the PCPS concentrations used, with incrementally changing values of Kᵦ. For these calculations, the following constants were used: Kᵦ = 2.7 nM; Kᵥ = 1 nM; n = 42; anisotropy in the absence of PCPS = 0.1716 (measured), and anisotropy at saturating PCPS = 0.1760 (measured). RMSD was calculated from the deviation of the fitted data at each value of Kᵦ. The “best fit” was chosen from a series of calculations (Kᵦ varied between 1 μM and 0.3 nm) providing the lowest RMSD.

Stopped-flow Measurements—Stopped-flow traces were fitted according to a single or two-exponential process as described previously (16). For single exponentials, the fitted constants corresponded to the first-order rate constant (k), the amplitude of the change (A), and the baseline offset. Fitting was accomplished using Fortran programs (KINFIT, On-Line Systems, Jefferson, GA) using the rapidly converging method of successive integration (39).

RESULTS

Experimental Design and Basis for Binding Studies—A model for prothrombinase assembly based on stopped-flow kinetic studies (16) is illustrated in Scheme 1. The model depicts complex assembly proceeding through three steps. On the basis of the rate-limiting steps observed in kinetic studies, factor Va and factor Xa must initially bind to separate combining sites (L) present on the phospholipid surface (reactions 1 and 2) to yield the Va-L and Xa-L binary complexes. The presence of subsequent reaction step(s) was only inferred from the kinetic studies since the step(s) following the initial phospholipid-binding events were never rate limiting. Hence, for lack of additional information, the reaction between the Va-L and Xa-L binary complex to form prothrombinase is depicted as a single reaction step (reaction 3). The lipid-combining sites L are indicated as identical and noninteracting for the factor Xa-dependent and factor Va-dependent reactions. This representation is based on the following evi-
dence. (i) Factor Va and factor Xa can mutually exclude each other in the lipid-combining steps (10); (ii) the mole of monomeric phospholipid required to bind 1 mol of factor Va or factor Xa at saturation are essentially identical (16, 19); (iii) prothrombin fragment 1 is capable of displacing lipid-bound factor Va or factor Xa (16); and (iv) no obvious evidence for cooperativity has been obtained for the binding of either of these proteins to PCPS (13, 19). Therefore, the lipid-combining sites for both of these reactions can be considered identical or overlapping. Finally, the prothrombinase complex is illustrated as being composed of factors Va and Xa associated with each other and with their independent phospholipid-combining sites. This representation is based on the lack of any direct evidence for the dissociation of either protein from the membrane surface after complex formation.

According to Scheme 1, the overall process of complex assembly is governed by the following equilibrium expressions:

\[ K_{d1} = \frac{[Va] \cdot [L]}{[Va \cdot L]} \]  
\[ K_{d2} = \frac{[Xa] \cdot [L]}{[Xa \cdot L]} \]  
\[ K_{d3} = \frac{[Va \cdot L] \cdot [Xa \cdot L]}{[Va \cdot L] + [Xa \cdot L] + 2 \cdot [L]} \]  

and the following conservation expressions:

\[ [Va] = [Va] + [Va \cdot L] + [L \cdot Va \cdot Xa \cdot L] \]  
\[ [Xa] = [Xa] + [Xa \cdot L] + [L \cdot Va \cdot Xa \cdot L] \]  
\[ [L] = [L] + [Va \cdot L] + [Xa \cdot L] + 2 \cdot [L \cdot Va \cdot Xa \cdot L] \]

where Va, Xa, L, Va \cdot Xa, and L represent the free and total concentrations of the respective reactants; Va \cdot L and Xa \cdot L represent the concentrations of the protein-phospholipid binary complexes; and L \cdot Va \cdot Xa \cdot L represents the concentration of prothrombinase. The term L refers to the concentration of lipid-binding sites (protein-binding sites on the vesicle surface) and is related to the concentrations of PCPS by the term L = [PCPS]/n, where n is the number of mol of monomeric phospholipid combining/mol of protein, with a measured value of 42 (16, 19). The known values of Kd1, Kd2, and Kd3 used in calculations corresponded to Kd1 = 2.7 \times 10^{-9} \text{ M} and Kd3 = 1.1 \times 10^{-7} \text{ M} (16, 19).

At saturating concentrations of PCPS (and therefore L), all added Xa is present as Xa \cdot L or L \cdot Va \cdot Xa \cdot L, and all added Va is present as Va \cdot L or L \cdot Va \cdot Xa \cdot L. Hence, the terms Va and Xa in Equations 5 and 6 are negligible, and the only pertinent equilibrium expression is given by Equation 4. This system of equations is ideal for analysis using the intensity changes in DEGR-Xa since this property of fluorophore has been shown to correspond to complex formation and is not influenced by the formation of DEGR-Xa-PCPS or DEGR-Xa-Va binary complexes. Therefore, fluorescence titrations performed at a fixed concentration of DEGR-Xa, a fixed and saturating concentration of PCPS, and variable concentrations of Va will provide constants (Kd4 and stoichiometry) relevant to reaction step 3 shown in Scheme 1. Under these conditions, dependence of fluorescence intensity on factor Va can be solved by algebraic manipulation of Equations 4–7 to yield an expression similar to the equation described previously (19). Although Scheme 1 represents the simplest case of prothrombinase assembly and does not incorporate any linkage between the individual protein-phospholipid combining events, the presence of linked interactions in complex formation would have no effect on the experimental isolation of step 3 (Scheme 1) provided PCPS was present at saturating concentrations.

At limiting concentrations of PCPS, all terms become significant, and the dependence of fluorescence intensity (of DEGR-Xa) on the concentration of factor Va cannot be solved explicitly. With previous knowledge of Kd4, however, the concentration of L \cdot Va \cdot Xa \cdot L (prothrombinase) formed as a function of factor Va concentrations can be evaluated along with the other five unknown parameters (Va \cdot L, Xa \cdot L, Va, Xa, and L) by iterative numerical solution of Equations 2–7. These conditions are useful to test experimentally for the presence of linkages between the formation of Va \cdot L and Xa \cdot L, which are not accounted for by Scheme 1.

**Fluorescence Measurements of Prothrombinase Formation at Saturating PCPS**—Using the rationale described above, fluorescence titration curves for prothrombinase complex formation were obtained at saturating concentrations of PCPS in order to investigate the equilibrium parameters governing reaction 3 illustrated in Scheme 1. Fluorescence titration experiments were therefore conducted at 50 \text{ M} PCPS, two fixed concentrations of DEGR-Xa, and variable concentrations of factor Va (Fig. 1). At both concentrations of DEGR-Xa, fluorescence intensity increased saturably to a maximum value of F/Fo = 1.56, consistent with previous values observed with this reporter group (16). The equivalent fluorescence change observed at both fluorophore concentrations and the obvious saturation of the data illustrate the well behaved nature of this reaction system at the high level of detection sensitivity required for these measurements. The data could be adequately fit according to the equations described above to extract a value of Kd4 = 1 \times 10^{-9} \text{ M} and a stoichiometry of approximately 1 mol of Va bound/mol of DEGR-Xa at saturation.

At the concentration of PCPS used to obtain the data in Fig. 1, calculations using the known values for Kd4 and Kd3 indicated that essentially all (>94%) of added DEGR-Xa, or added Va was bound to the vesicle surface. However, in order to demonstrate the validity of the approach used to isolate

![Fig. 1. Fluorescence intensity measurements of complex formation at saturating concentrations of PCPS. Reaction mixtures contained either 15 nM (○) or 6 nM (●) DEGR-Xa and 50 \text{ M} PCPS in 20 \text{ M} Hepes, 0.15 \text{ M} NaCl, 2.0 \text{ M} CaCl2, pH 7.4. Titrations were obtained at 370 nm and λexc = broadband + 500 nm as described under "Experimental Procedures." The lines are drawn according to the fitted constants Kd4 = 1.0 ± 0.2 \times 10^{-9} \text{ M}, stoichiometry = 1.13 ± 0.02 mol of Va/mol of Xa, and Fmax/Fo = 1.06 ± 0.02. The residuals to the fitted lines are illustrated in the upper panel.](http://www.jbc.org/)
step 3 in Scheme 1 experimentally, titrations were repeated at different fixed saturating concentrations of PCPS. The constants obtained from these studies are listed in Table I. All fitted parameters obtained between 50 and 300 μM PCPS were essentially identical, indicating that the determined dissociation constant and stoichiometry for the interaction between Va-L and Xa-L were independent of PCPS, provided that this reactant was present at saturating concentrations.

Measurement of Complex Formation by Kinetic Studies of Prothrombin Activation—Titration curves, generated by the dependence of the initial rate of prothrombin activation (rate/ [Xa]) on the concentration of factor Va at two fixed concentrations of factor Xa and a fixed and saturating concentration of PCPS, are illustrated in Fig. 2. Similar titration curves have been used previously to estimate the equilibrium constants for prothrombinase complex formation (20) but have been criticized for ignoring the possibility that the presence of substrate could influence the association between enzyme and cofactor (22). Since the concentration of PCPS was saturating, in the absence of an effect due to substrate, complex formation would be governed by the same criteria used in the fluorescence studies. Analysis of the data using the quadratic equation described previously (20) therefore yields equilibrium constants for interaction between PCPS-bound Va and Xa. The fitted constants from these data correspond to $K_d = 0.66 \times 10^{-7}$ μM and a stoichiometry of 0.9 mol of factor Va bound/mol of factor Xa at saturation. These values are very similar to the constants reported previously in kinetic studies of human and bovine prothrombinase (6, 20) and are within experimental error of the values obtained by direct fluorescence measurements in the absence of prothrombin (see Table I).

To investigate the effect of prothrombin on the constants determined from kinetic studies, titration curves similar to those illustrated in Fig. 2 were obtained at different fixed concentrations of prothrombin. The dissociation constants obtained from these experiments (Table II) indicate that the determined value for $K_d$ is independent of prothrombin concentrations within the range of 0.5–5.0 μM. These data eliminate the possibility that the binding energy of the prothrombin-enzyme interaction is utilized to stabilize the complex further in the physiological concentration range of the substrate.

The close correspondence between the constants obtained from the kinetic studies and fluorescence binding measurements (Tables I and II) indicates that functional complex formation and perturbations in the active site environment of DEGR-Xa leading to fluorescence enhancement are coincident.

**Table I**

<table>
<thead>
<tr>
<th>[PCPS]</th>
<th>$K_d \pm$ S.E.</th>
<th>Stoichiometry $\pm$ S.E.</th>
<th>$F_{max}/F_0$</th>
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</thead>
<tbody>
<tr>
<td>μM</td>
<td>nM</td>
<td>mol Va/mol Xa</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.0 $\pm$ 0.02</td>
<td>1.13 $\pm$ 0.02</td>
<td>1.58</td>
</tr>
<tr>
<td>100</td>
<td>1.0 $\pm$ 0.08</td>
<td>1.15 $\pm$ 0.03</td>
<td>1.55</td>
</tr>
<tr>
<td>200</td>
<td>0.9 $\pm$ 0.13</td>
<td>1.14 $\pm$ 0.04</td>
<td>1.55</td>
</tr>
<tr>
<td>300</td>
<td>0.8 $\pm$ 0.10</td>
<td>1.18 $\pm$ 0.02</td>
<td>1.59</td>
</tr>
</tbody>
</table>

* The fixed concentration of PCPS.
* Fitted constants are provided $\pm$ standard error.
* Fitted amplitude of fluorescence change at saturation.

**Fig. 2.** Measurement of functional prothrombinase complex formation at saturating PCPS using initial velocity studies of prothrombin activation. Reaction mixtures containing 1.2 μM prothrombin, 3.0 μM DAPA, 30 μM PCPS, and variable concentrations of factor Va in 20 mM Heps, 0.16 M NaCl, 2.0 mM CaCl₂, pH 7.4, were initiated with 1.0 nM Xa (C) or 2.0 nM Xa (O). The initial steady-state rate of prothrombin activation (measured rate/Xa) was measured for the concentration of factor Va and fluorescence intensity as a function of time. All the lines are drawn according to the fitted constants $K_d = 0.66 \pm 0.10 \times 10^{-7}$ μM and stoichiometry of 0.9 ± 0.06 mol of Va/mol of Xa. The residuals to the fitted lines are illustrated in the upper panel.

**Table II**

Equilibrium constants for prothrombinase assembly at saturating PCPS obtained from kinetic studies of prothrombin activation

<table>
<thead>
<tr>
<th>[Prothrombin]</th>
<th>$K_d \pm$ S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>nM</td>
</tr>
<tr>
<td>0.53</td>
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<tr>
<td>1.20</td>
<td>1.28 $\pm$ 0.46</td>
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<tr>
<td>1.60</td>
<td>0.66 $\pm$ 0.10</td>
</tr>
<tr>
<td>5.00</td>
<td>0.87 $\pm$ 0.06</td>
</tr>
</tbody>
</table>

* Fixed dissociation constants.
* Fitted stoichiometry $\pm$ standard error.
cofactor to bind to membranes with high affinity \((K_d = 10^{-9} \text{ M})\) and the fact that saturation of the surface with factor Va leads to a reduction in combining sites available for complex formation.

Experimental data obtained at the same concentrations of reactants used for the simulations are illustrated in Fig. 3B. There is good agreement between the experimental and simulated data at saturating concentrations of PCPS. However, at limiting concentrations of PCPS, the observed data show obvious saturation with no evidence for a decrease in the fluorescence signal at concentrations of factor Va as high as 90 nM. In these cases, fluorescence intensity remained constant during measurements made over a 4-h period, ruling out the possibility of a slow attainment of equilibrium at high concentrations of factor Va. Further, in all cases, the fluorescence increase could instantaneously and completely be reversed by the addition of 4.0 mM EDTA to the reaction mixture. The results of these control experiments are in agreement with the reported requirement of calcium ions for prothrombinase formation (13, 14) and rule out contributions from irreversible processes such as vesicle fusion which are irrelevant to complex assembly.

The discordance between the simulated data according to the model in Scheme 1 and the observed behavior of complex assembly at limiting concentrations of PCPS suggests the presence of additional steps in the process of prothrombinase formation. The lack of inhibition at high concentrations of factor Va suggests that factor Va is not capable of displacing DEGR-Xa from the membrane surface. One reasonable interpretation of the results is that the presence of the Va-PCPS complex qualitatively or quantitatively alters the ability of factor Xa to interact with the membrane surface. A qualitative change in the nature of the combining site would preclude competition by factor Va, whereas an increase in the affinity for the Xa-L interaction induced by factor Va would require higher concentrations of factor Va to inhibit complex formation.

**Verification of Binding Studies by Fluorescence Polarization**—The unexpected behavior of the assembly reaction at limiting concentrations of PCPS was independently verified by fluorescence polarization measurements. The measurement of this parameter provides lower sensitivity when compared with the intensity measurements, but the choice of excitation wavelength (330 nm) permits titrations over a broader range of factor Va concentrations without the complicating problems due to the inner filter effect.

Polarization titrations observed using 200 nM DEGR-Xa, either 5 \(\mu\text{M}\) (limiting) or 25 \(\mu\text{M}\) (saturating) PCPS, and increasing concentrations of factor Va are illustrated in Fig. 4. At the saturating concentration of PCPS, fluorescence polarization increased saturably from a value characteristic of the DEGR-Xa-PCPS complex \((0.245)\) to a value representing the incorporation of the fluorophore into prothrombinase \((0.303)\) (13). The constants obtained by fitting these data along with a titration curve obtained at 150 nM DEGR-Xa \((\text{data not shown})\) yielded equilibrium constants \((K_{d} = 4.8 \times 10^{-9} \text{ M and stoichiometry} = 1.2 \text{ mol of Va/mol of Xa})\) that are consistent with those obtained from the intensity measurements.

At the limiting concentration of PCPS used in this experiment, the titration curve is also saturable with no evidence for a decrease in complex formation at concentrations of factor Va as high as 600 nM. Simulations performed according to Equations 2–7 predicted a substantial decrease in signal as the Va concentration was increased from 100 to 600 nM (data not shown). These data support the conclusions obtained from the measurements of fluorescence intensity and suggest an altered interaction between DEGR-Xa and the membrane surface in the presence of factor Va.

**Fig. 4.** Fluorescence polarization measurements of prothrombinase assembly. Reaction mixtures contained 200 nM DEGR-Xa, fixed PCPS concentrations of 50 \(\mu\text{M}\) \((\circ)\) or 5.0 \(\mu\text{M}\) \((\bullet)\), and variable concentrations of factor Va in 20 mM Hepes, 0.15 M NaCl, 2.0 mM CaCl\(_2\), pH 7.4. Fluorescence polarization was measured at 25 °C as described under "Experimental Procedures" using \(\lambda_{ex} = 330 \text{ nm and } \lambda_{em} = 545 \text{ nm. The line drawn through the data obtained at 50 \(\mu\text{M}\) PCPS corresponds to fitted constants of } K_{d} = 4.8 \pm 0.37 \times 10^{-9} \text{ M, stoichiometry} = 1.2 \pm 0.1 \text{ mol of Va/mol of Xa, polarization in the absence of Va} = 0.244 \pm 0.001, \text{ and polarization at saturating Va} = 0.303 \pm 0.002. \text{ The line through the data obtained at 5.0 \(\mu\text{M}\) PCPS was arbitrarily drawn.}
Measurement of the Reversibility of Complex Assembly—As the equilibrium binding data suggest that factor Xa could not be displaced from the prothrombinase complex by high concentrations of factor Va, experiments were conducted to verify the reversibility of the incorporation of factor Xa into prothrombinase. Polarization titrations were performed by pre-forming prothrombinase (L-Va-Xa-L) using DEGR-Xa at saturating PCPS and adding increasing concentrations of FPR-Xa as a competitor (Fig. 5). Fluorescence polarization decreased with increasing concentrations of FPR-Xa, indicating that complex formation is freely reversible. Assuming that DEGR-Xa and FPR-Xa compete with identity for the formation of prothrombinase, the fitted values for $K_M$ and for polarization at saturating concentrations of competitor are in good agreement with the independently determined constants. Based on the reasonable fit of the competition data (Fig. 5) and the internally consistent constants obtained, the data suggest that prothrombinase assembly is freely reversible and that DEGR-Xa and FPR-Xa are incorporated into prothrombinase with near identical affinities.

In separate experiments (data not shown), the lipid-binding subunit of factor Va (VaLC), was used to test for its ability to displace prothrombinase at limiting concentrations of PCPS. In these experiments, concentrations of VaLC as high as 1.8 $\mu$M produced no change in the signal, indicating that prothrombinase could not be displaced from the vesicle surface by depletion of lipid-combining sites in the range predicted by Scheme 1.

Stopped-flow Studies of the Influence of Membrane Availability on the Rate and Extent of Prothrombinase Assembly—The equilibrium binding studies performed at limiting PCPS (above) have all tested the ability of high concentrations of factor Va or VaLC to displace factor Xa already incorporated into prothrombinase. The influence of vesicle-bound factor Va on the incorporation of factor Xa into prothrombinase was tested by stopped-flow fluorescence measurements initiated by reacting the Va-PCPS binary complex with DEGR-Xa. For these experiments, two concentrations of factor Va were chosen at constant concentrations of all other reactants to present DEGR-Xa with the Va-PCPS complex with additional free lipid-combining sites (Fig. 6A) or with no additional combining sites (Fig. 6B). When the reaction is initiated by reacting with the Va-PCPS complex at nonsaturating levels of factor Va with DEGR-Xa (Fig. 6A), complex assembly proceeds rapidly ($t_{1/2} = 47$ ms) and is well described by a single exponential. Average constants obtained from six repetitive traces were $k = 14.5 \pm 0.8$ s$^{-1}$ and $A = 0.56 \pm 0.03$. As determined from previous stopped-flow kinetic studies (16), the rate of complex assembly under these conditions corresponds to the initial rate-limiting combination of DEGR-Xa with free combining sites on the vesicle surface (reaction 2, Scheme 1). The rate constant determined for these experimental conditions is consistent with the expected rate on the basis of the second-order rate constant for the association of Xa with PCPS. In addition, the normalized amplitude of the fluorescence change is consistent with the incorporation of all added DEGR-Xa into prothrombinase (see Table 1).
When the concentration of factor Va is increased to saturate the vesicle surface completely prior to reaction with DEGR-Xa (Fig. 6B), the progress curve for complex formation can still be fitted by the equation for a single exponential process. However, the rate of complex assembly is decreased 15-fold ($k = 700$ nm). Average constants obtained from eight repetitive traces were $k = 1.04 \pm 0.04$ s$^{-1}$ and $A = 0.49 \pm 0.04$. As determined previously (16), the rate-limiting step under these conditions corresponds to the dissociation of vesicle-bound factor Va from the membrane surface, which provides sites for the initial and required interaction between DEGR-Xa and PCPS prior to complex assembly. These data clearly indicate that factor Va and DEGR-Xa can mutually exclude each other for the membrane combination event. Most importantly, the amplitude of the fluorescence change under these conditions (50.5%) is also consistent with the slow but complete incorporation of all added DEGR-Xa into prothrombinase, whereas calculations according to Scheme 1 predicted a maximum amplitude of 32%. The absence of any effect of saturating concentrations of factor Va on the final concentration of prothrombinase provides additional evidence for an altered interaction between DEGR-Xa into prothrombinase in the presence of a vast excess of lipid binding competitor.

The results of the stopped-flow experiments indicate that although factors Xa and Va compete for identical or overlapping combining sites on the vesicle surface in the formation of protein-PCPS binary complexes (reactions 1 and 2, Scheme 1), saturation of the vesicle surface with factor Va does not alter the final concentration of prothrombinase formed. Taken together with the binding data obtained at nonsaturating PCPS and limiting concentrations of factor Xa, these studies indicate that although the rate of complex assembly is determined by the concentration of free combining sites on the vesicle surface, the determinants for the concentration of prothrombinase formed at equilibrium are the total concentration of these combining sites and the concentration of the limiting protein component in the system.

Fluorescence Polarization Measurements of the Membrane Requirement for Complex Formation The phospholipid requirement for complex formation (mol of PCPS/mol of prothrombinase at saturation) can be inferred from the data illustrated in Figs. 3B and 4. However, since this parameter determines an important aspect of the initial model (see Scheme 1) and dictates our understanding of whether both proteins are associated with the membrane surface in the assembled complex, further studies were undertaken to determine this value directly. A titration curve obtained from polarization measurements performed using a fixed concentration of DEGR-Xa, a molar excess of factor Va (with respect to DEGR-Xa), and increasing concentrations of PCPS is illustrated in Fig. 7. Fluorescence polarization increased saturably from a value characteristic of free (0.236) to a value typical of prothrombinase (0.311). This titration curve therefore describes the phospholipid dependence of the incorporation of DEGR-Xa into prothrombinase at saturating concentrations of factor Va. However, the partitioning of DEGR-Xa between the solution phase and the prothrombinase complex is a complex function of at least two separate reaction steps under these experimental conditions. Hence, the stoichiometry for the PCPS requirement for the saturable assembly of prothrombinase could only be estimated graphically using the intersection point of linear extrapolations from the limits of the polarization data (Fig. 7). This interpolation provides a reasonable approximation of the stoichiometry because the fixed concentrations of DEGR-Xa and factor Va are significantly above the $K_d$ for the overall process and yields a stoichiometry of 88 mol of PCPS require/mol of prothrombinase. When compared with the measured stoichiometries of 42-44 for the Va-PCPS or the Xa-PCPS interactions (16, 19), this value suggests that both factor Va and factor Xa are bound to the vesicle surface when incorporated into prothrombinase.

No evidence for sigmoidicity was observed under the conditions used to obtain the data illustrated in Fig. 7. In addition, essentially identical titration curves were obtained when the fixed concentration of factor Va was increased to 800 nM (data not shown). Both these criteria provide further indications that prothrombinase assembly cannot be described adequately by Scheme 1. Equations 2-7 predict a sigmoid dependence of polarization on PCPS when the fixed concentration of factor Va is in excess of the fixed concentration of DEGR-Xa (see below). Further, these equations predict that the titration curve illustrated in Fig. 7 would show a strong dependence on the fixed concentration of factor Va. These data also suggest that the incorporation of DEGR-Xa into prothrombinase results in a qualitative or quantitative change in the Xa-PCPS interaction such that the complex cannot be disrupted by the presence of high concentrations of factor Va.

Studies of the interaction between DEGR-Xa and PCPS in the presence of factor Va would provide information regarding the extent of the change, if any, in the Xa-PCPS interaction in the presence of factor Va. However, interpretations of these measurements are complicated because of the presence of at least one additional step following the Xa-PCPS combination event and the potential of heterogenous combination events at nonsaturating levels of factor Va. In order to simplify interpretations, polarization titrations were conducted using a fixed concentration of DEGR-Xa, a saturating concentration of Va, and increasing concentrations of PCPS (Fig. 8). Concentrations of reactants were chosen such that the factor Va was sufficient to saturate the vesicle surface completely at all PCPS concentrations. As a consequence of this experimental design, the pertinent reactions of complex assembly would involve: (i) the binding of DEGR-Xa to PCPS in the presence of prebound factor Va; and (ii) the reaction between Xa-L and Va-L to form prothrombinase. Concentrations were also chosen such that the concentration of the intermediate species Xa-I was negligible throughout the titration curve. Hence, the data illustrated in Fig. 8 describe the partitioning DEGR-Xa between solution and the pro-
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These conditions, fluorescence titrations indicated that the isolated equilibrium constants for complex formation which posed by the model (Scheme 1, reaction 3) was experimentally thrombin activation. The steady-state kinetic studies proceeded to investigate the mechanism of prothrombinase complex assembly using a model proposed on the basis of rapid reaction kinetic studies (see Scheme 1) (16). Particular emphasis was placed on the reaction steps involving membrane-bound proteins to investigate further interactions that could only be inferred previously.

The reaction between membrane-bound proteins as proposed by the model (Scheme 1, reaction 3) was experimentally isolated using saturating concentrations of PCPS. Under these conditions, fluorescence titrations indicated that the formation of prothrombinase (L-Va-Xa-PCPS) from the protein-lipid binary complexes is characterized by a $K_d = 110$ nM and the stoichiometry of approximately 1 mol of Va/mol of Xa at saturation. Binding studies using DEGR-Xa were correlated with the formation of the functional prothrombinase by investigating the same interaction using the kinetics of prothrombin activation. The steady-state kinetic studies provided equilibrium constants for complex formation which were indistinguishable from fluorescence studies using activated factor Xa and were independent of the concentration of prothrombin used. Experiments performed at nonsaturating concentrations of PCPS showed significant departure from the expected behavior of complex formation based on the initial model (Scheme 1). Saturation of the membrane surface with high concentrations of factor Va could not displace prothrombinase from the vesicle, although competition studies using FPR-Xa indicated that complex formation was readily reversible. These data provided evidence that both Xa and factor Va are associated with the vesicle surface in the prothrombinase complex and that the DEGR-Xa-PCPS interaction was quantitatively altered in the presence of factor Va. Both conclusions were examined further by fluorescence polarization measurements and by stopped-flow kinetic studies of complex formation. These studies showed that although factors Xa and Va could exclude each other in the initial membrane-binding event, the presence of factor Va on the vesicle surface significantly increased the affinity of the Xa-membrane interaction. Hence, much higher concentrations of factor Va would be required to displace prothrombinase from the vesicle surface.

The determined dissociation constant at saturating PCPS indicates that lipid-bound factors Xa and Va interact tightly to form prothrombinase. Some qualitative and quantitative evidence exists for an interaction between these proteins in the absence of phospholipid (40–42). Estimates for the dissociation constant for the solution-phase interaction are in the $10^{-5}$–$10^{-6}$ M range on the basis of kinetic studies (41, 42) and on the basis of direct hydrodynamic measurements. Two reasonable explanations exist for the increased affinity for Xa-Va interaction on the vesicle surface. (i) The membrane-binding events alter protein conformation, leading to an increased affinity for the protein-protein interactions. (ii) The formation of the protein-lipid complexes significantly enhances the efficiency of productive collisions between proteins, leading to an increased association rate constant. Although both possibilities could contribute to the process of complex formation, some evidence already exists for the latter explanation. Stopped-flow kinetic studies indicated that the reaction between membrane-bound proteins probably occurs under conditions approximating two-dimensional space and proceeds at a rate that is significantly greater than the calculated three-dimensional diffusion-limited rate (16). Further, the formation of the protein-lipid complex would significantly reduce the degrees of freedom in the orientation of the molecules and hence increase the frequency of productive collisions between reacting species.

The studies of complex assembly utilizing the kinetics of prothrombin activation demonstrate that the process of fluorescence enhancement in the reporter group (assumed to represent complex formation) is coincident with the assembly of functional prothrombinase. Although this technique has been used previously to estimate the equilibrium constant for complex formation (6, 20), the interpretations were compromised by the lack of a reasonable model describing the process. In addition, previous studies of complex formation using steady-state kinetic measurements of prothrombin activation have been proposed to be incorrect because the linkage between complex formation and substrate binding was not considered (22). The data obtained in the present study indicate that the substrate-binding term does not contribute to the assembly of the functional complex in the range of prothrombin concentrations of 0–5.0 μM. The physiological concentration of prothrombin is 1.4 μM (5). Although a "substrate effect" may be evident at much higher concentrations of prothrombin or at lower concentrations of factors Va and Xa, this effect is not observed in the physiologically relevant concentration range of reacting components. This behavior of prothrombinase is quite distinct from the observed properties of at least one of the other enzyme complexes of coagulation. Kinetic evidence exists for the stabilization of

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**Fig. 8. Determination of the dissociation constant for the DEGR-Xa-PCPS interaction at saturating concentrations of factor Va.** The reaction mixture contained 100 nM DEGR-Xa, 600 nM Va, and variable concentrations of PCPS in 20 mM Hepes, 0.15 M NaCl, 2.0 mM CaCl$_2$, pH 7.4. Fluorescence polarization was measured at 25 °C using $\lambda_{ex} = 330$ nm and $\lambda_{em} = 545$ nm. The lines were drawn following numerical solution of Equations 2–7 as described under "Data Analysis." The dashed line represents the results of calculations obtained by setting $K_d = 110$ nM, and the solid line was drawn according to $K_d = 0.9$ nM (RMSE = 0.0059).

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E. Pryzdial and K. G. Mann, unpublished results.
the interaction between factor VIIa and tissue factor in the presence of the substrate, factor X (40).

The discrepancy between the observed results at limiting PCPS and the expected behavior according to Scheme 1 suggests the presence of additional reaction steps in the kinetic model. Several possibilities could explain the observed results. (i) Factor Xa is no longer associated with the membrane surface in the prothrombinase complex. (ii) The determination of $K_d$, on which the model is heavily dependent, is incorrect. (iii) The Xa combination site on the vesicle surface is qualitatively altered by the presence of factor Va. (iv) The presence of factor Va alters (increases the affinity for) the Xa-PCPS interaction.

The first possibility seems unlikely on the basis of the observed membrane requirement for prothrombinase assembly. Further, the postulation of a readily reversible mechanism in which factor Xa must combine with PCPS prior to the reaction with factor Va, followed by the disruption of the Xa-PCPS interaction within prothrombinase would require complicated steps that could potentially violate microscopic reversibility.

The incorporation of DEGR-Xa into prothrombinase according to Scheme 1 is determined by reaction steps 2 and 3. Hence, displacement of enzyme by increasing concentrations of factor Va is determined by all three reaction steps in Scheme 1 (see Fig. 3A). If this mechanism is correct, the lack of observed displacement (Fig. 3B) could be accounted for by an incorrect determination of one of the equilibrium constants, particularly $K_{d_a}$. Simulations according to Equations 2-7 using the data illustrated in Fig. 8 with fixed values of $K_{d_b} = 2.7$ nM, $K_{d_a} = 110$ nM and varying values of $K_{d_a}$ indicated that reasonable fits could be obtained at values of $K_{d_a} = 0.008$ nM in comparison with the measured value of 1 nM (simulations not shown). This discrepancy is considerably outside the range of experimental error in the present work. In addition, the close correspondence between $K_{d_a}$ determined from direct fluorescence measurements and by activity measurements and the independence of this constant on the fixed saturation concentrations of PCPS all suggest that the assumptions used in the experimental isolation of step 3 for the determination of $K_{d_a}$ are reasonably valid.

One potential source of error in the determination of $K_{d_a}$ is the application of three-dimensional terms to describe a reaction that involves vesicle-bound proteins reacting with dimensional restrictions. Based on the techniques used in the present work, it is only feasible to infer the properties of these surface-localized reactions in the context of bulk solution dynamics. As a consequence, although $K_{d_a}$ may describe accurately the behavior of this enzyme system from the standpoint of bulk solution measurements, it may not completely describe this surface-dependent reaction step partly because the reactant concentration terms increase dramatically as the dimensions of the subspace under consideration are reduced. As described previously for the catalytic properties of prothrombinase (8), this increase in local concentration terms would yield a lower than expected dissociation constant when measured with bulk solution techniques. Hence, these criteria suggest that in the worst cases, $K_{d_a}$ would represent a lower limit estimate of the intrinsic equilibrium constant for step 3.

The present data cannot distinguish between possibilities 3 and 4 as explanations for the observed results. However, all the data are consistently consistent with the interpretation of the polarization measurements that indicate a substantial decrease in the $K_d$ for the Xa-L interaction in the presence of factor Va.

A revised kinetic model is consistent with these conclusions and with previous observations is illustrated in Scheme 2. This model represents an expanded form of Scheme 1 and incorporates steps describing the linked formation of protein-phospholipid binary complexes. The saturation of the membrane surface by the binding of factor Va or factor Xa is governed by reaction steps 1 and 5 or 3 and 6. In the absence of any observed cooperativity in these individual binding interactions (13, 16, 19), both reactions in each pair can be considered to be identical. Hence the equilibria for reactions 1 and 5 (Va-L formation) are governed by $K_{d_a}$ and for reactions 3 and 6 (Xa-L formation) by $K_{d_a}$. The formation of prothrombinase from membrane-bound reactants (reaction 7) is governed by $K_{d_a}$, which has been measured in the present study. The formation of Xa-L in the presence of factor Va (reaction 2) is quantitatively different from that observed in the absence of the cofactor (reaction 3) with a $K_d \approx 10^{-9}$ M. If Scheme 2 describes all reaction steps involved in complex assembly, the data imply that the binding of factor Va to the vesicle is also altered by the presence of prebound factor Xa (i.e. reaction steps 4 and 1 are not identical). On the basis of the estimated 100-fold decrease in the $K_d$ for the Xa-PCPS interaction in the presence of vesicle-bound Va, reaction step 4 (Scheme 2) would be characterized by a $K_d \approx 10^{-11}$ M in comparison with the observed $K_d$ of $10^{-9}$ M for the Va-PCPS interaction (reactions 1 and 5).

Previous reports on the binding of pyrene maleimide-modified factor Va to PCPS in the presence or absence of factor Xa provided no evidence for an increased affinity in the Va-L reaction in the presence of factor Xa (19). However, due to the absence of a reasonable kinetic model and partly due to the nature of the fluorescent signal used in these measurements, the binding of the cofactor to PCPS in the presence of factor Xa was considered to represent a single step. The lack of observable effect of factor Xa on the binding of factor Va to PCPS could be explained by this oversimplification and the experimental design used in those studies. Mathematical simulations performed under comparable conditions with the appropriate level of experimental noise indicated that the model used to describe the Va-membrane interaction (19) performed adequately in the absence of factor Xa. However, fits obtained using simulated data in the presence of the concentrations of factor Xa used in those studies indicated that the single-step model could not reliably distinguish a further decrease in the dissociation constant of the Va-membrane interaction.

Linked binding interactions in surface-dependent ternary complex formation have been characterized rigorously using rapid reaction kinetics and equilibrium binding studies for the interactions of thrombin and antithrombin III on heparin.
(44). In the case of membrane-dependent events such as prothrombinase assembly, the simplest explanation for linkage is provided by a mechanism involving a change in the membrane surface upon the binding of the first reactant. Hence, the presence of factor Va on the vesicle surface could lead to a rearrangement of the phospholipids in the outer leaflet, resulting in an increased affinity for the formation of the Xa-PCPS binary complex. Some evidence exists for the rearrangement phospholipids upon binding of some coagulation proteins (45).

In summary, prothrombinase assembly on the membrane surface involves a high affinity interaction between the Va-PCPS and Xa-PCPS binary complexes. Complex formation is quantitatively independent of the expression of the active site of factor Xa and is not further stabilized by the presence of prothrombin. The formation of the Xa-PCPS and Va-PCPS complexes appears to be linked such that the presence of factor Va on the vesicle surface has a significant effect on the combination of factor Xa with PCPS, resulting in an increased affinity at much lower concentrations than would be predicted from knowledge of the individual binary interactions.

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