Membrane-mediated assembly of the prothrombinase complex

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1991 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 266, No. 28, Issue of October 5, pp. 18720–18725, 1991
Printed in U.S.A.

Membrane-mediated Assembly of the Prothrombinase Complex*

(Received for publication, March 19, 1991)

Peter L. A. Giesen, George M. Willems, H. Coenraad Hemker, and Wim Th. Hermens‡

From the Cardiovascular Research Institute Maastricht, University of Limburg, 6200 MD Maastricht, The Netherlands

Prothrombinase assembly was studied on macroscopic planar bilayers consisting of 20% dioleoyl-phosphatidylserine (DOPS) and 80% dioleoyl-phosphatidylcholine (DOPC). The dissociation constant for the binding of factor Xa to the bilayer, measured by ellipsometry, was K_d = 47 ± 8 nM (mean ± S.D.) and this value was lowered to K_d = 2.2 ± 0.3 pM by preadsorption of factor Va. This latter value was determined from direct measurement of steady-state thrombin production. A comparable value of K_d = 1.0 ± 0.1 pM was found by repeating these experiments in suspensions of phospholipid vesicles, and it was verified that prothrombinase assembly was not influenced by the addition of prothrombin.

Using a minute amount (0.094 fmol cm^{-2}) of preadsorbed factor Va, it was found that the rate of prothrombinase assembly exceeds the rate of collisions between Xa molecules from the buffer and the sparse Va molecules on the bilayer. Apparently, factor Xa adsorbs first to the membrane and then associates rapidly with factor Va by lateral diffusion. The data indicate almost instantaneous equilibrium of this complex formation on the surface with a lower limit for the bimolecular rate constant of k_{on} = 2.8 × 10^{13} (mol/cm^2)$^{-1}$ s^{-1}.

In suspensions of small phospholipid vesicles, prothrombinase assembly is collisionally limited and the value of k_{on} should be proportional to vesicle diameter. This was verified with a method for estimation of k_{on} values from thrombin generation curves. Values of 0.36 × 10^9 and 1.6 × 10^9 M^{-1} s^{-1} were found for vesicles of 20–30- and 60–80-nm diameter, respectively.

After it had been established (1, 2) that adsorption of the coagulation factors Xa, Va, and prothrombin on phospholipid membranes is essential for efficient conversion of prothrombin by factor Xa, it was intuitively assumed that the membrane facilitates this process. Such facilitation could be the result of concentration of proteins on the membrane surface and restriction of molecular movement to the plane of the membrane. This concept has been called "reduction of dimensionality" (3). Not only the directions of movement are restricted, but also molecular orientation, and this may enhance collisional efficiency.

Reduction of dimensionality was not only supposed to promote prothrombinase assembly but, by the same mechanism, also to enhance the rate of prothrombin conversion. A number of studies, however, challenged this concept (4–6). It seemed as if the converted molecules arrived directly from the buffer solution, without previous adsorption to the membrane (free substrate model). As a result it was also doubted whether the principle played a role in prothrombinase assembly.

Recently, however, it was shown that binding of factors Xa and Va to phospholipid vesicles indeed precedes the assembly of the prothrombinase complex (7). The rate of this assembly appeared to be limited by the frequency of collisions between Xa-containing vesicles and Va molecules in the bulk solution. Because of this collisional limitation it was not possible to estimate the assembly rate constant of membrane-bound factors Xa and Va.

In the present study, this assembly is studied on planar macroscopic bilayers. Using less than 0.1 fmol cm^{-2} of adsorbed factor Va and picomolar buffer concentrations of factor Xa, we directly demonstrate that the rate of prothrombinase production exceeds the frequency of collisions between Xa molecules from the buffer and Va molecules on the bilayer. Any significant contribution of free factor Xa molecules to prothrombinase assembly can thus be excluded. Moreover, under these circumstances the total flux of factor Xa molecules toward the surface exceeds the rate of prothrombinase formation, which allows estimation of a lower limit for the bimolecular rate constant of the assembly process on the membrane.

MATERIALS AND METHODS

Proteins and Lipids—Bovine prothrombin (factor II) and coagulation factors X and V were obtained as described, respectively, in Refs. 8–10. Factor X was activated with Russell's viper venom (Sigma) (11) and factor V with thrombin (10). Concentrations of factor Xa were determined by active-site titration with *p*-nitrophenyl-*p*′-guanidinobenzoate hydrochloride (ICN Nutritional Biochemicals) (12). Concentrations of prothrombin were determined similarly after complete activation to thrombin with *Echis carinata* venom (Sigma). Concentrations of factor Va were determined by titration with factor Xa in a kinetic assay (10). Thrombin concentrations were measured at 37 °C as described (13), using the chromogenic substrate S2238 (Kabi Diagnostica) and a turnover number of thrombin for S2238 of k_{cat} = 162 s^{-1}.

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)[1] was purchased from Sigma and converted to the corresponding phosphatidylserine (DOPS) by enzymatic conversion (14). Phospholipid concentrations were determined by phosphate analysis (15). Planar phospholipid bilayers were deposited on silicon slides (Wacker Chemie, type n) as described (13). After preconditioning of the surface, an area of 0.5 cm^2 was covered with a 20% DOPS, 80% DOPC bilayer by immersion of the slide in a vesicle suspension. Bilayer mass, measured by ellipsometry, was 0.42 ± 0.03 μg cm^{-2} (mean ± S.D.). Suspensions of small (20–30-nm diameter) vesicles were prepared by sonication of a 20% DOPS, 80% DOPC mixture (16). Suspensions of larger (60–80-nm diameter) vesicles were prepared by lipid extrusion, using polycarbonate filters of 80-nm pore size (17). Unless stated otherwise, all

* This work was supported by Grant 900-526-079 from the Netherlands Organization for the Advancement of Scientific Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Cardiovascular Research Institute Maastricht, Biomedical Center, University of Limburg, P. O. Box 616, 6200 MD Maastricht, The Netherlands.

[1] The abbreviations used are: DOPC, dioleoyl-phosphatidylcholine; DOPS, dioleoyl-phosphatidylserine.

experiments were performed in 0.05 M Tris-HCl buffer, pH = 7.5, containing 0.1 M NaCl, 3 mM $CaCl_2$ and 0.5 g liter^{-1} ovalbumin (Sigma).

Experiments with Planar Bilayers—Adsorption of factor Xa on planar phospholipid bilayers was measured with an automated ellipsometer as described (18, 19). Experiments were performed at 25 °C in a cuvette containing 4 ml of buffer with a rotating stirrer at 2 mm from the slide. Initial adsorption rates of factors II and Va are transport-limited, that is, proportional to the protein concentration in the buffer and a constant Δ, determined by the diffusion constant of the protein, the buffer viscosity and the flow conditions (19, 20). For prothrombin, a value of $\Delta = (5.2 \pm 0.2) \times 10^{-4}$ cm s^{-1} was estimated from steady-state thrombin production of a bilayer containing excess prothrombinase (13). The diffusion constant of prothrombin is $D_{II} = 4.8 \times 10^{-7}$ cm^2 s^{-1} (21). For factor Va, a Stokes radius of $R = 5.05$ nm has been determined from gel filtration (22). By using the Stokes-Einstein relation $D = kT/6\pi\eta R$, a diffusion constant of $D_{Va} = 4.3 \times 10^{-7}$ cm^2 s^{-1} is found at 25 °C. This small difference in diffusion constants for two molecules with molecular weights of respectively 72,000 and 168,000 is thought to reflect the highly asymmetrical form of prothrombin (23). The value of $\Delta = 5.2 \times 10^{-4}$ cm s^{-1} was therefore also used for factor Va. The diffusion constant of factor Xa was estimated from ellipsometric measurements of initial adsorption rates of factors II and Xa on 100% DOPS bilayers, as described (19). For identical buffer concentrations, factor Xa adsorbed 1.22 times faster than prothrombin, and a value of $\Delta = 6.3 \times 10^{-4}$) cm s^{-1} was used for factor Xa. The initial adsorption rate is proportional to $D^{2/3}$ (24), thus $1.22 = (D_{Xa}/D_{II})^{2/3}$. Inserting D_{II} one obtains $D_{Xa} = 6.5 \times 10^{-7}$ cm^2 s^{-1} and, from the Stokes-Einstein equation, $R_{Xa} = 3.5$ nm.

Experiments with Vesicle Suspensions—Proteins were added to vesicle suspensions in 1.5-ml sealable plastic tubes (Eppendorf type 3810) and mechanically mixed for 5 s (Janke & Kunkel Vibrofix VF1). Final reaction volumes were 0.4–1 ml. During the experiments tubes were kept in a waterbath at 25 °C. Fresh vesicle suspensions were prepared each day and stored at −20 °C until use.

The Maximal Rate Constant for the Assembly of Adsorbed Factor Va and Factor Xa from the Buffer—The maximal, collisional-limited steady-state rate constant of assembly of factor Va and factor Xa molecules in buffer solution is $k_{max} = 4\pi N(D_{Xa} + D_{Va})(R_{Xa} + R_{Va})$ mol^{-1} cm^3 s^{-1} (25), with N for Avogadro's number. For the relatively immobilized factor Va molecules on the lipid surface, D_{Va} can be neglected and a factor of 1/2 must be added because Xa molecules can only approach from one side. Inserting $N = 6.0 \times 10^{23}$ and the values of D and R mentioned before, one obtains $k_{max} = 2.0 \times 10^9$ M^{-1} s^{-1}.

The Maximal Rate Constant for the Assembly of Adsorbed Factors Va and Xa—It is assumed that for a factor Va molecule, situated at the origin $r = 0$, a free circular lipid area is available with radius R_o. The steady-state equation for diffusion of factor Xa on the lipid surface toward the factor Va molecule is $D' r^{-1}\partial/\partial r[r\partial\Gamma(r)/\partial r] = 0$, with $\Gamma(r)$ the surface concentration of factor Xa at a distance r from the factor Va molecule and D' the sum of the diffusion constants for lateral diffusion of factor Xa and factor Va on the lipid bilayer. The maximal flow toward the factor Va molecules is obtained by assuming that the factor Va molecule acts as a perfect sink for factor Xa or $\Gamma(R_c) = 0$, with $R_c = R_{Xa} + R_{Va}$. By using this condition the diffusion equation has the solution $\Gamma(r) = \Gamma_o \ln(r/R_c)$. The arbitrary parameter Γ_o can be expressed in the average surface concentration of factor Xa, defined as $\Gamma_{av} = (\int 2\pi r\Gamma(r)dr)/(\pi R_o^2)$. Performing this integration from $r = R_c$ to $r = R_o$ and using the condition $R_c << R_o$ one obtains $\Gamma_{av} \approx \Gamma_o \cdot \ln(R_o/R_c)$. The solution of the diffusion equation thus becomes $\Gamma(r) = \{\Gamma_{av}/\ln(R_o/R_c)\}\ln(r/R_c)$.

The maximal transport rate of factor Xa toward the factor Va molecule then becomes $2\pi R_c \cdot D'[\partial\Gamma(r)/\partial r]_{r_{R_c}} = 2\pi D'\Gamma_{av}/\ln(R_o/R_c)$. Multiplication with Avogadro's number N and division by Γ_{av} gives the maximal assembly rate constant/mol of adsorbed factor Va $k_{max} = 2\pi ND'/\ln(R_o/R_c)$ mol^{-1} cm^2 s^{-1}. For a surface concentration of 0.094 fmol·cm^{-2}, the mean distance between factor Va molecules is 1400 nm and $R_o = 700$ nm. Inserting the values $R_{Xa} = 3.5$ nm, $R_{Va} = 5.05$ nm, $N = 6.0 \times 10^{23}$, and $D' \approx 10^{-8}$ cm^2 s^{-1} (26, 27), one obtains $k_{max} \approx 10^{16}$ mol^{-1} cm^2 s^{-1}.

Analysis of Data—Values of the dissociation constant K_d and maximal surface concentration Γ_{max} were obtained from the equilibrium surface concentrations Γ_{eq}, as a function of buffer concentrations C_b of protein, using the equation

$$\Gamma_{eq} = \Gamma_{max} \cdot C_b/(C_b + K_d). \quad (1)$$

If a low concentration of factor Va in excess lipid is equilibrated with various concentrations of factor Xa and excess prothrombin is added, thrombin production will be proportional to the amount of factor Xa bound to factor Va on the lipid surface. This is only true, of course, if production of thrombin by factor Xa not included in prothrombinase complexes can be neglected. In that case one has

$$dIIa/dt = v_{max} \cdot Xa/(Xa + K_d) \quad (2)$$

with v_{max} the rate of thrombin production for excess factor Xa. Equation 2 was used to estimate K_d from steady-state thrombin production.

The kinetic constants k_{on} and k_{off} of prothrombinase complex formation in vesicle suspensions were estimated as follows. If a low concentration of factor Xa is added to excess lipid, prothrombin, and factor Va, the concentration of prothrombinase complexes changes according to the following.

$$dP/dt = k_{on} \cdot Va(Xa - P) - k_{off} \cdot P.$$

Integration of this expression gives the amount of prothrombinase formed up to time t: $P(t) = P_{eq}\{1 - \exp(-kt)\}$ with $k = k_{on}Va + k_{off}$ and $P_{eq} = k_{on}VaXa/k$. Multiplication with the turnover number of prothrombinase k_{cat} and integrating again, one obtains the amount of thrombin formed up to time t:

$$IIa(t) = v_{eq}\{t - 1/k + \exp(-kt)/k\} \quad \text{with} \quad v_{eq} = k_{cat}P_{eq} \quad (3)$$

Parameters were obtained from non-linear least square fits of pooled data to the model Equations 1–3, using a standard computer program (BMDP Statistical Software Inc., procedure BMDP-3R, version 1988). Errors are expressed as standard error of estimate, that is, the standard deviation of the estimated parameter.

RESULTS

Influence of Preadsorbed Factor Va on the Binding of Factor Xa to Planar Bilayers—Equilibrium concentrations of adsorbed factor Xa as a function of the buffer concentrations are presented in Fig. 1. Fitting Equation 1 (see "Materials and Methods") to these data resulted in values of $K_d = 46.5 \pm 7.9$ nM and $\Gamma_{max} = 10.3 \pm 0.8$ pmol cm^{-2} or 0.45 ± 0.035 μg cm^{-2} (mean ± S.D.). Preadsorption of factor Va largely increases the binding affinity for factor Xa and K_d drops below the nanomolar range. Such low values are difficult to measure directly by ellipsometry because of the extremely long adsorption times for such low concentrations. For instance, for a buffer concentration of 1 nM (= 1 pmol cm^{-3}) factor Xa and a value of $\Delta = 6.3 \times 10^{-4}$ cm s^{-1} (see "Materials and Methods") it would take 10^4 s before 6.3 pmol cm^{-2} has adsorbed. Therefore K_d was measured from steady-state rates of thrombin production as explained under "Materials and Methods." In order to prevent transport limitation, the bilayer should contain very low prothrombinase activity. To this end, the bilayer was exposed during 1 min to a buffer solution containing 3 pM of factor Va and the cuvette was then rapidly flushed with

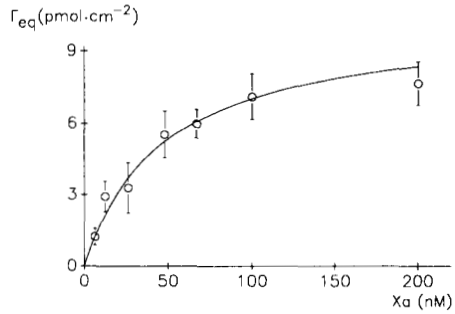

FIG. 1. **Binding isotherm of factor Xa on planar 20% DOPS, 80% DOPC bilayers.** Mean values of six experiments with best fit of the model equation are shown and S.D. is indicated.

fresh buffer. Initial adsorption of factor Va is transport limited (20) and the amount adsorbed in 1 min will thus be $60 \times \Delta \times C_b = 60 \times 5.2 \times 10^{-4} \times 3 \times 10^{-15} = 0.094$ fmol·cm^{-2}. Desorption of factor Va during and after the flushing of the cuvette is negligeable (20). Excess (200 nM prothrombin and various concentrations (1–20 pM) of factor Xa were now added. The minute amount of factor Va is rapidly equilibrated with factor Xa, even for factor Xa concentrations in the picomolar range (see Fig. 3), and Fig. 2 shows the resulting steady-state rates of thrombin production. Fitting Equation 2 (see "Materials and Methods") to these data one finds $K_d = 2.15 \pm 0.30$ pM. For a final prothrombinase concentration on the surface equal to the total factor Va concentration of 0.094 fmol·cm^{-2}, it follows from these data that $k_{cat} = 32.3 \pm 1.3$ s^{-1}. This result is only valid if the contribution to thrombin production of free factor Xa, not complexed to factor Va, can be neglected. From the values of $K_d = 46.5$ nM and $\Gamma_{max} = 10.3$ pmol cm^{-2} for the binding of factor Xa to the lipid bilayer it follows that for the highest concentration of 20 pM factor Xa used in Fig. 2 about 4 fmol cm^{-2} of factor Xa will be lipid-bound. This is about 40 times more factor Xa than present in the form of prothrombinase, but k_{cat} for the factor Xa-lipid complex is about 1000 times lower than for prothrombinase (28, 29). The contribution of lipid-bound free factor Xa to thrombin production in Fig. 2 will thus be less than 4%.

The Rate of Prothrombinase Assembly by Lateral Diffusion of Factor Va and Factor Xa on Planar Bilayers—Fig. 3 presents the initial phase of the experiments with 1 pM factor Xa shown in Fig. 2. The thrombin production rate increases during about 4 min until the steady-state rate is obtained. If v_c is the initial rate of formation of prothrombinase complexes, the amount of prothrombinase formed at time t will be $v_c \cdot t$ and the rate of thrombin production $k_{cat} \cdot v_c \cdot t$ (excess prothrombin). Integrating this expression, it is found that total thrombin production up to time t, as shown in Fig. 3, equals $0.5 \cdot k_{cat} \cdot v_c \cdot t^2$. Fitting the inital 3 min of Fig. 3 to this expression and using the value of $k_{cat} = 32.3$ s^{-1} we found that $v_c = 0.20 \times 10^{-3}$ fmol cm^{-2} s^{-1}. For the factor Va concentration of 0.094 fmol·cm^{-2} and the factor Xa concentration of 1 pM, this result implies a value of 2.1×10^9 M^{-1} s^{-1} for the bimolecular rate constant of complex formation. However, this value is an underestimation by more than one order of magnitude because, due to the gradient in the boundary layer, the factor Xa concentration close to the surface is much lower than the buffer concentration. During the initial adsorption phase, the relation between the buffer concentration C_b and the concentration close to the surface C_0 is given by Ref. 19: $C_0/C_b = \Delta/(\Delta + k_{on}\,\Gamma_{max})$. For factor Xa we have $\Delta = 6.3 \times 10^{-4}$ cm s^{-1} (*cf.* "Materials and Methods"), $\Gamma_{max} = 10.3$ pmol cm^{-2} and $k_{on} = 0.4 \times 10^9$ M^{-1} s^{-1} (*cf.* "Results"). The latter value was expressed per mole of factor Va or per mole of vesicles because each vesicle will carry (less than) one factor Va molecule. From the vesicle radius of 12.5 nm and the value of Γ_{max}, a value of 118 binding sites for factor Xa/vesicle is calculated. So, expressed per mol of binding sites, $k_{on} = 3.4 \times 10^6$ M^{-1} s^{-1} $= 3.4 \times 10^9$ (mol/cm^3)$^{-1}$ s^{-1}. Inserting these values, one finds $C_0/C_b = 0.02$. The rate of complex formation thus exceeds the maximal theoretically possible value of 2.0×10^9 M^{-1} s^{-1} (see "Materials and Methods") by a factor of about 50. It is concluded that direct binding of factor Xa molecules, from the buffer to factor Va molecules adsorbed on the lipid surface, contributes only marginally to the observed rate of prothrombinase formation (see "Discussion").

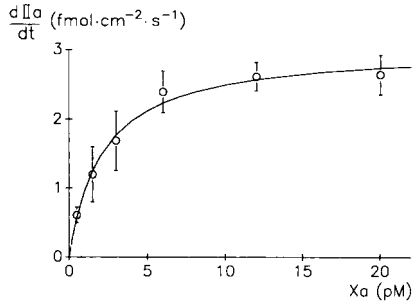

FIG. 2. **Steady-state rates of thrombin production after addition of factor Xa to planar 20% DOPS, 80% DOPC bilayers containing 0.094 fmol cm^{-2} of factor Va and incubated in 200 nM of prothrombin.** Mean results of four experiments with best fit of the model equation are shown and S.D. is indicated.

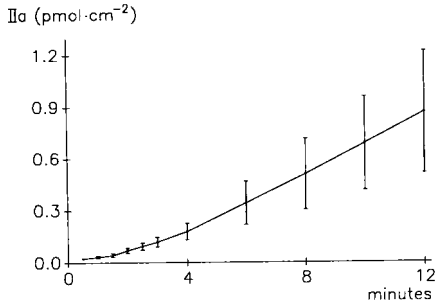

FIG. 3. **Kinetics of thrombin production by planar 20% DOPS, 80% DOPC bilayers containing 0.094 fmol cm^{-2} of factor Va.** Bilayers were incubated for 5 min in 200 nM of prothrombin and, at zero time, 1 pM of factor Xa was added to the buffer. Mean results of three experiments are shown and S.D. is indicated.

The only alternative mechanism of prothrombinase formation is complex formation between molecules meeting by lateral diffusion on the bilayer. An estimate of the bimolecular rate constant for complex formation between lipid-bound molecules can be obtained as follows. After 2 min, the total amount of factor Xa adsorbed on the surface is $120 \times 6.3 \times 10^{-4} \times 10^{-15} = 0.076$ fmol cm^{-2}. From the factor Va concentration of 0.094 fmol·cm^{-2} and the observed rate of prothrombinase formation of $v_c = 0.2 \times 10^{-3}$ fmol cm^{-2} s^{-1}, a bimolecular reaction rate constant of 2.8×10^{13} mol^{-1} cm^2 s^{-1} is calculated. This is an underestimation because, due to formation of prothrombinase complexes, the actual concentrations of free factor Va and factor Xa on the surface will be lower than indicated (see "Discussion").

It is interesting to calculate the surface concentration of factor Xa corresponding to a buffer concentration of 2 pM, that is for half-saturated bound factor Va. By applying Equation 1 with $\Gamma_{max} = 10.3$ pmol cm^{-2}, $K_d = 46.5$ nM, and $C_b = 2$ pM, one obtains $\Gamma_{eq} = 0.44$ fmol cm^{-2}, which is the K_d value expressed in the surface concentration.

Estimation of the Dissociation Constant from Steady-state Thrombin Production in Vesicle Suspensions—The value of $K_d = 2.15$ pM for the dissociation constant of the prothrombinase complex obtained in the preceding section could be influenced by the physical state of the lipids deposited on a solid surface. Similar measurements were therefore performed in vesicle suspensions. A low concentration of factor Va was incubated for 10 min with excess lipid and various concentrations of factor Xa. The reaction was started by addition of excess prothrombin. The results, presented in Fig. 4, were similar if factors Xa and Va were interchanged. Fitting these results again to Equation 2, a value of $K_d = 1.0 \pm 0.1$ pM was obtained, after correction for the fraction of factor Xa (50%) bound to the lipid.

As demonstrated by the constant rate of thrombin production in the upper curve of Fig. 5, adsorption of prothrombin

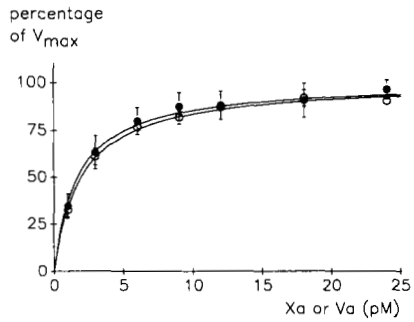

FIG. 4. **Steady-state rate of thrombin production in suspensions of 20% DOPS, 80% DOPC vesicles of 20–30-nm diameter.** *Open* and *closed circles* indicate, respectively, 0.5 pM of factor Va titrated with factor Xa, and 0.5 pM of factor Xa titrated with factor Va. Mixtures were incubated for 10 min in 2 μM of lipid before 400 nM of prothrombin was added. Mean results of eight experiments with best fits of the model equation are shown and S.D. is indicated.

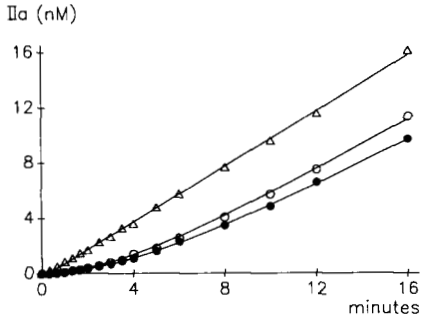

FIG. 5. **Thrombin production in suspensions of 20% DOPS, 80% DOPC vesicles with 20–30 nm diameter, containing 1 pM of factor Xa, 5 pM of factor Va, 400 nM of prothrombin and 2 μM of lipid.** Mixtures were incubated for 10 min before the reaction was started with factor Va (●), factor Xa (○) or prothrombin (△).

to the lipid surface does not influence the assembly of prothrombinase. Although the lipid will almost instantaneously be saturated with prothrombin, formation of extra prothrombinase activity from the very low factor Xa and Va concentrations in the buffer would require several minutes, as shown in the lower curves of Fig. 5. These latter curves show little effect of starting the reaction with either factor Xa or factor Va. The number of vesicles is more than 100 times larger than the number of Va molecules and factor Va will bind rapidly. In both cases one essentially observes the much slower binding of factor Xa to the relatively few vesicles containing a factor Va molecule.

Collisionally Limited Kinetics of Prothrombin Assembly in Vesicle Suspensions—As explained under "Materials and Methods," the values of k_{on} and k_{off} for the formation of prothrombinase can be obtained from measurement of thrombin production after addition of a low factor Xa concentration to vesicles containing factor Va. It also follows from the preceding sections that, for vesicles with small surface areas ($\ll$1 μm^2), k_{on} is determined by the frequency of collisions between vesicles and factor Xa molecules and by the collisional efficiency, that is the probability that the colliding factor Xa molecule will stick to the vesicle. If the collision indeed results in adsorption of the factor Xa molecule to the lipid surface, it will almost immediately be converted to prothrombinase, even if the vesicle contains only a single factor Va molecule. This implies that k_{on} will be strongly dependent on vesicle size because, as explained under "Materials and Methods," the frequency of collisions is proportional to vesicle radius. Moreover, by comparison of calculated

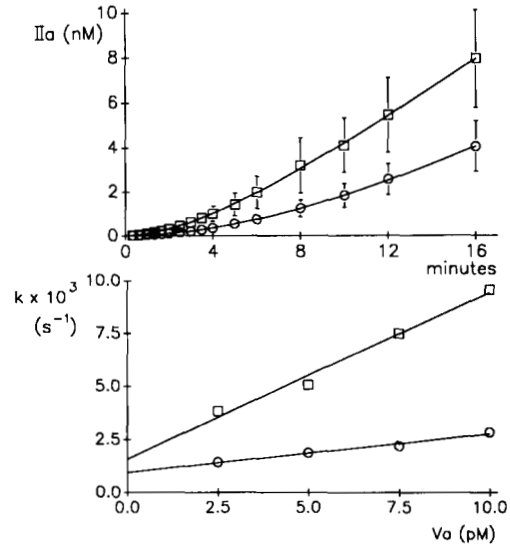

FIG. 6. **Thrombin production in suspensions of 20% DOPS, 80% DOPC vesicles of 20–30 nm (○) and 60–80-nm (□) diameter.** The *upper part* shows mean results with best fitting model curves and S.D. of five experiments with 1 pM of factor Xa added to a mixture of 2.5 pM factor Va and 400 nM prothrombin, incubated for 10 min in 2 μM lipid. The lower part shows values of $k = k_{on}[Va] + k_{off}$, as a function of factor Va concentrations (see text).

collision rates and observed values of k_{on}, one may directly estimate the collisional efficiency.

These predictions were checked in suspensions of vesicles of 20–30- and 60–80-nm diameter. A mixture of 2 μM vesicles and 400 nM prothrombin was incubated for 10 min with 2.5, 5.0, 7.5, and 10 pM of factor Va and production of thrombin was started by addition of 1 pM of factor Xa. The upper part of Fig. 6 shows the results of these experiments. Fitting these curves to Equation 3 ("Materials and Methods") one obtains values of $k = k_{on} \cdot Va + k_{off}$. The lower part of Fig. 6 shows a plot of k as a function of factor Va concentrations from which the slope k_{on} and the cut-off k_{off} were estimated by linear regression. Values obtained were $k_{on} = (0.36 \pm 0.03) \times 10^9$ M^{-1} s^{-1} for 25-nm vesicles and $k_{on} = (1.6 \pm 0.19) \times 10^9$ M^{-1} s^{-1} for 70-nm vesicles, using again a correction of 50% lipid-bound factor Xa. Values of k_{off} were, respectively, $(1.0 \pm 0.4) \times 10^{-3}$ s^{-1} and $(1.6 \pm 2.3) \times 10^{-3}$ s^{-1} and values of $K_d = k_{off}/k_{on}$, respectively, 2.6 and 1.0 pM. It is concluded that larger vesicles indeed have higher values of k_{on} and that values of K_d obtained by this kinetic method are in agreement with the values found from steady-state thrombin production. The theoretically maximal value of k_{on} can be estimated as (*cf.* "Materials and Methods") $4\pi N(D_{Xa} + D_{vesicle})$ $(R_{Xa} + R_{vesicle})$ mol^{-1} cm^3 s^{-1}. Using the Stokes-Einstein relation with $R_{vesicle} = 12.5$ nm, one finds $D_{vesicle} = 1.7 \times 10^{-7}$ cm^2 s^{-1}, and inserting also the values $D_{Xa} = 6.2 \times 10^{-7}$ cm^2 s^{-1} and $R_{Xa} = 3.5$ nm (*cf.* "Materials and Methods"), a value of $k_{max} = 9.5 \times 10^9$ mol^{-1} cm^3 s^{-1} is obtained. Comparison of this value with k_{on} demonstrates a collisional efficiency of about 4%, that is, about six times lower than observed in binding experiments with stopped-flow light scattering (7). This difference is probably caused by the coverage of vesicle surface with prothrombin because a 2.5 times higher value of k_{on} was found when the prothrombin concentration was lowered to 50 nM, instead of 400 nM (result not shown).

DISCUSSION

The Influence of Preadsorbed Factor Va on the Adsorption of Factor Xa—The value of $K_d = 47$ nM for the dissociation

constant of factor Xa on a 20% DOPS, 80% DOPC planar bilayer is in reasonable agreement with the values of 100–114 nM that were obtained under comparable conditions (2–3 mM $CaCl_2$ and 25% phosphatidylserine, 75% phosphatidylcholine) for factor X and factor Xa binding to small unilamellar vesicles as measured by light scattering (7, 30). For planar bilayers as well as for vesicles, the K_d value of factor X(a) is three to five times lower than for prothrombin (30, 31). The maximal surface concentration of 0.45 μg cm^{-2} for factor Xa is more than twice as high as for prothrombin (31). This high surface mass implies that the factor Xa molecules are protruding relatively far from the surface, as also concluded in studies using light scattering (23) and fluorescence energy transfer (32).

In contrast to the situation for factor X or Xa and prothrombin, there is much confusion in the literature about the binding parameters of factor V and Va. It seems as if, even under comparable conditions such as 1–3 mM calcium and mixtures of phosphatidylcholine with 20–25% phosphatidylserine, different techniques give different answers. Values of $K_d = 10^{-7}$-10^{-8} M have been calculated from data on protein depletion (33) and steady-state light scattering (34). Values of $K_d = 3 \times 10^{-9}$ M were obtained from fluorescence polarization (35) and stopped-flow light scattering in the presence of excess prothrombin fragment 1 (7). Values of $K_d \approx 10^{-11}$ M were found from stopped-flow light scattering in buffer (33) and ellipsometry (20).

Part of these discrepancies may be explained by some inherent limitations of the various techniques used (37), but the main differences are probably caused by large variation in surface concentrations. It has been shown that, due to surface exclusion effects and molecular interactions, K_d values will generally increase rapidly for increasing surface concentrations and will be strongly influenced by the presence of other adsorbed proteins (38). For instance, the saturation value of 42 mol of lipid/mol of Va, reported in some studies (7, 35), corresponds to about 2 μg of Va/cm^2 of lipid surface, which is six times more than the value of $\Gamma_{max} = 0.35$ μg cm^{-2} found for factor Va in the present study (data not shown) and more than five orders of magnitude higher than the 0.094 fmol cm^{-2} of adsorbed Va used for the prothrombinase assembly experiments.

It follows from the present study that, for such low surface concentrations, preadsorption of factor Va lowers the value of K_d for the adsorption of factor Xa from 47 nM to 2.2 pM, that is, with a factor of 25,000. This "receptor" effect is much stronger than reported in two recent studies by Krishnaswamy *et al.* In those studies, prothrombinase assembly was measured from assembly induced fluorescence changes in dansyl-glutamyl-glycylarginyl chloromethyl ketone-Xa (7), and from the kinetics of thrombin production (39). Both studies indicate values of $K_d \approx 10^{-9}$ M for the dissociation of the prothrombinase complex, that is almost three orders of magnitude higher than presently reported. This is consistent with the difference in the dissociation constant of factor Va, just mentioned, and could thus also be caused by the large differences in surface concentrations. The fact that thrombin production yielded a similar value of K_d (39) as direct measurement of complex formation in the absence of prothrombin (7) supports the conclusion, obtained from Fig. 5, that prothrombin does not contribute to the binding affinity of the prothrombinase complex. The conversion process does not involve a so-called conformational cage effect (40).

Analogy with Membrane-mediated Conversion of Prothrombin—The formation of the prothrombinase complex from the activated coagulation factors Xa and Va, phospholipids, and calcium increases the catalytic efficiency of factor Xa with five to six orders of magnitude (28,29) and this tremendous effect strongly suggests that the principle of reduction of dimensionality is operative in the conversion of prothrombin (41). Some doubts were raised, however, by several studies (4, 5) presenting evidence for direct conversion of prothrombin from the buffer. It was shown that the conversion could be described as a bimolecular rate process in bulk solution.

Recently, these seemingly conflicting results were reconciled. It was shown (13) that reduction of dimensionality indeed occurs. This makes the conversion process so efficient that even a single prothrombinase complex will convert almost instantaneously all prothrombin molecules landing on a small phospholipid vesicle. This implies that in suspensions of small unilamellar vesicles, as usually studied, the conversion rate is limited by the arrival of new prothrombin molecules on the vesicle surface and thus will appear to be a bimolecular bulk reaction between prothrombin molecules and prothrombinase-containing vesicles. Only for larger surface areas per prothrombinase complex, for instance on macroscopic planar bilayers (13), it may be possible to prevent such transport limitation.

A similar situation exists with respect to prothrombinase assembly (7). The assembly will usually appear to be a collisionally limited bimolecular rate process between vesicles containing one of the components and the other component in bulk solution. Only by bringing the surface area/adsorbed factor Va molecule in the range of square micrometers, as in the present study, can one obtain a situation in which the total transport of factor Xa molecules towards the membrane surface may exceed the rate of complex formation.

Quantitative Aspects of Membrane-mediated Prothrombinase Assembly—The values of K_d = 47 nM and Γ_{max} = 10.3 pmol cm^{-2} for the binding of factor Xa to the lipid membrane allow estimation of the membrane-induced increase of the rate of prothrombinase generation. For a given bulk concentration of C_b = 1 pM factor Xa, it follows from Equation 1 that $\Gamma_{eq} = 0.2 \times 10^{-3}$ pmol cm^{-2}. From the values for the maximal rate constants, given under "Materials and Methods," it then follows that per mole of adsorbed factor Va, the maximal rates will be 2.0×10^{-3} s^{-1} for assembly from bulk solution and 1.6 s^{-1} for assembly by lateral diffusion on the membrane. Thus, the maximal rate of prothrombinase generation will be increased by three orders of magnitude. This implies that the lateral diffusion constant of factor Xa could be lowered by about two orders of magnitude, for instance due to lower fluidity of the bilayer, without losing the membrane-induced enhancement.

It should be realized that the value calculated for the bimolecular rate constant on the surface $k_{on} = 2.8 \times 10^{13}$ mol^{-1} cm^2 s^{-1}, is probably an underestimation. One min after addition of 1 pM (= 10^{-15} mol cm^{-3}) of factor Xa to the buffer a quantity of $10^{-15} \times 60 \times 6.3 \times 10^{-4} = 0.038$ fmol cm^{-2} of factor Xa has adsorbed. If this adsorbed factor Xa were in equilibrium with the 0.094 fmol cm^{-2} of adsorbed factor Va, it follows from the value of 0.44 fmol cm^{-2} calculated for the surface concentration of factor Xa at half-saturation (see "Results") that the prothrombinase concentration would be approximately 0.007 fmol cm^{-2}. But in 1 min, and for the observed rate of prothrombinase assembly of 0.2×10^{-3} fmol cm^{-2} s^{-1}, a quantity of 0.012 fmol cm^{-2} is formed, and this is even more than the estimated equilibrium concentration. Thus, the data are roughly consistent with almost instantaneous equilibrium of adsorbed factors Xa and Va, which would imply a much higher bimolecular association constant.

These conclusions are only valid, of course, for the condi-

tions used in the present study. Reduction of the residence time of proteins on the surface, for instance by lowering of the percentage of acidic phospholipids, or "freezing" of the proteins on the surface by use of lipids with a high melting temperature, could change the situation. This may explain effects of phospholipid composition on the generation of prothrombinase activity (42,43). Such effects could also be produced more indirectly. For instance, a change in phospholipid composition could alter protein-binding parameters and thereby, depending on the lipid concentration, the bulk concentration of proteins. Use of different phospholipid preparations could also cause differences in vesicle size and thereby, as illustrated in the present study, influence the kinetics of prothrombinase assembly.

REFERENCES

1. Hemker, H. C., Esnouf, M. P., Hemker, P. W., Swart, A. C. W., and MacFarlane, R. G. (1967) *Nature* **215,** 248–251
2. Jobin, F., and Esnouf, M. P. (1967) *Biochem. J.* **102,** 666–674
3. Adam, G., and Delbrück, M. (1968) in *Structural Chemistry and Molecular Biology* (Rich, A., and Davidson, N., eds) pp. 198–215., W. H. Freeman & Co., San Francisco
4. Pusey, M. L., and Nelsestuen, G. L. (1983) *Biochem. Biophys. Res. Commun.* **114,** 526–532
5. Van Rijn, J. L. M. L., Govers-Riemslag, J. W. P., Zwaal, R. F. A., and Rosing, J. (1984) *Biochemistry* **23,** 4557–4564
6. Forman, S. D., and Nemerson, Y. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4675–4679
7. Krishnaswamy, S., Jones, K. C., and Mann, K. G. (1988) *J. Biol. Chem.* **263,** 3823–3834
8. Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 594–605
9. Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11,** 4882–4891
10. Lindhout, Th., Govers-Riemslag, J. W. P., Van de Waart, P., Hemker, H. C., and Rosing, J. (1982) *Biochemistry* **21,** 5494–5502
11. Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11,** 4892–4899
12. Smith, R. L. (1973) *J. Biol. Chem.* **248,** 2418–2423
13. Giesen, P. L. A., Willems, G. M., and Hermens, W. T. (1991) *J. Biol. Chem.* **266,** 1379–1382
14. Comfurius, P., and Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta* **488,** 36–42
15. Böttcher, C. J. F., Van Gent, C. M., and Pries, C. (1962) *Anal. Chim. Acta* **24,** 203–207
16. De Kruijff, B., Cullis, P. R., and Radda, G. K. (1975) *Biochim. Biophys. Acta* **406,** 6–20
17. Hope, M. J., Bally, M. B. Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta* **812,** 55–65
18. Cuypers, P. A., Corsel, J. W., Janssen, M. P., Kop, J. M. M., Hermens, W. T., and Hemker, H. C. (1983) *J. Biol. Chem.* **258,** 2426–2431
19. Corsel, J. W., Willems, G. M., Kop, J. M. M., Cuypers, P. A., and Hermens, W. T. (1986) *J. Colloid Interface Sci.* **111,** 544–554
20. Kop, J. M. M., Willems, G. M., and Hermens, W. T. (1989) *J. Colloid Interface Sci.* **133,** 369–376
21. Nelsestuen, G. L., Resnick, R. M., Wei, G. J., Pletcher, C. H., and Bloomfield, V. A. (1981) *Biochemistry* **20,** 351–358
22. Mann, K. G., Nesheim, M. E., and Tracy, P. B. (1981) *Biochemistry* **20,** 28–33
23. Lim, T. K., Bloomfield, V. A., and Nelsestuen, G. L. (1977) *Biochemistry* **16,** 4177–4181
24. Levich, V. G. (1962) *Physicochemical Hydrodynamics,* Prentice-Hall, Englewood Cliffs, NJ
25. Smoluchowsky, V. M. (1917) *Z. Phys. Chem.* **92,** 129–168
26. Clegg, R. M., and Vaz, W. L. C. (1985) in *Progress in Protein-Lipid Interactions* (Watts, A., and De Pont, J. J. H. M., eds) Vol. 1, pp. 173–229, Elsevier, Amsterdam
27. Tamm, L. K. (1988) *Biochemistry* **27,** 1450–1457
28. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) *J. Biol. Chem.* **254,** 10952–10962
29. Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., and Hemker, H. C. (1980) *J. Biol. Chem.* **255,** 274–283
30. Nelsestuen, G. L., and Broderius, M. (1977) *Biochemistry* **16,** 4172–4176
31. Kop, J. M. M., Cuypers, P. A., Lindhout, Th., Hemker, H. C., and Hermens, W. Th. (1984) *J. Biol. Chem.* **259,** 13993–13998
32. Husten, E. J., Esmon, Ch. T., and Johnson, A. E. (1987) *J. Biol. Chem.* **262,** 12953–12961
33. Van de Waart, P., Bruls, H., Hemker, H. C., and Lindhout, Th. (1983) *Biochemistry* **22,** 2427–2432
34. Bloom, J. W., Nesheim, M. E., and Mann, K. G. (1979) *Biochemistry* **18,** 4419–4425
35. Krishnaswamy, S., and Mann, K. G. (1988) *J. Biol. Chem.* **263,** 5714–5723
36. Pusey, M. L., Mayer, L. D., Wei, J., Bloomfield, V. A., and Nelsestuen, G. L. (1982) *Biochemistry* **21,** 5262–5269
37. Hermens, W. Th., Kop, J. M. M., and Willems, G. M. (1989) in *Coagulation and Lipids* (Zwaal, R. F. A., ed) pp. 73–97, CRC Press, Inc., Boca Raton
38. Willems, G. M., Hermens, W. T., and Hemker, H. C. (1991) *J. Biomater. Sci. Polymer Edn.* **2,** 217–226
39. Krishnaswamy, S. (1990) *J. Biol. Chem.* **265,** 3708–3718
40. Nemerson, Y., and Gentry, R. (1986) *Biochemistry* **25,** 4020–4033
41. Nesheim, M. E., Eid, S., and Mann, K. G. (1981) *J. Biol. Chem.* **256,** 9874–9882
42. Tans, G., Van Zutphen, H., Comfurius, P., Hemker, H. C., and Zwaal, R. F. A. (1979) *Eur. J. Biochem.* **95,** 449–457
43. Higgins, D. L., Callahan, P. J., Prendergast, F. G., Nesheim, M. E., and Mann, K. G. (1985) *J. Biol. Chem.* **260,** 3604–3612