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Citation for published version (APA):

Document status and date:
Published: 10/01/1980

Document Version:
Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Download date: 27 Sep. 2023
The Role of Phospholipids and Factor Va in the Prothrombinase Complex

(Received for publication, December 5, 1978, and in revised form, May 17, 1979)

Jan Rosing, Guido Tans, José W. P. Govers-Rijnslag, Robert F. A. Zwaal, and H. Coenraad Hemker

From the Department of Biochemistry, Biomedical Centre, State University of Limburg, Maastricht, The Netherlands

The kinetic parameters of the conversion of bovine prothrombin into thrombin by activated bovine blood clotting factor X (Xa) have been determined in the absence and presence of Ca2+, activated bovine factor V (Va), and phospholipid (dioleoylphosphatidylcholine/dioleoylphosphatidylserine, 1:1; mol/mol). In the absence of accessory components, the $K_a$ for prothrombin is 131 μM, which is well above its concentration in bovine plasma of about 1.5 μM. The $V_{max}$ of thrombin formation is 0.61 mol min$^{-1}$ mol of $X_a$ under these conditions. In the presence of 7.5 μM phospholipid, the $K_a$ drops to 0.058 μM and the $V_{max}$ slightly increases to 2.25 mol min$^{-1}$ mol of $X_a$. For the complete prothrombinase complex ($X_a$, $V_a$, Ca2+, and 7.5 μM phospholipid), a $K_a$ for prothrombin of 0.21 μM and a $V_{max}$ of 1919 mol min$^{-1}$ mol of $X_a$ is found. The $V_{max}$ of thrombin formation slightly increases when more phospholipid is present in our experiments and there is a considerable increase of the $K_a$ for prothrombin at higher phospholipid concentrations. Preliminary calculations show that the prothrombin density at the phospholipid surface in the presence of factor Va is independent of the phospholipid concentration. This indicates that the $K_a$ measured in the presence of phospholipid has to be regarded as an apparent $K_a$ and the local prothrombin concentration determines the kinetics of activation.

Prothrombin activation by prothrombinase complexes of different compositions was followed by gel electrophoresis in the presence of sodium dodecyl sulfate. Both in the absence and presence of phospholipid, $V_{max}$ of prothrombin 2 is the main product formed during the initial stages of steady state prothrombin activation. In the presence of factor Va, thrombin is the main end product and minute amounts of prothrombin 2 are formed. This shift in the reaction pathway of prothrombin activation caused by factor Va will contribute to the observed increase of the $V_{max}$ measured in the presence of factor Va.

One of the key reactions in blood coagulation and hemostasis is the formation of thrombin by limited proteolysis of its zymogen, prothrombin. Several proteolytic enzymes can bring about this reaction, but under physiological conditions the serine protease factor Xa is the activating enzyme. In the absence and presence of factor Va, prothrombin 2 is the main product formed during the initial stages of steady state prothrombin activation. In the presence of factor Va, thrombin is the main end product and minute amounts of prothrombin 2 are formed. This shift in the reaction pathway of prothrombin activation caused by factor Va will contribute to the observed increase of the $V_{max}$ measured in the presence of factor Va.

The purpose of the experiments described in this paper was to assess the kinetic parameters ($K_a$ for prothrombin and $V_{max}$ of thrombin formation) for different prothrombin activating mixtures (i.e., factor Xa either in the absence or presence of Ca$^{2+}$, factor Va, and/or phospholipid). This enables a precise quantification of the observed rate enhancements and allows a first attempt to explain the role of the accessory components in the mechanism of prothrombin activation.

Silverberg et al. (14) have reported an example of the effect of an accessory component on the kinetics of a proteinase-catalyzed activation of a clotting factor. They studied the effect of tissue factor on the activation of factor X by factor VII. Tissue factor, a preparation which contains both phospholipids and protein components decreased the $K_a$ for factor X about 10-fold and increased the $K_a$ 3000-fold. Our kinetic approach allows a separate assessment of the effects of phospholipid and the protein accessory component (factor Va) of...
the prothrombinase complex on the kinetic parameters of prothrombin activation. The data presented in this paper likely have implications for the role of phospholipid and factor VIII in the factor X-activating complex (factor IX, factor VIII, Ca²⁺, and phospholipid) of the intrinsic pathway of blood coagulation.

EXPERIMENTAL PROCEDURES

Materials—S 2238 and S 2222 were purchased from AB Kabi Diagnostica, Stockholm, Sweden. p-NPGB was from Nutritional Biochemicals. Russell’s viper venom, soybean trypsin inhibitor, and ovomucoid were obtained from Sigma. DEAE-Sephadex A-50, QAE-sulfonated sephadex C-50, Sephadex G-100, Sephadex 4B and 6B, and CNBr-activated Sepharose 4B were obtained from Pharmacia. QAE-Cellulose was a product of Schleicher and Schuell. p-Aminobenzamidine obtained from Merck was coupled to Sepharose 4B according to the procedure described by Di Stefano et al. (15). STI was coupled to CNBr-activated Sepharose 4B following the method of Cuatrecasas (16). All reagents used were of the highest grade commercially available.

Proteins—Bovine prothrombin was prepared according to the method of Owen et al. (2). Before storage at -80°C the prothrombin preparations were passed through a column (0.9 x 20 cm) of Sephadex and STI-Sepharose 4B to reduce the small amounts of thrombin and factor X, which might be present in these preparations. Factor V, the prothrombinase, could be detected in our final prothrombin preparations using an assay with the chromogenic substrate S 2222 and S 2222. Prothrombin concentrations were calculated from the E₅₀₀ using E₅₀₀ = 15.5 (2) and 72,000 for the molecular weight of prothrombin (2). Bovine factors X and X₂ were purified as described by Fujikawa et al. (17). Bovine factor V was purified from prothrombin preparations using an assay with the chromogenic substrate S 2222 and S 2222. Factor X, using RVV-X according to the method of Fujikawa et al. (18). Factor X₄ concentrations were calculated after active site titration according to Smith (19).

Prothrombin, factor X, and factor X₂ preparations were homogenous and pure as determined by sodium dodecyl sulfate. The specific activities attained were equal to those reported in Ref. 2 (for prothrombin) and Ref. 17 (for factor X, and X₄).

RVV-X was purified from the crude venom by the method of Schifferman et al. (20). Factor V was purified according to the procedure of Smith and Hanahan (21) with minor modifications. The final preparation had a specific activity of 40 U/mg assuming 1 unit of the factor X present to be per mg of normal bovine plasma. Factor V (0.4 mg/ml) was activated at 37°C for 15 min in 200 mM Tris-HCl, 50 mM NH₄Cl, 10% glycerol (pH 7.5) with thrombin (1.31 U/ml), and the amount of factor X present for all different compositions of the prothrombin-activating mixtures used. That this is the case is shown in Fig. 1 where are plotted the amounts of thrombin formation in the incubation mixture is calculated from the amounts of thrombin present in the samples taken after different time intervals. Pure prothrombin I and prothrombin 2, respectively, have negligible amidase activity toward S 2238 and, therefore, do not contribute to the absorbance changes measured. From the absorbance changes measured in all protein solutions and incubations in order to prevent inactivation of the proteins.

For the construction of Lineaweaver-Burk plots the rate of thrombin formation was averaged from three independent determinations. Kₘ, Vₘₐₓ, and the relevant standard errors were determined using the weighted and nonlinear regression method described by Wilkinson (29). Lines were drawn accordingly.

Gel Electrophoretic Analysis of Prothrombin Activation—Gel electrophoresis was carried out as described by Laemmli (8) on gels containing 10% acrylamide, 0.27% N,N'-methylenebisacrylamide, and 0.1% sodium dodecyl sulfate. After electrophoresis in the presence of sodium dodecyl sulfate, the gels were stained with 0.05% Coomassie blue. The gels were scanned using a densitometer (Gilford). The optical density measurements were made at 600 nm for the bands of interest.

RESULTS

Introductory Experiments to Determine the Conditions for the Measurements of the Kinetic Parameters—To allow a kinetic approach it is necessary to confirm that the rate of thrombin formation is constant in time and proportional to the amount of factor X, present for all different compositions of the prothrombin-activating mixtures. That this is the case is shown in Fig. 1 where are plotted the amounts of thrombin formation in the incubation mixture is calculated from the amounts of thrombin present in the samples taken after different time intervals. Pure prothrombin I and prothrombin 2, respectively, have negligible amidase activity toward S 2238 and, therefore, do not contribute to the absorbance changes measured. From the absorbance changes measured in all protein solutions and incubations in order to prevent inactivation of the proteins.

The abbreviations used are: S 2238, H-D-phenylalanyl-l-pipec- tolyl-l-arginine-p-nitroanilide dihydrochloride; S 2222, N-benzyol-l-iso-leucyl-p-thrombin and factor IX, Ca²⁺, and phospholipid) of the intrinsic pathway of blood coagulation.

Phospholipids and Phospholipid Vesicle Preparations—1,2-Di-octanoyl-sn-glycero-3-phosphocholine (18.1ₜₐₐ.,18.1ₜₐ.,phosphatidyldi- choline) was prepared by reacylating the cadmium chloride adduct of sn-1,2-dioleyl-sn-glycero-3-phosphocholine with appropriate fatty acyl chloride according to the method of Bear and Buchnea (24). 1,2-Dioleoyl-sn-glycero-3-phosphoerine (18.1ₜₐ.,18.1ₜₐ.,phosphatidylethanolamine) was prepared from the respective phosphatidylolethanolamine by enzymatic synthesis as described by Comfurius and Zwaal (25). Single bilayer vesicles were used to protect and solubilize the thrombin preparation described by De Kruijff et al. (26) for sonication for 10 min in 20 mM Tris, 100 mM NaCl buffer at pH 7.5 at 0°C (above the phase transition of the lipids used). Sonication was performed using a MSK Mark II 150-wat ultrasonic disintegrator set at 10 μm to peak amplitude. After sonication no pH adjustment was needed. Phospholipid concentrations were determined by phosphate analysis according to the method of Böttcher et al. (27). The vesicle preparations described above were used throughout our experiments and were chosen because they exhibited excellent anticoagulant promoting activity (28).

Measurement of Rates of Thrombin Formation—Factor X, either alone or in the presence of phospholipids, CaCl₂, and/or factor V, was incubated for 3 to 5 min at 37°C in a buffer containing 20 mM Tris, 100 mM NaCl, and 0.5 mg/ml of ovomucoid at pH 7.5. Prothrombin was added and after different time intervals samples were taken and added to a cuvette (thermostated at 37°C). It does, however, not affect the rate of conversion of S 2238 by thrombin. From the absorbance change at 405 nm, recorded on a Gilford spectrophotometer, the amount of thrombin in the incubation mixture is calculated from the calibration curve made with known amounts of thrombin. The calibration curve was determined under the assay conditions described above. The rate of thrombin formation in the incubation mixture is calculated from the amounts of thrombin present in the samples taken after different time intervals. Pure prothrombin I and prothrombin 2, respectively, have negligible amidase activity toward S 2238 and, therefore, do not contribute to the absorbance changes measured. From the absorbance changes measured in all protein solutions and incubations in order to prevent inactivation of the proteins.
factor \( X_a \) is bound to the phospholipid vesicles, while at the higher phospholipid concentrations almost all factor \( X_a \) added is bound. The linearity of thrombin formation with time was preserved for all prothrombin concentrations used throughout.

It is well known that the rate of thrombin formation is influenced by the presence of factor \( V_a \) and \( \text{Ca}^{2+} \) (7, 13). In order to compare rates in the presence or absence of the accessory compounds it is necessary that the experiments be carried out under optimal conditions with respect to the amounts of these components. This was accomplished by measuring thrombin formation at different factor \( V_a \) and \( \text{Ca}^{2+} \) concentrations at constant amounts of factor \( X_a \) and prothrombin. Fig. 2 shows the \( \text{Ca}^{2+} \) titration of thrombin formation when factor \( X_a \) is converting prothrombin in solution in the presence and absence of factor \( V_a \). The shapes of the titration curves and the optimal \( \text{Ca}^{2+} \) concentrations are independent of prothrombin, factor \( X_a \), and factor \( V_a \) in the concentration range used in our further experiments (not shown). The significant inhibition at \( \text{Ca}^{2+} \) concentrations above 4 mM observed in the presence of factor \( V_a \) remains as yet unexplained. Similar \( \text{Ca}^{2+} \) titrations have been carried out for the prothrombinase complex consisting of phospholipids and factor \( X_a \) either in the absence or presence of factor \( V_a \). Fig. 3 shows the \( \text{Ca}^{2+} \) dependence of the rate of thrombin formation at two different phospholipid concentrations (7.5 and 75 \( \mu \)M) in the absence of factor \( V_a \). The same experiment carried out in the presence of factor \( V_a \) is shown in Fig. 4. It is interesting to note that in the presence of factor \( V_a \) and phospholipid the \( \text{Ca}^{2+} \) titration curves are sigmoidal.

The dependence of the thrombin formation rate on the amount of factor \( V_a \) present was measured at the optimal \( \text{Ca}^{2+} \) concentrations determined in the above mentioned experiments. When factor \( X_a \) converts prothrombin in solution in the presence of 3 mM \( \text{CaCl}_2 \), large amounts of factor \( V_a \) have to be added in order to obtain the optimal rate of thrombin formation (Fig. 5). The true maximal rate cannot be obtained experimentally, however, since thrombin formation is inhibited at high factor \( V_a \) concentrations. Much lower amounts of factor \( V_a \) are required to obtain an optimal rate of thrombin formation in the presence of phospholipids (Fig. 6). In this case no inhibitory effect of factor \( V_a \) is found, so the rates of thrombin formation in the presence of phospholipid and saturating amounts of factor \( V_a \) can be regarded as true optimal rates of prothrombin activation. In the presence of a 10-fold higher phospholipid concentration the amount of factor \( V_a \) needed to obtain half-saturation is increased about 5 times, and the optimal rate attained is about 15% lower, which is caused by a difference in the \( K_m \) for prothrombin at high and low phospholipid concentrations (see below).

\textbf{Determination of the Kinetic Constants of Prothrombin Conversion with Different Prothrombin Activation Mixtures}—Rates of thrombin formation were measured at varying prothrombin concentrations with different activation mixtures. When \( \text{Ca}^{2+} \) or factor \( V_a \), or both, are present, the data

\textbf{Fig. 1.} Time course of thrombin formation with different amounts of factor \( X_a \). Prothrombin (30 \( \mu \)M) was incubated with varying amounts of factor \( X_a \) in 1 ml of a buffer containing 20 mM Tris-HCl, 100 mM \( \text{NaCl} \), 20 mM \( \text{CaCl}_2 \), and 0.5 mg/ml of ovalbumin (pH 7.5) at 37°C. After the time intervals indicated a sample was taken and assayed for the amount of thrombin as described under "Experimental Procedures." The amounts of factor \( X_a \) present were: \( \text{---} \), 9.1 x 10^{-5} \( \text{mol/ml} \); \( \text{---} \), 18.2 x 10^{-5} \( \text{mol/ml} \); and \( \text{---} \), 45.6 x 10^{-5} \( \text{mol/ml} \).

\textbf{Fig. 2.} The \( \text{Ca}^{2+} \) dependence of thrombin formation in the absence and presence of factor \( V_a \). Prothrombin (30 \( \mu \)M) was incubated at 37°C with factor \( X_a \) (9.1 x 10^{-5} \( \text{mol/ml} \)) in 1 ml of a buffer containing 20 mM Tris-HCl, 100 mM \( \text{NaCl} \), 0.5 mg/ml of ovalbumin (pH 7.5), and varying amounts of \( \text{CaCl}_2 \). The rate of thrombin formation was calculated from the amounts of thrombin present after 5 and 10 min of incubation. \( \text{---} \), prothrombin (1.12 \( \mu \)M) was incubated with factor \( X_a \) (7.75 x 10^{-7} \( \text{mol/ml} \)) and factor \( V_a \) (3.6 units/ml). Further experimental conditions as described above.

\textbf{Fig. 3.} The \( \text{Ca}^{2+} \) dependence of the rate of thrombin formation at two different phospholipid concentrations (7.5 and 75 \( \mu \)M) in the absence of factor \( V_a \). The same experiment carried out in the presence of factor \( V_a \) is shown in Fig. 4. It is interesting to note that in the presence of factor \( V_a \) and phospholipid the \( \text{Ca}^{2+} \) titration curves are sigmoidal.

\textbf{Fig. 4.} The \( \text{Ca}^{2+} \) dependence of thrombin formation in the presence of factor \( V_a \) and phospholipid (7.5 \( \mu \)M). Factor \( X_a \) (3.1 x 10^{-5} \( \text{mol/ml} \)) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM \( \text{NaCl} \) and 75 \( \mu \)M phospholipid (A-A) or 75 \( \mu \)M phospholipid (---), and varying amounts of \( \text{CaCl}_2 \) at pH 7.5. After 3 min the reaction was started by adding 40 \( \mu \)l of prothrombin, resulting in a final concentration of 0.25 \( \mu \)M. From the amounts of thrombin present after 5 and 10 min the rate of thrombin formation was calculated. The phospholipid vesicles were prepared as described under "Experimental Procedures."
Fig. 4. The Ca$^{2+}$ dependence of the rate of thrombin formation in the presence of phospholipid and factor V$. Factor X$_a$ (3.1 x 10$^{-8}$ μmol/ml) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 0.96 unit of factor V$^a$, 7.5 μM phospholipid (A——A) or 75 μM phospholipid (—O), and varying amounts of CaCl$_2$ at pH 7.5. After 3 min the reaction was started by adding 40 μl of prothrombin giving a final concentration of 3 μM. For further experimental details see Fig. 3.

Fig. 5. The factor V$^a$ dependence of the rate of thrombin formation in solution. Factor X$_a$ (7.75 x 10$^{-9}$ μmol/ml) was incubated at 37°C in 1 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 3 mM CaCl$_2$, and varying amounts of factor V$^a$ at pH 7.5. After 5 min the reaction was started by addition of 10 μl of prothrombin to give a final concentration of 1.12 μM. From the amounts of thrombin present after 5 and 10 min of reaction time the rate of thrombin formation was calculated.

from Figs. 2 to 6 have been used to select concentrations that give an optimal rate of thrombin formation. In order to make a direct comparison of the different Lineweaver-Burk plots possible, rates of thrombin formation were expressed as moles of thrombin formed per min per mol of factor X$_a$ present. From the kinetic data the K$_m$ for prothrombin (in μM) and the V$_{max}$ of thrombin formation (in moles of thrombin per min per mol of X$_a$) were determined. Fig. 7 shows the Lineweaver-Burk plot of factor X$_a$ conversion of prothrombin into thrombin in the absence of accessory components. From this plot a K$_m$ of 131 μM and a V$_{max}$ of 0.61 mol min$^{-1}$ mol X$_a^{-1}$ can be calculated. When the same experiment is carried out in the presence of 20 mM Ca$^{2+}$ there is a small decrease of the K$_m$, while the V$_{max}$ is not affected. With a prothrombin activation mixture consisting of factor X$_a$, phospholipid (7.5 μM), and Ca$^{2+}$, a V$_{max}$ of 2.25 mol of thrombin min$^{-1}$ mol X$_a^{-1}$ and a K$_m$ of 0.058 μM are found (Fig. 9). Increasing the phospholipid concentration slightly increases the V$_{max}$, and a much higher K$_m$ value is measured. Compared with the kinetic parameters measured under the same conditions, but in the absence of phospholipids, the considerable decrease of K$_m$ values is most striking. Fig. 10 shows the Lineweaver-Burk plot of the complete prothrombinase complex (factor X$_a$, phospholipid, Ca$^{2+}$, and factor V$^a$). As expected this prothrombin activation mixture yields kinetic constants which greatly favor thrombin formation. A low K$_m$ value for prothrombin, which is again dependent on the phospholipid concentration, and a high V$_{max}$ of thrombin formation in the presence of phospholipid are shown in Fig. 9 and 10. With a prothrombin activation mixture consisting of factor X$_a$, phospholipid (7.5 μM), and Ca$^{2+}$, a V$_{max}$ of 2.25 mol of thrombin min$^{-1}$ mol X$_a^{-1}$ and a K$_m$ of 0.058 μM are found (Fig. 9). Increasing the phospholipid concentration slightly increases the V$_{max}$, and a much higher K$_m$ value is measured. Compared with the kinetic parameters measured under the same conditions, but in the absence of phospholipids, the considerable decrease of K$_m$ values is most striking. Fig. 10 shows the Lineweaver-Burk plot of the complete prothrombinase complex (factor X$_a$, phospholipid, Ca$^{2+}$, and factor V$^a$). As expected this prothrombin activation mixture yields kinetic constants which greatly favor thrombin formation. A low K$_m$ value for prothrombin, which is again dependent on the phospholipid concentration, and a high V$_{max}$ of

Fig. 6. The effect of factor V$^a$ on the rate of thrombin formation in the presence of phospholipid. Factor X$_a$ (2.3 x 10$^{-9}$ μmol/ml) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 7.5 μM phospholipid (O——O) or 75 μM phospholipid (A——A), 5 mM CaCl$_2$, and varying amounts of factor V$^a$ at pH 7.5. After 3 min the reaction was started by adding 60 μl of prothrombin resulting in a final concentration of 0.95 μM. From the amounts of thrombin present after 4 and 8 min of reaction time, the rate of thrombin formation was calculated.

Fig. 7. Lineweaver-Burk plot of factor X$_a$ converting prothrombin into thrombin in the absence and presence of CaCl$_2$. Thrombin formation at varying concentrations of prothrombin was measured at 37°C in 1 ml of a reaction mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), factor X$_a$ (9.1 x 10$^{-9}$ μmol/ml), and varying amounts of factor V$^a$, at pH 7.5. The reaction was started by addition of factor X$_a$. After 7 and 15 min samples were taken and assayed for thrombin as described under "Experimental Procedures." From the amounts of thrombin found the rate of thrombin formation was calculated. The kinetic constants calculated are summarized in Table I.
1919 mol of thrombin min$^{-1}$ mol X$^{-1}$ at 7.5 µM phospholipid can be calculated from the experimental data. Table I summarizes the kinetic constants for thrombin formation with different activation mixtures calculated from the data presented in Figs. 7 to 10.

The dependence of the kinetic parameters on the amount of phospholipid present is studied in more detail for the prothrombin-activating complex consisting of factor X_n, phospholipid, and Ca$^{2+}$ (Table II). The gradual increase of the $K_m$ for prothrombin with increasing phospholipid concentrations is obvious. The increase of the $V_{max}$ of the thrombin formation at higher amounts of phospholipid is much less pronounced. This is likely due to the fact that the $V_{max}$ is determined by the amount of factor X_n actually bound to the phospholipid at the different phospholipid concentrations. The kinetic parameters of free factor X_n (Table I) are such that any nonbound factor X_n has no detectable contribution to thrombin formation in the presence of phospholipid. Thus variation of $V_{max}$ with the phospholipid concentration is determined by the binding isotherm of factor X_n to varying amounts of phospholipid. Extrapolation of a double reciprocal plot ($1/V_{max}$ versus $1/[tHb]$) at constant factor X_n to infinite phospholipid concentration yields the $V_{max}$ for the case that all factor X_n is bound (Fig. 11). The $V_{max}$ calculated from this plot is 4 mol min$^{-1}$ mol X$^{-1}$, which shows that even at the lowest phospholipid concentration used in our experiments about 25% of the added factor X_n is bound to the phospholipid vesicles.

**Fig. 10.** Lineweaver-Burk plot of factor X_n converting prothrombin into thrombin in the presence of phospholipid and factor V_n. The experimental details are described in the legend of Fig. 9 except the amount of factor X_n was 7.75 x 10$^{-9}$ µmol/ml, and 0.9 U/ml of factor V_n was present. The CaCl$_2$ concentrations were 7.5 mM and 5.0 mM at, respectively, 7.5 µM phospholipid (A-A) and 75 µM phospholipid (O-O).

**Table I**

<table>
<thead>
<tr>
<th>Prothrombin-activating mixture</th>
<th>$K_m$ for prothrombin (µM) ± S.E.</th>
<th>$V_{max}$ (mol Hb min$^{-1}$ mol X$^{-1}$) ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor X_n</td>
<td>131 ± 24</td>
<td>0.61 ± 0.08</td>
</tr>
<tr>
<td>Factor X_n CaCl$_2$</td>
<td>84 ± 11</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>Factor X_n CaCl$_2$, factor V_n</td>
<td>34 ± 5</td>
<td>373 ± 30</td>
</tr>
<tr>
<td>Factor X_n CaCl$_2$, phospholipid (2.6)</td>
<td>0.032 ± 0.003</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td>Factor X_n, CaCl$_2$, phospholipid (7.5)</td>
<td>0.058 ± 0.005</td>
<td>2.25 ± 0.06</td>
</tr>
<tr>
<td>Factor X_n, CaCl$_2$, phospholipid (75)</td>
<td>0.35 ± 0.03</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>Factor X_n, CaCl$_2$, phospholipid (7.5), factor V_n</td>
<td>0.21 ± 0.02</td>
<td>1919 ± 63</td>
</tr>
<tr>
<td>Factor X_n, CaCl$_2$, phospholipid (75), factor V_n</td>
<td>1.7 ± 0.6</td>
<td>2748 ± 580</td>
</tr>
</tbody>
</table>

for prothrombin with increasing phospholipid concentrations is obvious. The increase of the $V_{max}$ of the thrombin formation at higher amounts of phospholipid is much less pronounced. This is likely due to the fact that the $V_{max}$ is determined by the amount of factor X_n actually bound to the phospholipid at the different phospholipid concentrations. The kinetic parameters of free factor X_n (Table I) are such that any nonbound factor X_n has no detectable contribution to thrombin formation in the presence of phospholipid. Thus variation of $V_{max}$ with the phospholipid concentration is determined by the binding isotherm of factor X_n to varying amounts of phospholipid. Extrapolation of a double reciprocal plot (1/$V_{max}$ versus 1/[phospholipid]) at constant factor X_n to infinite phospholipid concentration yields the $V_{max}$ for the case that all factor X_n is bound (Fig. 11). The $V_{max}$ calculated from this plot is 4 mol min$^{-1}$ mol X$^{-1}$, which shows that even at the lowest phospholipid concentration used in our experiments about 25% of the added factor X_n is bound to the phospholipid vesicles.

**Time Course of Prothrombin Activation by Different Prothrombin Activation Mixtures Visualized by Sodium Dodecyl Sulfate Gel Electrophoresis**—The experiments of the group of Jackson (2-7) have shown that different partial prothrombin activation products accumulate during activation of prothrombin with different activation mixtures. It can be argued...
it is possible to follow prothrombin activation by different prothrombin-activating mixtures using sodium dodecyl sulfate gel electrophoresis (Fig. 12).

Prothrombin and its activation products are identified in the figures. The relative migration distances and the sequence of prothrombin and activation products on the gels are consistent with earlier published gel data (2-6). In the absence of factor V (with or without phospholipid) prothrombin 2 is the main end product during the initial phase of prothrombin activation (Fig. 12, A and C). At a later stage of the activation process sufficient thrombin is formed to be detectable on the gels. With factor V, in the absence of phospholipid, thrombin is generated in excess of prothrombin 2 (Fig. 12B), whereas in the presence of phospholipid and factor V, formation of prothrombin 2 cannot be detected (Fig. 12D). These data point out that in the presence of factor V, a shift in the pathway of prothrombin activation occurs (see under "Discussion"). Formation of prothrombin 1 during prothrombin activation will take place as a result of thrombin-catalyzed proteolysis of prothrombin. Indeed prothrombin 1 is formed during the time course of prothrombin activation. In particular, it is found in those experiments where considerable amounts of thrombin are formed.

DISCUSSION

The studies of Esmon et al. (7) and Jobin and Esnouf (13) have shown that both phospholipid and factor V increase the rate of conversion of prothrombin to thrombin by factor Xa. Since their experiments were carried out at a single concentration of prothrombin, factor Xa, factor Va, and phospholipid, no insight in the mechanistic function of factor V, and phospholipid in the prothrombinase complex could be obtained. Several explanations can be proposed for the rate enhancements observed in the presence of the accessory components of the prothrombinase complex (cf. Ref. 1) e.g.: 1. a change in the reaction pathway of prothrombin activation; 2. an increase of the proteolytic capacity of factor Xa upon interaction with phospholipid, factor V, and/or Ca2+; 3. a conformational change of prothrombin upon binding to phospholipid, factor V, and/or Ca2+; 4. an increase of local prothrombin and factor Xa concentration after binding to phospholipid and/or factor V; 5. binding of prothrombin, factor V, and factor Xa to phospholipid in a favorable steric position. It is obvious that these different mechanisms may act simultaneously. None of these possibilities can be excluded on the basis of the properties of the prothrombinase complex reported in the literature.

Knowledge of the effects of phospholipid and factor V on the kinetic parameters of prothrombin activation will be a prerequisite in order to elucidate their role in the mechanism of the prothrombinase complex.

The kinetic parameters of different prothrombin-activating mixtures are summarized in Table I. In the absence of accessory components prothrombin is a very poor substrate for factor Xa. The high $K_a$ value (131 $\mu$M) indicates that prothrombin has a low affinity for factor Xa under these conditions. Taking into account a plasma prothrombin concentration of about 1.5 $\mu$M, which is far below the $K_a$, and the low $V_{\text{max}}$ of thrombin formation measured under these conditions, it is unlikely that physiologically significant thrombin formation can take place without involvement of phospholipid and factor V.

The small decrease of the $K_a$ for prothrombin occurring when the same experiment is carried out in the presence of Ca2+ may be the result of Ca2+ binding to $\gamma$-carboxyglutamic acid residues present in factor Xa and prothrombin. Since

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**Table II**

**Dependence of $K_v$ and $V_{\text{max}}$ on the phospholipid concentration**

Prothrombin was activated by factor $X_a$ in the presence of Ca2+ and phospholipid. Reactions were carried out in a mixture containing 4.6 $\times$ 10$^{-6}$ mol of $X_a$/ml, 100 mm NaCl, 25 mm Tris, 0.5 mg of ovalbumin/ml at 37°C and pH 7.5. Amounts of phospholipid and CaCl2 present are indicated in the table. Further experimental details are described in the legend to Fig. 9 and under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>CaCl2$^+$</th>
<th>$K_v$ $\mu$M</th>
<th>$V_{\text{max}}$ mol II per min per mol Xa</th>
<th>$X_{\text{a}}^+$</th>
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</tr>
</thead>
<tbody>
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<td>$X_{\text{a}}^+$</td>
<td>$V_{\text{max}}$ mol II per min per mol Xa</td>
</tr>
</tbody>
</table>

$^a$ For each phospholipid concentration a Ca2+ titration was performed to obtain the optimal Ca2+ concentration.

**Fig. 11.** Double reciprocal plot of $V_{\text{max}}$ as a function of the phospholipid concentration. The prothrombin activating mixture consisted of factor $X_a$, CaCl2, and phospholipid. This plot contains data summarized in Table II.

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that the kinetic parameters measured in the experiments described above are in fact those for a mixture of prothrombin and its partial activation products liberated during the time course of the reaction. However, gel electrophoretic analysis of the products formed during prothrombin activation (Fig. 12, A and C). At a later stage of the activation process sufficient thrombin is formed to be detectable on the gels. With factor V, in the absence of phospholipid, thrombin is generated in excess of prothrombin 2 (Fig. 12B), whereas in the presence of phospholipid and factor V, formation of prothrombin 2 cannot be detected (Fig. 12D). These data point out that in the presence of factor V, a shift in the pathway of prothrombin activation occurs (see under "Discussion"). Formation of prothrombin 1 during prothrombin activation will take place as a result of thrombin-catalyzed proteolysis of prothrombin. Indeed prothrombin 1 is formed during the time course of prothrombin activation. In particular, it is found in those experiments where considerable amounts of thrombin are formed.

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FIG. 12. Time course of activation of prothrombin with different activating mixtures. A, prothrombin (13.4 μM) was activated with factor X, (1.82 μM) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate and 10 mM CaCl₂ at 37°C and pH 7.5. B, prothrombin (13.4 μM) was activated with factor X, (6.2 × 10⁻³ μM) and factor V, (19 units/ml) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate, and 3 mM CaCl₂ at 37°C and pH 7.5. C, prothrombin (2.68 μM) was activated...
Ca$^{2+}$ has no effect on the $V_{\text{max}}$ of thrombin formation we conclude that the catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) of factor Xa is virtually not changed upon Ca$^{2+}$ binding.

Important changes of kinetic parameters take place when phospholipid or factor V is added to both, form part of the prothrombinase complex. Phospholipid causes a profound decrease of the $K_{\text{m}}$ for prothrombin. In the presence of phospholipid the $K_{\text{m}}$ decreases to values below 1 $\mu$M, which is lower than the plasma prothrombin concentration.

The $K_{\text{m}}$ for prothrombin is, however, dependent on the amount of phospholipid present (Table II). Higher $K_{\text{m}}$ values are measured at increasing phospholipid concentrations. The $K_{\text{m}}$ increases from 0.032 $\mu$M at 2.8 $\mu$M phospholipid to 1.08 $\mu$M at a phospholipid concentration of 240 $\mu$M. Therefore, a $K_{\text{m}}$ determined in the presence of phospholipid has to be regarded as an apparent $K_{\text{m}}$. This is not surprising since the $K_{\text{m}}$ value is calculated using the concentrations of total added prothrombin. However, the thrombin formed at the phospholipid surface is in fact generated from bound prothrombin. The prothrombin concentration at the $K_{\text{m}}$ should, therefore, be expressed in terms of surface concentration in those experiments where $K_{\text{m}}$ values at different phospholipid concentrations are compared. To calculate the amount of bound prothrombin, binding parameters (e.g. available binding sites and dissociation constants) have to be known for prothrombin binding to the phospholipid vesicles under our experimental conditions (temperature, pH, ionic strength, and [Ca$^{2+}$]). Although the appropriate binding data are not available, we used as a first approximation prothrombin binding parameters of Nelsestuen and Broderius (31). For conditions approaching those of our experiments as close as possible (0.05 M Tris, 0.1 M NaCl, 5 mM CaCl$_2$ at pH 7.5 and 25°C) they reported a dissociation constant of 10$^{-7}$M for the prothrombin-phospholipid complex and 17 $\mu$mol of phospholipid binding sites per $g$ of phospholipid for vesicles prepared from a 1/1 (mol/mol) mixture of bovine brain phosphatidylserine and egg yolk phosphatidylcholine. Using these binding data we calculated the prothrombin density at the phospholipid surface, expressed as micromoles of prothrombin bound per g of phospholipid, at the $K_{\text{m}}$ measured at different phospholipid concentrations (Table III). Although the apparent $K_{\text{m}}$ increases when increasing amounts of phospholipid are present, the prothrombin density at the phospholipid surface at the $K_{\text{m}}$ is independent of the phospholipid concentration. This means that it is the local prothrombin concentration at the phospholipid surface which determines the kinetics in this case. Our experiments allow no conclusion as to what extent an increase of the $K_{\text{m}}$ observed in the presence of phospholipid.

This implies also that the possibility that phospholipid brings prothrombin and factor Xa together in a more favorable orientation, the so-called juxtaposing effect, cannot be evaluated. Legitimate conclusions in this connection have to be based on a theoretical treatment of enzyme kinetics in solution and heterogeneous enzyme catalysis taking place at the phospholipid surface. This requires, for instance, knowledge of diffusion constants in solution and at the phospholipid surface, appropriate binding data, and orientation at the phospholipid surface of the proteins involved.

The role of factor V, in the complete prothrombinase complex (factor Xa, factor V, Ca$^{2+}$, and phospholipid) is mainly restricted to an effect on the $V_{\text{max}}$ of thrombin formation. A 700-fold increase of $V_{\text{max}}$ is observed in the presence of factor V.

With respect to the mode of action of factor V, in the prothrombinase complex one has to consider whether factor V interacts with prothrombin, changing its properties as a substrate or whether it forms a complex with factor Xa with a catalytic capacity different from free factor Xa. We exclude the possibility that factor V exerts its stimulatory action, independent of factor X, or prothrombin, by trapping potential inhibitory activation peptides released during prothrombin activation. Neither fragment 1, fragment 2, nor fragment 1.2 inhibit thrombin formation at concentrations generated during prothrombin activation in the time course of our experiments. Since the factor $V_{\text{a}}$ concentration in our experiments is always much lower than the prothrombin concentration a prothrombin-factor V, complex, if present, will be a small fraction of the total amount of prothrombin added. This combined with the fact that a further increase of the amount of factor V, does not affect the kinetics of the reaction makes it very unlikely that a prothrombin-factor V, complex acts as a substrate for factor Xa. Therefore, it seems plausible to assume that a factor Xa-factor V, complex is the catalytic unit in the prothrombinase complex.

In solution factor V, also exhibits a stimulating effect on thrombin formation. However, from the experiment shown in Fig. 5 it is clear that it is impossible to achieve complete saturation of factor Xa with factor V. At high concentrations factor V, even inhibits thrombin formation. This phenomenon, for which we have no explanation yet, prevents adding saturating amounts of factor V. This leaves open the question whether phospholipid actually has an additional effect on the rate enhancement of thrombin formation brought about by factor V.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>$K_{\text{a}}$</th>
<th>Prothrombin density at phospholipid surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>µM</td>
<td>µmol/g ± S.E.</td>
</tr>
<tr>
<td>2.6</td>
<td>0.032</td>
<td>3.4 ± 0.3</td>
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<td>4.0</td>
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<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>5.3</td>
<td>0.054</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>7.5</td>
<td>0.068</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>8.0</td>
<td>0.068</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>10.5</td>
<td>0.14</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>16.0</td>
<td>0.164</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>26.3</td>
<td>0.23</td>
<td>5.7 ± 0.6</td>
</tr>
<tr>
<td>40.0</td>
<td>0.25</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>52.6</td>
<td>0.35</td>
<td>5.1 ± 0.4</td>
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<tr>
<td>75</td>
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</tr>
<tr>
<td>80</td>
<td>0.48</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>105</td>
<td>1.08</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>240</td>
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</table>
Comparison of the factor Vₐ dependence of thrombin formation in the absence and presence of phospholipid (Figs. 5 and 6) draws attention to another function of phospholipids in the prothrombinase complex. Since much lower amounts of factor Vₐ are required in the presence of phospholipid to maximize the rate of thrombin formation, we conclude that phospholipids promote the formation of the factor Vₐ-Xₐ complex. This may be caused by increased local factor Vₐ and factor Xₐ concentrations at the phospholipid surface after binding of both proteins to the vesicles. This will shift the equilibrium of formation of the factor Vₐ-Xₐ complex in the direction of association. For a discussion of the actual mechanism that causes the increased \( V_{max} \) of thrombin formation in the presence of factor Vₐ it is helpful to consider Scheme 1, depicting a minimal mechanism for the conversion of prothrombin and other activation products.

The different proteins and complexes represented in this scheme are: E, prothrombin activating complex; PT, prothrombin; PT1, prethrombin 1; PT2, prethrombin 2; T, thrombin; F1, prothrombin fragment 1; F2, prothrombin fragment 2; F1.2, prothrombin fragment 1.2.

The reactions represented in this scheme are based on results reported in the literature (2-7, 32). Although thrombin formation can take place with free prethrombin 1 or prethrombin 2 as substrate (5), electrophoretic analysis shows that in the course of our kinetic experiments such small amounts of partial activation products accumulate that conversion of intermediates released from the prothrombinase complex or formed by the action of thrombin cannot contribute to the amount of thrombin formed. Therefore, thrombin is generated in our kinetic experiments via the pathway indicated by the solid arrows (Steps 1, 2, 3, 4).

An intriguing explanation for the mechanism of rate enhancement by factor Vₐ can be put forward on the basis of the experiments (Fig. 12) in which the activation of prothrombin with different prothrombin-activating mixtures is followed by sodium dodecyl sulfate gel electrophoresis. The experimental setup is a refinement of similar experiments carried out by the group of Jackson (2, 4, 5, 6). Our reaction conditions, prothrombin concentration, and composition of activating mixtures are chosen on the basis of knowledge obtained from our kinetic experiments. When factor Xₐ converts prothrombin in the absence and presence of phospholipid, but in the absence of factor Vₐ, mainly prethrombin 2 is formed, while with the complete prothrombinase complex (factor Xₐ, factor Vₐ, phospholipid, and Ca²⁺) thrombin is the main end product, and no prethrombin 2 is detectable. Trace amounts of prethrombin 2 are formed when factor Xₐ activates prothrombin in the presence of factor Vₐ and Ca²⁺. Since it is not possible to saturate factor Xₐ with factor Vₐ under these conditions (Fig. 5) the small amounts of prethrombin 2 are likely formed by free factor Xₐ. Although such experiments are carried out with larger amounts of factor Xₐ and for longer time periods, it seems justified to correlate the implications of these findings with those of the kinetic experiments. In that case we make the following proposal for the mode of factor Vₐ action. The main pathway occurring during prothrombin activation in the absence of factor Vₐ, either in the absence or presence of phospholipid, is that giving rise to prethrombin 2 formation (steps 1, 2, 5). Only a small fraction of prothrombin is converted into thrombin (Steps 1, 2, 3, 4) and is measured in the kinetic experiments. Prethrombin 2 is the main end product since it easily dissociates from factor Xₐ (step 5). Reassociation with factor Xₐ, which offers a second chance to be converted to thrombin, is inhibited by the large excess of prethrombin present. Factor Vₐ changes the pathway of prothrombin activation from one resulting in prethrombin 2 (steps 1, 2, 5) into one giving rise to thrombin (steps 1, 2, 3, 4). Apparently the presence of factor Vₐ prevents the dissociation of prethrombin 2 from the prothrombinase complex. The tight association between fragment 1.2 and both prethrombin 2 and factor Vₐ (5) can explain why prethrombin 2 does not dissociate from the prothrombinase complex. Dissociation of prethrombin 2 can, however, also be prevented when factor Vₐ increases a limiting rate constant occurring in the reaction scheme after formation of the PT2.E complex. This will decrease the steady state concentration of the latter intermediate, with a consequent drop of the rate of dissociation of prethrombin 2. It must be emphasized that more complex mechanisms may be devised to accommodate our observations. However, the proposed explanation for the observed shift in the pathway brought about by factor Vₐ is consistent with our results and data available in the literature. A study on the kinetics of activation of partial prothrombin activation products with various activating mixtures and the effects of prothrombin fragments thereon, in progress in our laboratory, will lead to a more detailed description of the mechanism of prothrombin activation.

Our findings may have important implications for the mechanism of other phospholipid-protein complexes that participate in blood coagulation. The role of factor VIII and phospholipid in the factor X-activating complex consisting of factor IXₐ, factor VIIIₐ, phospholipid, and Ca²⁺ and by extension, the role of the protein and phospholipid component of tissue thromboplastin in the extrinsic factor X activator (factor VIIₐ, tissue thromboplastin, and Ca²⁺) may be identical with those of phospholipid and factor Vₐ in the prothrombinase complex. Indeed Silverberg et al. (14) reported that tissue factor increases the \( k_{cat} \) of factor X activation by factor VIIₐ about 2900-fold and decreased the \( K_{m} \) for factor X 10-fold. Since tissue factor contains both the phospholipid and protein accessory component it is not possible to separate their effect on the kinetic parameters. A kinetic study of the activation of factor X by factor IXₐ in the absence and presence of factor VIIIₐ, phospholipid, and Ca²⁺ is in progress in our laboratory.

Acknowledgments—We would like to thank Dr. H. v. Zutphen for synthesis of the phospholipids, Dr. G. v. Deyten for preparation of benzamidine-Sepharose and Mr. H. Bruls for technical assistance.

REFERENCES