The role of phospholipids and factor Va in the prothrombinase complex

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 1, Issue of January 10, pp. 274-283, 1980
Printed in U.S.A.

The Role of Phospholipids and Factor V_a 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-Riemslag, 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 (X_a) have been determined in the absence and presence of Ca^{2+}, activated bovine factor V (V_a), and phospholipid (dioleoylphosphatidylcholine/dioleoylphosphatidylserine, 1:1; mol/mol). In the absence of accessory components, the K_m 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^{-1} under these conditions. In the presence of 7.5 μM phospholipid, the K_m 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, Ca^{2+}, and 7.5 μM phospholipid), a K_m for prothrombin of 0.21 μM and a V_{max} of 1919 mol min^{-1} mol of X_a^{-1} 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_m for prothrombin at higher phospholipid concentrations. Preliminary calculations show that the prothrombin density at the phospholipid surface at the K_m is independent of the phospholipid concentration. This indicates that the K_m measured in the presence of phospholipid has to be regarded as an apparent K_m 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 but without factor V_a, prethrombin 2 is the main product formed during the initial stages of steady state prothrombin activation. In the presence of factor V_a, thrombin is the main end product and minute amounts of prethrombin 2 are formed. This shift in the reaction pathway of prothrombin activation caused by factor V_a will contribute to the observed increase of the V_{max} measured in the presence of factor V_a.

One of the key reactions in blood coagulation and hemostasis is the formation of thrombin by limited proteolysis of its zymogen, prothrombin.[1] Several proteolytic enzymes can bring about this reaction, but under physiological conditions the serine protease factor X_a is the activating enzyme. In the last 25 years many papers have appeared in the literature describing the detection and isolation of partial proteolysis products of prothrombin (see Ref. 1). They finally resulted in a series of papers by the group of Jackson *et al.* (2–7) which completed the description of the sites of peptide-bond splitting and order of bond cleavage during prothrombin activation. Papahadjopoulos and Hanahan (8) as well as several other authors (see Ref. 1) have shown that additional components are essential for prothrombin activation during *in vitro* blood coagulation. It is now generally accepted that Ca^{2+}, a phospholipid surface, and factor V_a are required for prothrombin activation under physiological conditions. It has been shown that the above mentioned clotting factors have to be absorbed on the phospholipid bilayer surface in order to acquire efficient interaction. Both prothrombin and factor X_a bind to the phospholipid surface via calcium bridges between γ-carboxyglutamic acids present in these proteins and polar head groups of the phospholipid molecules (9, 10), while hydrophobic interactions play an important role in the binding of factor V_a (11). For a recent review about phospholipid involvement in blood coagulation, see Ref. 12.

Not much is known, however, about the mechanism responsible for the enhancement of the rate of prothrombin activation by factor V_a and phospholipids. Esmon and Jackson (5) did not find evidence for a change of sites and orders of bond cleavage when the prothrombin molecule is activated by different combinations of the components of the prothrombinase complex. The enhancement of the rate of thrombin formation when phospholipid or factor V_a, or both, are added to a mixture of prothrombin, factor X_a, and $CaCl_2$ as such (7, 13), does not allow conclusions about the way they are involved in the enzymatic mechanism of prothrombin activation, since these experiments were carried out at single concentrations of the components of the prothrombinase complex.

The purpose of the experiments described in this paper was to assess the kinetic parameters (K_m for prothrombin and V_{max} of thrombin formation) for different prothrombin activating mixtures (*i.e.* factor X_a either in the absence or presence of Ca^{2+}, factor V_a, and/or phospholipid). This enables a precise quantitation 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_a. Tissue factor, a preparation which contains both phospholipids and protein components decreased the K_m for factor X about 10-fold and increased the K_{cat} 3000-fold. Our kinetic approach allows a separate assessment of the effects of phospholipid and the protein accessory component (factor V_a) of

* This investigation was partially supported by the Netherlands Organization for the Advancement of Pure Scientific Research (Z.W.O.). Part of the chromogenic substrates used in our laboratory were kindly donated by AB Kabi Diagnostica. 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.

[1] The nomenclature of the blood coagulation factors used is that recommended by the Task Force on Nomenclature of Blood Clotting Zymogens and Zymogen Intermediates.

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_a, factor VIII, Ca^{2+}, and phospholipid) of the intrinsic pathway of blood coagulation.

EXPERIMENTAL PROCEDURES

Materials—S 2238[2] 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 ovalbumin were obtained from Sigma. DEAE-Sephadex A-50, QAE-(quarternaryammoniumethyl) Sephadex A-50, SP (sulfopropyl) Sephadex C-50, Sephadex G-100, Sepharose 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 Scipio *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 × 20 cm) of SP-Sephadex and STI-Sepharose 4B to reduce the small amounts of thrombin and factor X_a which might be present in these preparations. No thrombin and factor X_a could be detected in our final prothrombin preparations using an assay with the chromogenic substrates S 2238 and S 2222. Prothrombin concentrations were calculated from the E_{280} using $E^{1\%}_{280} = 15.5$ (2) and 72,000 for the molecular weight of prothrombin (2). Bovine factors X_1 and X_2 were purified as described by Fujikawa *et al.* (17). Bovine factor X_a was prepared from factor X_2 using RVV-X according to the method of Fujikawa *et al.* (18). Factor X_a concentrations were calculated after active site titration according to Smith (19).

Prothrombin, factor X_1 and factor X_2 preparations were homogeneous and pure as determined by gel electrophoretic analysis in the presence of sodium dodecyl sulfate. The specific activities attained were equal to those reported in Ref. 2 (for prothrombin) and Ref. 17 (for factor X_1 and X_2).

RVV-X was purified from the crude venom by the method of Schiffman *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 factor V to be present per ml 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 NH_4Cl, 10% glycerol (pH 7.5) with thrombin (1.31 μg/ml). After this time interval the specific activity had risen to about 320 U/mg and did not increase upon longer incubation. Factor V_a was separated from thrombin on a benzamidine-Sepharose 4B column, which to our surprise also bound some of the factor V_a. The bound factor V_a could be eluted with 20 mM Tris, 200 mM NaCl (pH 7.5).

Thrombin was purified as a prothrombin activation product. Prothrombin (11.5 ml; 4.0 mg/ml) was activated with factor X_a (25 μg/ml) in a solution containing 20 mM Tris, 100 mM NaCl, 20 mM $CaCl_2$ (pH 7.5) at 37°C. After 20 min 3 ml of STI-Sepharose slurry was added to bind factor X_a. Ten minutes later this mixture was applied to QAE-Sephadex A-50 column (1.5 × 30 cm), and the prothrombin activation products were eluted with a linear gradient (2 × 150 ml) of 0.1 M to 0.6 M NaCl in 20 mM Tris at pH 7.5. The thrombin-containing fractions were applied to a benzamidine-agarose column (1.5 × 15 cm). Thrombin was eluted with a linear gradient (2 × 100 ml) of 0 to 0.3 M benzamidine in 0.1 M NaCl, 20 mM Tris at pH 7.5 (22). Benzamidine was removed by passing the thrombin-containing fractions through a Sephadex G-25 column (0.9 × 15 cm) followed by extensive dialysis against the same buffer without benzamidine at 5°C. The thrombin concentration was determined by active site titration with *p*-NPGB according to Chase and Shaw (23).

Phospholipids and Phospholipid Vesicle Preparations—1,2-Dioleoyl-*sn*-glycero-3-phosphocholine ($18:1_{cis}/18:1_{cis}$-phosphatidylcholine) was prepared by reacylating the cadmium chloride adduct of *sn*-glycero-3-phosphocholine with the appropriate fatty acyl chloride according the method of Bear and Buchnea (24). 1,2-Dioleoyl-*sn*-glycero-3-phosphoserine ($18:1_{cis}/18:1_{cis}$-phosphatidylserine) was prepared from the respective phosphatidylcholine by enzymatic synthesis as described by Comfurius and Zwaal (25). Single bilayer vesicle solutions were prepared according to the method described by De Kruijff *et al.* (26) by 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 MSE Mark II 150-watt ultrasonic disintegrator set at 10 μ peak to peak amplitude. After sonication no pH adjustment was needed. Phospholipid concentrations were determined by phosphate analysis according the method of Böttcher *et al.* (27). The vesicle preparations described above were used throughout our experiments and were chosen because they exhibit excellent clot-promoting activity (28).

Measurement of Rates of Thrombin Formation—Factor X_a either alone or in the presence of phospholipids, $CaCl_2$, and/or factor V_a 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 ovalbumin at pH 7.5. Prothrombin was added and after different time intervals samples were taken and added to a cuvette (thermostated at 37°C), containing 30 μg of STI and 0.47 μmol of S 2238 in such an amount of the above buffer that the final volume became 2 ml. The amount of STI is sufficient to inhibit further conversion of prothrombin and to inhibit the low amidase activity of factor X_a with S 2238. 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 is calculated from a 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 prethrombin 1 and prethrombin 2 have negligible amidase activity toward S 2238 and, therefore, do not contribute to the absorbance changes measured. Ovalbumin (0.5 mg/ml) was included in all protein solutions and incubations in order to prevent inactivation of the proteins.

For the construction of Lineweaver-Burk plots the rate of thrombin formation was averaged from three independent determinations. K_m, V_{max}, 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 in the presence of sodium dodecyl sulfate was carried out as described by Laemmli (30) on gels containing 10% acrylamide, 0.27% *N*,N^3-methylenebisacrylamide, and 0.1% sodium dodecyl sulfate. Aliquots (10 to 50 μl) of the incubation mixtures were added to 50 μl of 2% sodium dodecyl sulfate, 50 mM EDTA (pH 7.5) and kept for 4 min in a boiling water bath. Five per cent mercaptoethanol was present in disulfide-reduced gel samples.

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_a 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 formed at different time intervals after starting the reaction with varying known amounts of factor X_a. Also when phospholipid or factor V_a, or both, are present the rate of thrombin formation is constant for a long period (measured up to 20 min) and is proportional to the amount of factor X_a over the entire range of factor X_a concentrations used in the further experiments (data not shown). The latter result indicates that either a constant fraction of factor X_a or all factor X_a added is bound to the phospholipid vesicles. Since the maximal rate of thrombin formation increases only 4-fold when the phospholipid concentration is increased from 2.6 μM to 240 μM (Fig. 11, to be discussed below) we conclude that at the low phospholipid concentrations 25% of the added

[2] The abbreviations used are: S 2238, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; S 2222, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride; *p*-NPGB, *p*-nitrophenyl-*p*′-guanidinobenzoate hydrochloride; RVV-X, purified factor X activator from Russell's viper venom; STI, soybean trypsin inhibitor.

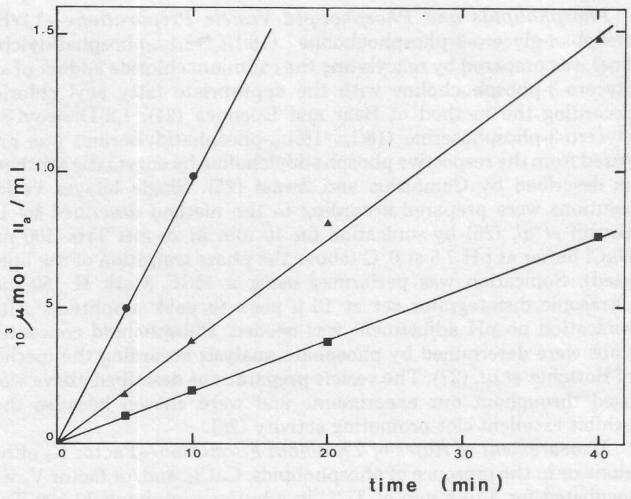


FIG. 1. **Time course of thrombin formation with different amounts of factor X_a.** Prothrombin (30 μM) was incubated with varying amounts of factor X_a in 1 ml of a buffer containing 20 mM Tris-HCl, 100 mM NaCl, 20 mM $CaCl_2$, and 0.5 mg/ml of ovalbumin (pH 7.5) at 37°C. After the time intervals indicated a sample was taken and assayed for the amount of thrombin as described under "Experimental Procedures." The amounts of factor X_a present were: ■——■, 9.1×10^{-5} μmol/ml; ▲——▲, 18.2×10^{-5} μmol/ml; and ●——●, 45.6×10^{-5} μmol/ml.

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 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 Ca^{2+} concentrations at constant amounts of factor X_a and prothrombin. Fig. 2 shows the 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 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 Ca^{2+} concentrations above 4 mM observed in the presence of factor V_a remains as yet unexplained. Similar 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 Ca^{2+} dependence of the rate of thrombin formation at two different phospholipid concentrations (7.5 and 75 μM) 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 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 Ca^{2+} concentrations determined in the above mentioned experiments. When factor X_a converts prothrombin in solution in the presence of 3 mM $CaCl_2$, 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).

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 Ca^{2+} or factor V_a, or both, are present, the data

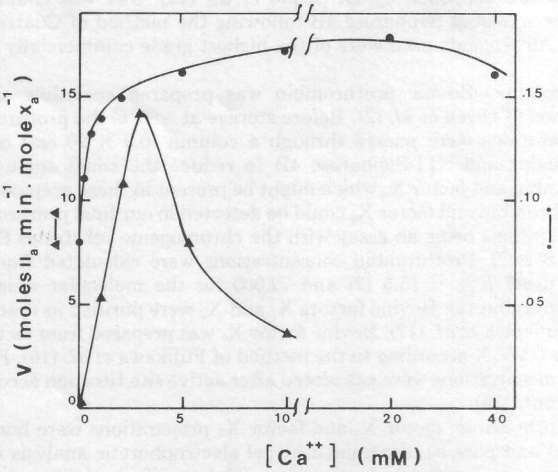


FIG. 2. **The Ca^{2+} dependence of thrombin formation in the absence and presence of factor V_a.** ●——●, prothrombin (30 μM) was incubated at 37°C with factor X_a (9.1×10^{-5} μmol/ml) in 1 ml of a buffer containing 20 mM Tris-HCl, 100 mM NaCl, 0.5 mg/ml of ovalbumin (pH 7.5), and varying amounts of $CaCl_2$. The rate of thrombin formation was calculated from the amounts of thrombin present after 5 and 10 min of incubation. ▲——▲, prothrombin (1.12 μM) was incubated with factor X_a (7.75×10^{-7} μmol/ml) and factor V_a (3.6 units/ml). Further experimental conditions as described above.

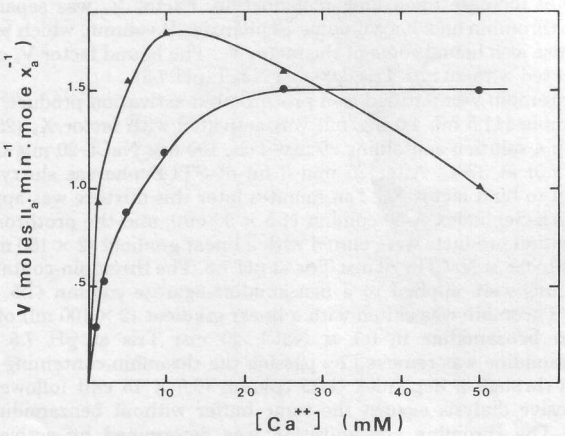


FIG. 3. **The Ca^{2+} dependence of the rate of thrombin formation in the presence of phospholipid.** Factor X_a (3.1×10^{-6} μmol/ml) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 7.5 μM phospholipid (▲——▲) or 75 μM phospholipid (●——●), and varying amounts of $CaCl_2$ at pH 7.5. After 3 min the reaction was started by adding 40 μl of prothrombin, resulting in a final concentration of 0.25 μM. 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."

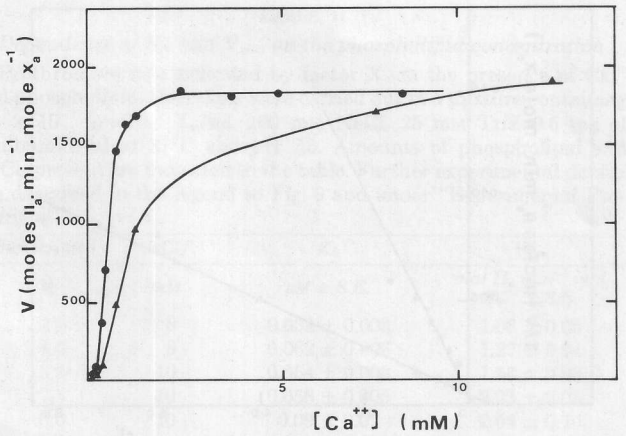


FIG. 4. **The Ca^{2+} dependence of the rate of thrombin formation in the presence of phospholipid and factor V_a.** Factor X_a (3.1×10^{-9} μmol/ml) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 0.95 unit of factor V_a, 7.5 μM phospholipid (▲——▲) or 75 μM phospholipid (●——●), 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.

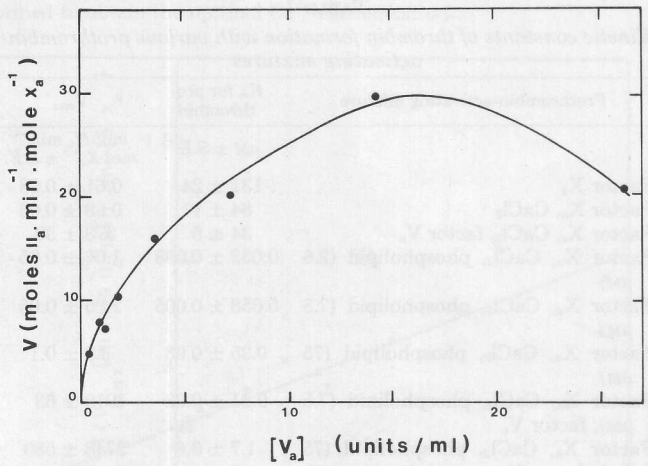


FIG. 5. **The factor V_a dependence of the rate of thrombin formation in solution.** Factor X_a (7.75×10^{-7} μmol/ml) was incubated at 37°C in 1 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 3 mM $CaCl_2$, and varying amounts of factor V_a at pH 7.5. After 5 min the 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, Ca^{2+}, and factor V_a, a K_m of 34 μM is found (Fig. 8). In the presence of factor V_a the maximal rate of thrombin formation is increased about 600-fold to a value of 373 mol min^{-1} mol X_a^{-1}. Lineweaver-Burk plots 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

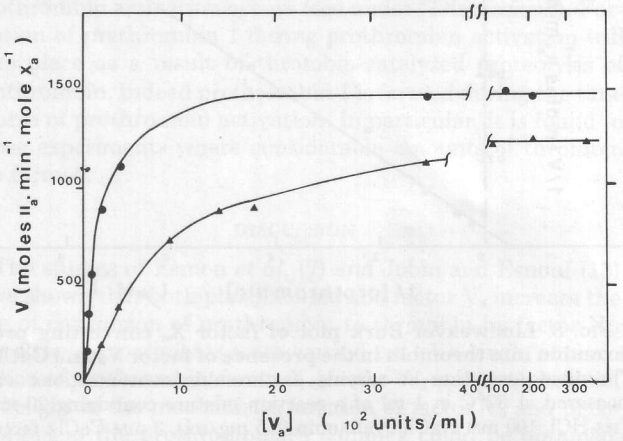


FIG. 6. **The effect of factor V_a on the rate of thrombin formation in the presence of phospholipid.** Factor X_a (2.3×10^{-9} μmol/ml) was incubated at 37°C in 4 ml of a mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 7.5 μM phospholipid (●——●) or 75 μM phospholipid (▲——▲), 5 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.

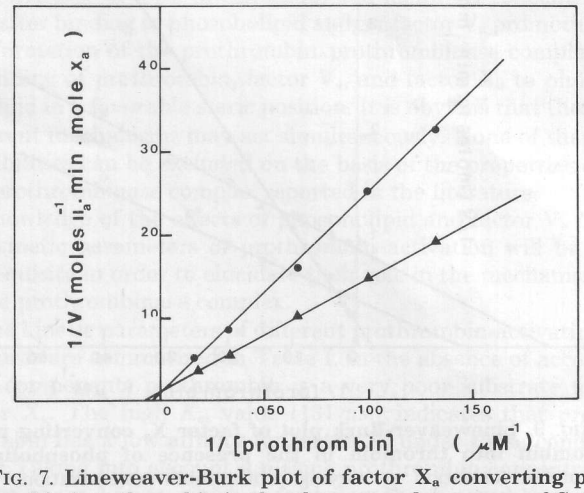


FIG. 7. **Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the absence and presence of $CaCl_2$.** Thrombin formation at varying concentrations of prothrombin was measured at 37°C in 1 ml of a reaction mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), factor X_a (9.1×10^{-5} μmol/ml) at pH 7.5 without $CaCl_2$ (●——●) and in the presence of 20 mM $CaCl_2$ (▲——▲). The reaction was started by addition of factor X_a. After 7½ 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_a^{-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_a, phospholipid, and Ca^{2+} (Table II). The gradual increase of the K_m

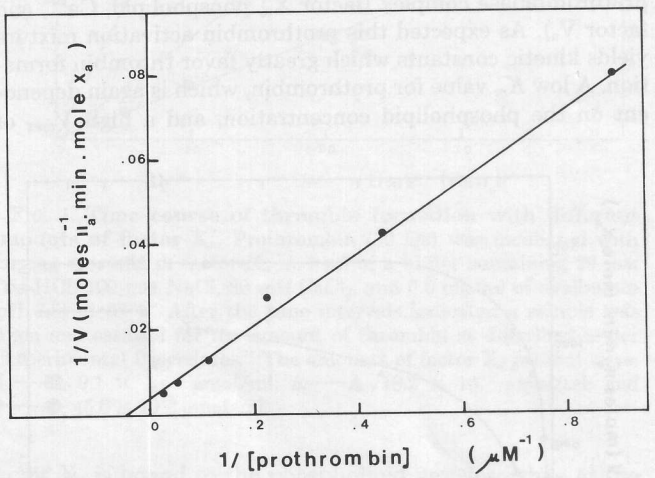


FIG. 8. **Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the presence of factor V_a and $CaCl_2$.** Thrombin formation at varying prothrombin concentrations was measured at 37°C in 1 ml of a reaction mixture containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), 3 mM $CaCl_2$, factor X_a (7.75×10^{-7} μmol/ml), and factor V_a (3.5 U/ml) at pH 7.5. The reaction was started by addition of prothrombin after 5 min of preincubation. Further experimental details are as in Fig. 7. The kinetic constants found are summarized in Table I.

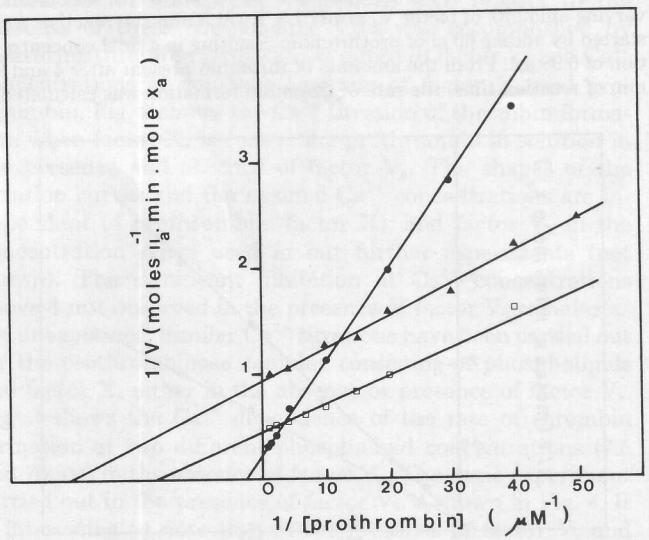


FIG. 9. **Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the presence of phospholipid.** Thrombin formation at varying prothrombin concentrations was measured at 37°C in 4 ml of a reaction volume containing 20 mM Tris-HCl, 100 mM NaCl, ovalbumin (0.5 mg/ml), factor X_a (3.87×10^{-7} μmol/ml), 2.6 μM phospholipid and 8 mM $CaCl_2$ (▲——▲), 7.5 μM phospholipid and 15 mM $CaCl_2$ (□——□) or 75 μM phospholipid and 25 mM $CaCl_2$ (●——●) at pH 7.5. The reaction was started after 3-min preincubation by the addition of prothrombin. At 4 and 8 min after prothrombin addition samples were taken from the reaction mixture and assayed for thrombin in order to calculate the rate of thrombin formation. The kinetic constants calculated are summarized in Table I.

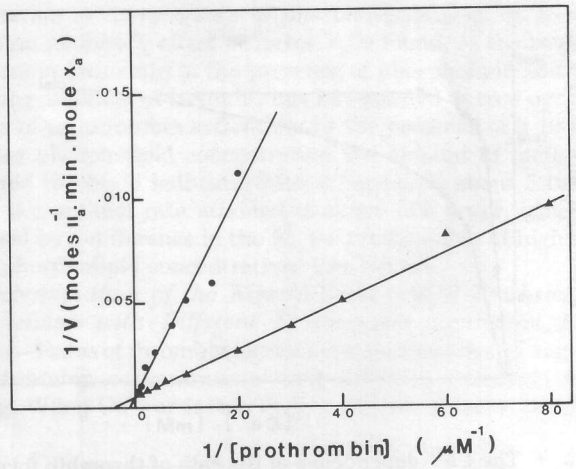


FIG. 10. **Lineweaver-Burk plot of factor X_a converting prothrombin into thrombin in the presence of phospholipid and factor V_a.** The experimental details are described in the legend of Fig. 9 except the amount of factor X_a was 7.75×10^{-10} μmol/ml, and 0.9 U/ml of factor V_a was present. The $CaCl_2$ concentrations were 7.5 mM and 5.0 mM at, respectively, 7.5 μM phospholipid (▲——▲) and 75 μM phospholipid (●——●).

TABLE I

Kinetic constants of thrombin formation with various prothrombin-activating mixtures

Prothrombin-activating mixture	K_m for prothrombin	V_{max}
	μM ± S.E.	*mol II_a min^{-1} mol X_a^{-1}* ± S.E.
Factor X_a	131 ± 24	0.61 ± 0.08
Factor X_a, $CaCl_2$	84 ± 11	0.68 ± 0.06
Factor X_a, $CaCl_2$, factor V_a	34 ± 5	373 ± 30
Factor X_a, $CaCl_2$, phospholipid (2.6 μM)	0.032 ± 0.003	1.06 ± 0.05
Factor X_a, $CaCl_2$, phospholipid (7.5 μM)	0.058 ± 0.005	2.25 ± 0.05
Factor X_a, $CaCl_2$, phospholipid (75 μM)	0.35 ± 0.03	3.9 ± 0.1
Factor X_a, $CaCl_2$, phospholipid (7.5 μM), factor V_a	0.21 ± 0.02	1919 ± 63
Factor X_a, $CaCl_2$, phospholipid (75 μM), factor V_a	1.7 ± 0.6	2748 ± 580

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_a actually bound to the phospholipid at the different phospholipid concentrations. The kinetic parameters of free factor X_a (Table I) are such that any nonbound factor X_a 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_a to varying amounts of phospholipid. Extrapolation of a double reciprocal plot ($1/V_{max}$ *versus* 1/[phospholipid] at constant factor X_a to infinite phospholipid concentration yields the V_{max} for the case that all factor X_a is bound (Fig. 11). The V_{max} calculated from this plot is 4 mol min^{-1} mol X_a^{-1}, which shows that even at the lowest phospholipid concentration used in our experiments about 25% of the added factor X_a 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

TABLE II

Dependence of K_m and V_{max} on the phospholipid concentration

Prothrombin was activated by factor X_a in the presence of Ca^{2+} and phospholipid. Reactions were carried out in a mixture containing 4.6×10^{-7} μmol 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 $CaCl_2$ present are indicated in the table. Further experimental details are described in the legend to Fig. 9 and under "Experimental Procedures."

Phospholipid	$CaCl_2$[a]	K_m	V_{max}
μM	mM	μM ± S.E.	mol II_a min^{-1} mol X_a^{-1} ± S.E.
2.6	8	0.032 ± 0.003	1.06 ± 0.05
4.0	8	0.062 ± 0.005	1.27 ± 0.04
5.3	10	0.054 ± 0.003	1.54 ± 0.03
7.5	10	0.058 ± 0.005	2.25 ± 0.05
8.0	10	0.09 ± 0.02	2.04 ± 0.20
10.5	10	0.068 ± 0.008	1.75 ± 0.09
16.0	10	0.14 ± 0.01	2.35 ± 0.08
26.3	15	0.164 ± 0.006	2.71 ± 0.04
40.0	15	0.23 ± 0.02	3.12 ± 0.10
52.6	25	0.25 ± 0.01	2.90 ± 0.07
75	25	0.35 ± 0.03	3.90 ± 0.10
80	25	0.46 ± 0.01	3.52 ± 0.03
105	32	0.48 ± 0.05	3.31 ± 0.20
240	40	1.08 ± 0.07	4.10 ± 0.10

[a] For each phospholipid concentration a Ca^{2+} titration was performed to obtain the optimal Ca^{2+} concentration.

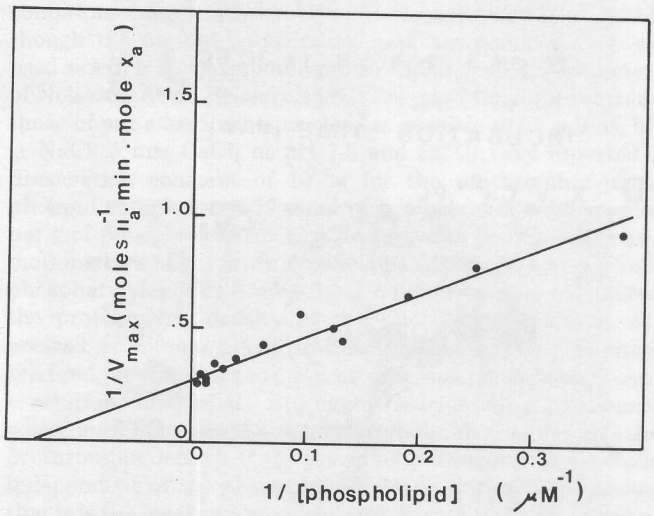


FIG. 11. **Double reciprocal plot of V_{max} as a function of the phospholipid concentration.** The prothrombin activating mixture consisted of factor X_a, $CaCl_2$, and phospholipid. This plot contains data summarized in Table II.

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 under the conditions of our experiments shows that only a negligible amount of prothrombin is converted into thrombin and other partial activation products. Preliminary experiments[3] on the kinetics of prethrombin 1 and prethrombin 2 point out that a possible conversion of liberated activation products cannot contribute to thrombin formation in our experiments. To investigate whether the changes of kinetic parameters are accompanied or caused by changes in the reaction pathway of prothrombin activation it is necessary to devise experiments in which sufficient activation products are formed. By increasing the amount of enzyme (factor X_a and factor V_a, if present) 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 gel are consistent with earlier published gel data (2–6). In the absence of factor V_a (with or without phospholipid) prethrombin 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_a in the absence of phospholipid, thrombin is generated in excess of prethrombin 2 (Fig. 12*B*), whereas in the presence of phospholipid and factor V_a formation of prethrombin 2 cannot be detected (Fig. 12*D*). These data point out that in the presence of factor V_a a shift in the pathway of prothrombin activation occurs (see under "Discussion"). Formation of prethrombin 1 during prothrombin activation will take place as a result of thrombin-catalyzed proteolysis of prothrombin. Indeed prethrombin 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_a increase the rate of conversion of prothrombin to thrombin by factor X_a. Since their experiments were carried out at a single concentration of prothrombin, factor X_a, factor V_a, and phospholipid, no insight in the mechanistic function of factor V_a 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 X_a upon interaction with phospholipid, factor V_a, and/or Ca^{2+}; 3. a conformational change of prothrombin upon binding to phospholipid, factor V_a, and/or Ca^{2+}, making it a more readily cleavable substrate; 4. an increase of local prothrombin and factor X_a concentration after binding to phospholipid and/or factor V_a promoting the formation of the prothrombin·prothrombinase complex; 5. binding of prothrombin, factor V_a, and factor X_a 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_a 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 X_a. The high K_m value (131 μM) indicates that prothrombin has a low affinity for factor X_a under these conditions. Taking into account a plasma prothrombin concentration of about 1.5 μM, which is far below the K_m, and the low V_{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_a.

The small decrease of the K_m for prothrombin occurring when the same experiment is carried out in the presence of Ca^{2+} may be the result of Ca^{2+} binding to γ-carboxyglutamic acid residues present in factor X_a and prothrombin. Since

[3] J. W. P. Govers-Riemslag and J. Rosing, unpublished observations.

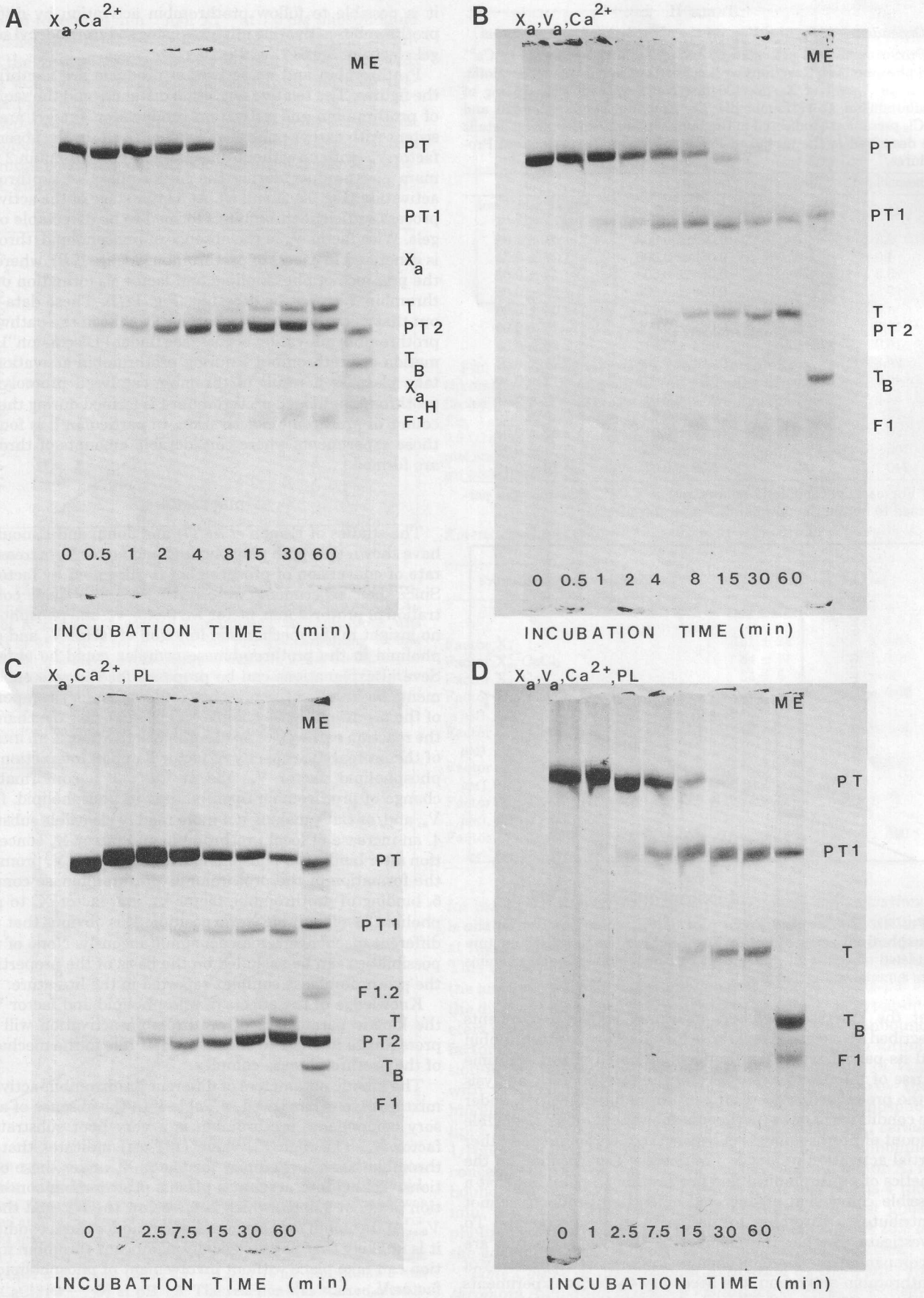


FIG. 12. **Time course of activation of prothrombin with different activating mixtures.** *A*, prothrombin (13.4 μM) was activated with factor X_a (1.82 μM) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate and 10 mM $CaCl_2$ at 37°C and pH 7.5. *B*, prothrombin (13.4 μM) was activated with factor X_a (6.2×10^{-3} μM) and factor V_a (19 units/ml) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate, and 3 mM $CaCl_2$ at 37°C and pH 7.5. *C*, prothrombin (2.68 μM) was activated

Ca^{2+} has no effect on the V_{max} of thrombin formation we conclude that the catalytic efficiency (k_{cat}/K_m) of factor X_a is virtually not changed upon Ca^{2+} binding.

Important changes of kinetic parameters take place when phospholipid or factor V_a, or both, form part of the prothrombinase complex. Phospholipid causes a profound decrease of the K_m for prothrombin. In the presence of phospholipid the K_m decreases to values below 1 μM, which is lower than the plasma prothrombin concentration.

The K_m for prothrombin is, however, dependent on the amount of phospholipid present (Table II). Higher K_m values are measured at increasing phospholipid concentrations. The K_m increases from 0.032 μM at 2.6 μM phospholipid to 1.08 μM at a phospholipid concentration of 240 μM. Therefore, a K_m determined in the presence of phospholipid has to be regarded as an apparent K_m. This is not surprising since the K_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_m should, therefore, be expressed in terms of surface concentration in those experiments where K_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 μmol of prothrombin 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_m measured at different phospholipid concentrations (Table III). Although the apparent K_m increases when increasing amounts of phospholipid are present, the prothrombin density at the phospholipid surface at the K_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 increased local prothrombin concentration explains the large decrease of the K_m observed in the presence of phospholipid. This implies also that the possibility that phospholipid brings prothrombin and factor X_a 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.

TABLE III

K_m for prothrombin and corresponding prothrombin density at the phospholipid surface at varying phospholipid concentrations

Phospholipid	K_m	Prothrombin density at phospholipid surface
μM	μM	*μmol/g ± S.E.*
2.6	0.032	3.4 ± 0.3
4.0	0.062	5.2 ± 0.5
5.3	0.054	4.4 ± 0.3
7.5	0.058	4.2 ± 0.4
8.0	0.090	5.7 ± 1.0
10.5	0.068	4.2 ± 0.5
16.0	0.14	6.2 ± 0.6
26.3	0.164	5.5 ± 0.2
40.0	0.23	5.7 ± 0.6
52.6	0.25	5.4 ± 0.3
75	0.35	5.1 ± 0.4
80	0.46	6.2 ± 0.1
105	0.48	5.2 ± 0.5
240	1.08	5.4 ± 0.4

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

With respect to the mode of action of factor V_a in the prothrombinase complex one has to consider whether factor V_a interacts with prothrombin, changing its properties as a substrate or whether it forms a complex with factor X_a with a catalytic capacity different from free factor X_a. We exclude the possibility that factor V_a exerts its stimulatory action, independent of factor X_a 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_a concentration in our experiments is always much lower than the prothrombin concentration a prothrombin·factor V_a 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_a does not affect the kinetics of the reaction makes it very unlikely that a prothrombin·factor V_a complex acts as a substrate for factor X_a. Therefore, it seems plausible to assume that a factor X_a·factor V_a complex is the catalytic unit in the prothrombinase complex.

In solution factor V_a 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 X_a with factor V_a. At high concentrations factor V_a even inhibits thrombin formation. This phenomenon, for which we have no explanation yet, prevents adding saturating amounts of factor V_a. This leaves open the question whether phospholipid actually has an additional effect on the rate enhancement of thrombin formation brought about by factor V_a.

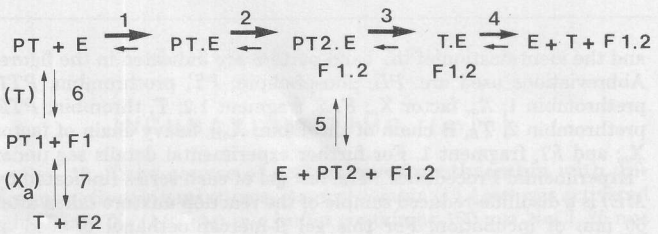
with factor X_a (5.5×10^{-3} μM) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate, 75 μM phospholipid, and 25 mM $CaCl_2$ at 37°C and pH 7.5. *D*, prothrombin (2.68 μM) was activated with factor X_a (6.2×10^{-5} μM) and factor V_a (10 units/ml) in a buffer containing 100 mM NaCl, 25 mM Tris, 2 mM diisopropylphosphorofluoridate, 75 μM phospholipid, and 5 mM $CaCl_2$ at 37°C and pH 7.5. In order to slow down prothrombin conversion by thrombin formed 2 mM diisopropylphosphorofluoridate was included in the activation mixture. Time points at which samples were taken and the identification of the band pattern are indicated in the figure. Abbreviations used are: *PL*, phospholipid; *PT*, prothrombin; *PT1*, prethrombin 1; X_a, factor X_a; *F1.2*, fragment 1.2; *T*, thrombin; *PT2*, prethrombin 2; T_B, B chain of thrombin; X_{aH}, heavy chain of factor X_a; and *F1*, fragment 1. For further experimental details see under "Experimental Procedures." The last gel of each series (indicated by *ME*) is a disulfide-reduced sample of the reaction mixture taken after 60 min of incubation. For this gel β-mercaptoethanol is used as reducing agent.

Comparison of the factor V_a 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 V_a are required in the presence of phospholipid to maximize the rate of thrombin formation, we conclude that phospholipids promote the formation of the factor $V_a \cdot X_a$ complex. This may be caused by increased local factor V_a and factor X_a concentrations at the phospholipid surface after binding of both proteins to the vesicles. This will shift the equilibrium of formation of the factor $V_a \cdot X_a$ 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 V_a 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 V_a 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 X_a converts prothrombin in the absence and presence of phospholipid, but in the absence of factor V_a, mainly prethrombin 2 is formed, while with the complete prothrombinase complex (factor X_a, factor V_a, phospholipid, and Ca^{2+}) thrombin is the main end product, and no prethrombin 2 is detectable. Trace amounts of prethrombin 2 are formed when factor X_a activates prothrombin in the presence of factor V_a and Ca^{2+}. Since it is not possible to saturate factor X_a with factor V_a under these conditions (Fig. 5) the small amounts of prethrombin 2 are likely formed by free factor X_a. Although such experiments are carried out with larger amounts of factor X_a 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 V_a action. The main pathway occurring during prothrombin activation in the absence of factor V_a, 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 X_a (step 5). Reassociation with factor X_a, which offers a second chance to be converted to thrombin, is inhibited by the large excess of prothrombin present. Factor V_a 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 V_a prevents the dissociation of prethrombin 2 from the prothrombinase complex. The tight association between fragment 1.2 and both prethrombin 2 and factor V_a (5) can explain why prethrombin 2 does not dissociate from the prothrombinase complex. Dissociation of prethrombin 2 can, however, also be prevented when factor V_a 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 V_a 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.



SCHEME 1

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 IX_a, factor $VIII_a$, phospholipid, and Ca^{2+} and by extension, the role of the protein and phospholipid component of tissue thromboplastin in the extrinsic factor X activator (factor VII_a, tissue thromboplastin, and Ca^{2+}) may be identical with those of phospholipid and factor V_a in the prothrombinase complex. Indeed Silverberg *et al.* (14) reported that tissue factor increases the k_{cat} of factor X activation by factor VII_a 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 IX_a in the absence and presence of factor $VIII_a$, phospholipid, and Ca^{2+} is in progress in our laboratory.

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

REFERENCES

1. Jackson, C. M., and Suttie, J. W. (1977) *Physiol. Rev.* **57,** 1–70
2. Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 594–605
3. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 606–611
4. Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 7782–7790
5. Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 7791–7797
6. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 7798–7807
7. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 8045–8047
8. Papahadjopoulos, D. P., and Hanahan, D. J. (1964) *Biochim. Biophys. Acta* **90,** 436–439

9. Papahadjopoulos, D. P., Hougie, C., and Hanahan, D. J. (1962) *Proc. Soc. Exp. Biol. Med.* **111,** 412–416
10. Barton, P. G., and Hanahan, D. J. (1969) *Biochim. Biophys. Acta* **187,** 319–327
11. Kandall, C. L., Shohet, S. B., Akinbarni, T. K., and Colman, R. W. (1975) *Thromb. Diath. Haemorrh.* **34,** 271–284
12. Zwaal, R. F. A. (1978) *Biochim. Biophys. Acta* **515,** 163–205
13. Jobin, F., and Esnouf, M. P. (1967) *Biochem. J.* **102,** 666–674
14. Silverberg, S. A., Nemerson, Y., and Zur, M. (1977) *J. Biol. Chem.* **252,** 8481–8488
15. Di Scipio, R. G., Hermodson, M. A., Yates, S. G., and Davie, E. W. (1977) *Biochemistry* **16,** 698–706
16. Cuatrecasas, P. (1970) *J. Biol. Chem.* **245,** 3059–3065
17. Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11,** 4882–4891
18. Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972) *Biochemistry* **11,** 4892–4899
19. Smith, R. L. (1973) *J. Biol. Chem.* **248,** 2418–2423
20. Schiffman, S., Theodor, J., and Rapaport, S. J. (1969) *Biochemistry* **8,** 1397–1405
21. Smith, C. M., and Hanahan, D. J. (1976) *Biochemistry* **15,** 1830–1838
22. Schmer, G. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353,** 810–814
23. Chase, T., Jr., and Shaw, E. (1969) *Biochemistry* **8,** 2212–2224
24. Bear, E., and Buchnea, D. (1959) *Can. J. Biochem. Physiol.* **37,** 953–959
25. Comfurius, P., and Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta* **488,** 36–42
26. De Kruijff, B., Cullis, P. R., and Radda, G. K. (1975) *Biochim. Biophys. Acta* **406,** 6–20
27. Böttcher, C. J. F., van Gent, C. M., and Pries, C. (1961) *Anal. Chim. Acta* **24,** 203–207
28. Tans, G., van Zutphen, H., Comfurius, P., Hemker, H. C., and Zwaal, R. F. A. (1979) *Eur. J. Biochem.* **95,** 449–457
29. Wilkinson, G. N. (1961) *Biochem. J.* **80,** 324–332
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685
31. Nelsestuen, G. L., and Broderius, M. (1977) *Biochemistry* **16,** 4172–4177
32. Mann, K. G., Heldenbrand, C. M., Fass, D. N., Bajaj, S. P., and Butkowski, R. J. (1974) *Thromb. Diath. Haemorrh. Suppl.* **57,** 179–196