The Conversion of Prothrombin to Thrombin

V. THE ACTIVATION OF PROTHROMBIN BY FACTOR X, IN THE PRESENCE OF PHOSPHOLIPID

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SUMMARY

As shown previously for activated Factor X and Ca$^{2+}$ alone, (ESMON, C. T., AND JACKSON, C. M. (1974) J. Biol. Chem. 249, 7782-7790) prothrombin activation by activated Factor X and Ca$^{2+}$ in the presence of phospholipid also proceeds via Intermediate 2 and Fragment 1-2, i.e.

\[
\text{PROTHROMBIN} \rightarrow \begin{array}{c}
\text{INTERMEDIATE 2} \\
\text{FRAGMENT 1-2}
\end{array} \rightarrow \begin{array}{c}
\text{THROMBIN} \\
\text{FRAGMENT 1} + \text{FRAGMENT 2}
\end{array}
\]

Unless rapidly inactivated, thrombin cleaves Fragment 1-2 into two smaller activation products, i.e.

\[
\text{FRAGMENT 1-2} \rightarrow \begin{array}{c}
\text{THROMBIN} \\
\text{FRAGMENT 1} + \text{FRAGMENT 2}
\end{array}
\]

The direct and concomitant formation of Intermediate 2 and Fragment 1-2 was demonstrated by activating prothrombin in the presence of iPr$_2$P-F. In the absence of this inhibitor, thrombin, in addition to catalyzing the reaction of Equation 2, cleaves Fragment 1 from prothrombin and in so doing eliminates the possibility of observing phospholipid catalysis from all but the earliest stages of reaction (GITTEL, S. N., OWEN, W. G., ESMON, C. T., AND JACKSON, C. M. (1973) Proc. Nat. Acad. Sci. U. S. A. 70, 1344-1348). The reaction of Equation 1 was shown to occur independent of any "special effect" of diisopropyl fluorophosphate (iPr$_2$P-F) by a direct demonstration of Intermediate 2 and Fragment 1-2 formation from tritium-labeled prothrombin. Both the formation of Intermediate 2 and its conversion to thrombin are accelerated by phospholipid. However, in the presence of phospholipid, Intermediate 2 was found to satisfy the necessary condition for an intermediate on a sequential activation path, i.e. that its rate of conversion to thrombin be greater than or equal to the rate of prothrombin conversion to thrombin, if and only if, Fragment 1-2 were also present in the activation mixture.

In view of this observation, the true activation intermediate of the phospholipid-catalyzed pathway must consist of both Intermediate 2 and Fragment 1-2. Fragment 1 plus Fragment 2 was not able to substitute for Fragment 1-2.

Intermediate 2 and Fragment 1-2 were demonstrated to associate noncovalently to form a product with gel electrophoretic properties indistinguishable from those of prothrombin. This association of Fragment 1-2 with Intermediate 2 apparently provides the mechanism by which Intermediate 2 can be retained on the phospholipid surface and thus be readily accessible to the activated Factor X for the final proteolysis which is required to form thrombin.

An alternative prothrombin activation pathway which maintains the integrity of the Intermediate 2 and Fragment 1-2 portions of the prothrombin molecule during the two-step activation process was proposed previously (ESMON, C. T., OWEN, W. G., AND JACKSON, C. M. (1974) J. Biol. Chem. 249, 606-611). No unambiguous evidence for the existence of the alternative activation route could be demonstrated and thus the reactions of Equation 1 appear to describe the principal, if not exclusive, pathway of prothrombin activation by Factor X, phospholipid, and Ca$^{2+}$.

Although optimum prothrombin activation requires a complex enzyme which consists of activated Factor X (X$_a$), Factor V (V$_a$), phospholipid, and Ca$^{2+}$ (2-14), considerable insight into the activation process has been obtained by investigating the inter-

1 The abbreviations used are: X$_a$, the enzymatically active form of blood clotting Factor X; (I-1), Intermediate 1; (I-2), Intermediate 2; (F-1), Fragment 1; (F-2), Fragment 2; (F-1-2), Fragment 1-2; and (F-1-2-A-S-S-B), Fragments 1 and 2 and the A chain of thrombin still linked by peptide bonds but attached to the B chain of thrombin only by a disulfide bridge.

2 Intermediates are defined by us as partial activation products which can be activated further to yield thrombin. An activation fragment is a non-thrombin-forming activation product. The relationship of the Fragments and Intermediates to the structure of prothrombin can be determined from the diagram above. Fragment 1 and Fragment 2 in this system of nomenclature appear to be equivalent to the products labeled Intermediate 3 and Intermediate 4 respectively by Mann and co-workers (1).
interactions that occur between pairs of the components (4-6, 8-10, 15-19). From such studies, it has been shown that Xs is the activating protease and alone can convert prothrombin to thrombin (1, 2, 7, 8, 10-14, 19). However, the rate of prothrombin activation by Xs alone is less than 0.1% of that obtained with the complex enzyme (8, 20).

Even though rapid activation requires the complex enzyme, a 40-fold increase in the rate of thrombin formation has been observed when phospholipid is added to Xs in the presence of Ca++. This ability of phospholipid to accelerate blood clotting in general and prothrombin activation specifically has been investigated extensively and has been reviewed (20-27).

Chemical characterization of thrombin (28) and the products of prothrombin activation (14, 29, 30) have demonstrated that prothrombin activation requires the cleavage of 2 peptide bonds. The relationships between the cleavages which occur and the polypeptides which are produced during prothrombin activation are indicated on the linear schematic model for the prothrombin molecule which is presented in the accompanying manuscript (31).

Recent work has demonstrated that Xs-catalyzed prothrombin activation in the presence of Ca++ occurs principally, if not exclusively via the sequence of reactions given in Equations 1 and 2 (Pathway II).

$$\text{PROTHROMBIN} \rightarrow \text{INTERMEDIATE } A/B$$

Although in free solution prothrombin activation occurs via this pathway, Pathway II is not necessarily suitable for explaining the increase in the activation rate that is observed in the presence of phospholipid. Specifically, because the phospholipid binding sites of prothrombin are in Fragment 1-2 (19), if activation in the presence of phospholipid occurs via Pathway II, the activation process occurs via an intermediate which is apparently no longer bound directly to the phospholipid surface. In view of the fact that the initial observation which identified the Fragment 1 region of prothrombin as the phospholipid binding region was a loss in the ability of phospholipid to activate the activation process after proteolytic cleavage of Fragment 1 from prothrombin, the adequacy of Pathway II for [Xs, phospholipid, Ca++] catalyzed prothrombin activation must be questioned.

An alternative to Pathway II has been proposed for prothrombin activation (30) which, although demonstrated not to contribute to a quantitatively significant manner to thrombin formation by [Xs, Ca++]], could not be ruled out. In this pathway (Pathway III) an intermediate designated Intermediate A/B is formed instead of Intermediate 2, as shown in Equations 3 and 4.

Because Intermediate A/B maintains the integrity of the entire prothrombin molecule in the activation intermediate via the A/B disulfide bridge, Pathway III is a teleologically attractive mechanism for prothrombin activation in the presence of phospholipid.

Because prothrombin activation is the formation of the protease thrombin, interpretation of prothrombin activation experiments must take into account the rapid proteolysis of prothrombin by thrombin (1, 28-30, 32-34), as shown in Equations 5 and 6.

$$\text{PROTHROMBIN} \rightarrow \text{THROMBIN} + \text{FRAGMENT } 1-2$$

Species 5, which is observed during prothrombin activation by [Xs, phospholipid, Ca++] and is not simply due to the accumulation of Intermediate 2 which has been formed by Factor Xs cleavage of prothrombin in bulk solution.

In precisely the same way as that with [Xs, Ca++] (31), the time course of prothrombin activation by [Xs, phospholipid, Ca++] was monitored by sodium dodecyl sulfate gel electrophoresis. The progress of the activation as a function of time was determined from the product distributions in the electrophoresis gels. Aliquots from the reaction mixture were taken at the times indicated in the appropriate figure, and the reaction product distributions were examined both before and after the reduction of the polypeptide disulfide bonds. The identity of each of the bands in the gels was determined from its relative migration distance and molecular weight and by co-electrophoresis with samples of each of the partial activation products that had been

**RESULTS**

The demonstration of direct formation of Intermediate 2 from prothrombin by [Xs, phospholipid, Ca++] is more complicated than that with only [Xs, Ca++] (31). First, the increased rate of thrombin formation in the presence of phospholipid makes it more difficult to ensure that thrombin inactivation by iPrzP-F is sufficiently rapid to limit adequately the formation of Intermediate 1. Second, it is also necessary to demonstrate that the Intermediate 2, which is observed during prothrombin activation by [Xs, phospholipid, Ca++]], is formed via the phospholipid-catalyzed pathway and is not simply due to the accumulation of Intermediate 2 which has been formed by Factor Xs cleavage of prothrombin in bulk solution.

The three pathways for prothrombin activation were previously designated A, B, and C (30). However, the mnemonic advantage of using Roman numerals to designate the pathways fully justifies a nomenclature change. With this revised system, Pathway I (previously A) commences with the cleavage of bond (4) and proceeds via Intermediate 1; Pathway II (previously B) commences with the cleavage of bond (4) and proceeds via Intermediate 2; and Pathway III (previously C) commences with the cleavage of bond (4) and proceeds via Intermediate A/B. i.e., F-1-2-A-S-S-B. Unfortunately, the intermediate of Pathway III cannot be designated Intermediate 3 because this name has been used previously by other workers.3 Numbers enclosed by the symbol (4) represent the specific bonds which are being cleaved in the particular step. (See the diagram in the accompanying manuscript (31)).
isolated and characterized (14). The time course of prothrombin activation by [Xn, phospholipid, Ca2+] in the presence of iPrzP-F is shown in Fig. 1A (disulfide bonds reduced prior to electrophoresis). Inspection of Fig. 1A shows the presence of seven components in the reaction mixture during the course of the activation process. From the top to the bottom of the gels, these components are: prothrombin (mol wt 72,000 to 74,000, single polypeptide chain) (1, 14); a trace of Intermediate 1 (mol wt 36,000, single polypeptide chain) (14); Fragment 1.2 (mol wt 33,000 to 37,000 including carbohydrate, single polypeptide chain) (30); Intermediate 2 (mol wt 37,000, single polypeptide chain) (1, 14); the B chain of thrombin (mol wt 31,000 to 32,000) (28); the heavy chain of Factor Xn (mol wt 27,000 to 28,000) (35, 36); and Fragment 1 (mol wt 22,000 to 24,000 including carbohydrate) (1, 14). Intermediate 1 can be seen to be present in small and approximately constant amounts throughout the entire course of the reaction. Significant quantities of Fragment 1, the second product formed by thrombin's action on prothrombin, or Fragment 1.2 is seen to be present in a readily detectable amount only near the end of the reaction time course.

**Fig. 1.** Time course of prothrombin activation by [Xn, phospholipid, Ca2+] in the presence and absence of iPrzP-F. Prothrombin (0.23 mg per ml) was activated by [Xn, 5 µg per ml, phospholipid, 40 µg per ml, CaCl2, 0.01 M] in 0.05 M Tris-HCl (pH 7.5)-0.1 M NaCl at room temperature. At the times given below, 100-µl aliquots were removed from the activation mixture and added to 10 µl of 10% sodium dodecyl sulfate, 0.05% ethylenediaminetetraacetic acid at 70°. In samples for electrophoresis after disulfide reduction, 10 µl of β mercaptoethanol were added to the protein-sodium dodecyl sulfate mixture. A, activation in the presence of iPrzP-F (0.01 M); disulfides reduced. 1, 0 min; 2, 15 s; 3, 1 min; 4, 2 min; 5, 3 min; 6, 4 min; 7, 5 min; 8, 8 min; 9, 12 min; 10, 20 min; 11, 30 min; and 12, 60 min. B, activation in the presence of iPrzP-F (0.01 M) disulfides intact. The time points are the same as in A. C, activation time course in the absence of iPrzP-F. Gel electrophoresis was carried out on the samples after reduction. The number in parenthesis is the percentage of the theoretical maximum amount of thrombin present as determined by bioassay at each time point. 1, 0 min; 2, 15 s; (not measured); 5, 1 min (3.3); 4, 2 min (6.8); 5, 3 min (8.4); 6, 4 min (10.2); 7, 5 min (12.0); 8, 8 min (15.5); 9, 12 min (17.4); 10, 20 min (20.0); 11, 30 min (23.9); and 12, 60 min (26.0). D, time course of prothrombin activation by [Xn, Ca2+] in the absence of iPrzP-F. Prothrombin (0.23 mg per ml) was activated by [Xn, 5 µg per ml, CaCl2, 0.01 M] at room temperature exactly as above, except that no phospholipid was present. Activation time course in the absence of iPrzP-F. Gel electrophoresis on samples after disulfide reduction. The number in parentheses is the percentage of the maximum theoretical amount of thrombin present as determined by bioassay at each time point. 1, 0 min (0); 2, 1 min (*); 3, 15 min (*); 4, 30 min (<0.8); 5, 60 min (1.5); 6, 120 min (4.5); 7, 180 min (7.5); 8, 240 min (11.2); 9, 300 min (13.5); 10, 360 min (17.4); 11, 420 min (22.0); and 12, 480 min (36).
Neither the A chain of thrombin (mol wt 6,000) (28) nor Fragment 2 (mol wt 13,000) (14), which must also exist after the cleavage of Fragment 1-2, are visible in the gels. Fig. 1B shows the gels obtained from the same time course as those in Fig. 1A, except that electrophoresis was run prior to the disulfide bond reduction. The components in the gels are identified in the figure. Significant quantities of Fragment 1-2 are seen clearly more in Fig. 1B than in the previous photograph. This large quantity of Fragment 1-2 relative to Fragment 1 during the initial stage of the reaction indicates unambiguously that the Intermediate 2 which is being formed in this activation mixture cannot be arising principally via Intermediate 1. Furthermore, because only a trace of Intermediate 1 and Fragment 1 is seen until most of the prothrombin has disappeared, it appears that, as long as thrombin action is limited effectively by the iPrzP-F, direct formation of Intermediate 2 from prothrombin can be observed throughout the entire reaction.

Fig. 1C shows the results of prothrombin activation under identical conditions with those of the above reactions except for the omission of iPrzP-F. As anticipated, both Intermediate 1 and Fragment 1 are formed. Thrombin was measured by bioassay during this activation reaction (Fig. 1C) and the amount of thrombin present in the mixture at each time point is given in the figure legend.

Although direct formation of Intermediate 2 and Fragment 1-2 has been demonstrated in the presence of phospholipid, what is not clear from the time course studies alone is that the Intermediate 2 and Fragment 1-2 are necessarily formed in the phospholipid-catalyzed process. A comparison of these gels with the gels obtained from a time course of prothrombin activation by [Xa, CaCl2] alone (Fig. 1D; see Ref. 31), shows that the same relative amounts of Intermediate 2 and prothrombin are present after 1 to 3 min of reaction in the presence of phospholipid as are present after 60 to 120 min in the absence of phospholipid. From this result, it can be estimated that the rate of formation of Intermediate 2 is increased as much as 40- to 60-fold by the phospholipid and thus, consistent with phospholipid-catalyzed prothrombin activation via Pathway II, the rate of Intermediate 2 formation is increased by the phospholipid. Similarly, the acceleration of thrombin formation by phospholipid can be calculated from the bioassay data which was obtained from the two reactions carried out in the absence of iPrzP-F (Fig. 1C and Fig. 2C; see Ref. 31). Under the conditions of these experiments, an increase in the rate of thrombin formation of approximately 100-fold occurs.

Quantitative confirmation of the conclusions derived from visual examination of the time courses of Fig. 1 was obtained by employing radiolabeled prothrombin as a substrate. Electrophoresis gels obtained from a 3H-labeled prothrombin activation mixture were sliced as described previously (31) and the relative amounts of the reaction products determined (Fig. 2 and Table

Fig. 2. Quantitation of prothrombin activation products. Tritiated prothrombin (0.125 mg per ml) was activated in both the presence and absence of iPrzP-F by [Xa, 5 μg per ml, phospholipid, 20 μg per ml, CaCl2, 0.010 M]. Reaction products were separated by sodium dodecyl sulfate gel electrophoresis, the gels sliced, and the distribution of radioactivity determined as described under “Materials and Methods.” A, distribution of radioactive products after activation for 1 min in the presence of iPrzP-F. Disulfide bridges were reduced prior to electrophoresis. B, distribution of radioactive products after activation for 10 min in the absence of iPrzP-F. Disulfide bridges were reduced prior to electrophoresis. C, distribution of radioactivity in an electrophoresis gel which was run on the same sample from the reaction mixture as in B above, but prior to disulfide reduction, is shown here for comparison (see text and Table 1).
I). Activation of tritium-labeled prothrombin was carried out both in the presence and absence of iPr,P-F.

The first requirement, i.e. that the major amount of the Intermediate 2 observed during this experiment arises directly from prothrombin, can be seen to be met from the following analysis

\[ I-2 = (F-1 (1 \text{ min}) - F-1 (\text{zero time})) - (I-1 (1 \text{ min}) - I-1 (\text{zero time})) \]

The radioactive product distribution during prothrombin activation by \([X, \text{ phospholipid, Ca}^{2+}]\)

Tritium-labeled prothrombin (0.125 mg per ml) was activated by \([X, \text{ 0.5 mg per ml, phospholipid, 20 mg per ml, CaCl}_2, 0.01 \text{ M in 0.05 \text{ M Tris-HCl, pH 7.8, as described under "Materials and Methods."}}\]

Parallel reactions were run, one in the presence of iPr,P-F (0.01 M) and the other without iPr,P-F. Product separation by sodium dodecyl sulfate gel electrophoresis, gel slicing, and 3H-counting are described under "Materials and Methods." The amount of each product at the different time points is the mole of that product which would be derived from 1 mol of prothrombin at zero time, i.e. (mole of the particular product per mol of prothrombin from which all of the products would be derived). By this convention, at any time point, the sum of all thrombin-region-containing polypeptides, i.e., prothrombin plus "Intermediate 1" + Intermediate 2 + thrombin = 1 and the sum of all activation fragment region-containing polypeptides, i.e. prothrombin plus Fragment 1.2 + Fragment 1 = 1. (Because Fragment 2 contains no radioactivity it is not counted in the summation of radiolabeled products.)

\[
\begin{align*}
\text{Prothrombin} & : 0.099 \pm 0.009, 0.951 \pm 0.092 \\
\text{Intermediate 1} & : 0.049 \pm 0.009, 0.062 \pm 0.082 \\
\text{Thrombin} & : 0.026 \pm 0.002 \\
\text{Fragment 1} & : 0.054 \pm 0.002, 0.009 \pm 0.099 \\
\text{Intermediate 2} & : 0.024 \pm 0.002, 0.022 \pm 0.066 \\
\text{F1-2-A} & : 0.008 \pm 0.002
\end{align*}
\]

\( A(A) \) ELECTROPHORESIS PERFORMED ON REACTION PRODUCTS PRIOR TO DISULFIDE REDUCTION; \( B \) ELECTROPHORESIS RUN AFTER DISULFIDE REDUCTION.

\( \text{Data from duplicate gels are given in order to "define" the precision of gel counting data.} \)

\( \text{Total radioactive material above background of the top of sodium dodecyl sulfate electrophoresis gel and the first radioactive material unambiguously identifiable as being associated with the peak of prothrombin (see Fig. 1B).} \)

\( \text{Extent of reaction is the total counts due to Fragment 1-2, Intermediate 2, and thrombin divided by 8 (see Methods).} \)

\( \text{Extinction of reaction plus Intermediate 1 and Intermediate 2 obtained from gel slice data. As a gel slice without significant radioactivity was never present between Fragment 1.2 and Intermediate 2 peaks, the counts present in this slice were divided equally between Fragment 1.2 and Intermediate 2.} \)

\( \text{Excess of Intermediate 1 over Fragment 1.} \)

\( \text{A negative value indicates that Fragment 1.2 is being cleaved to form Fragment 1 and Fragment 2.} \)

\( \text{Prothrombin activity as determined by bioassay from the reaction mixture which did not contain iPr,P,F, the value given is to the fraction of the amount expected from the starting prothrombin (14).} \)

\( \text{Mol of material in the Intermediate 1 region (counts/3) minus mol of Fragment 1. A positive value in gels run on reaction plus iPr,P,F prior to reduction indicates that Intermediate 1 present in the starting prothrombin is remaining as Intermediate 1; a negative value indicates that Fragment 1-2 is being cleaved to form Fragment 1 and Fragment 2.} \)

\( \text{Excess of reaction (mol of product) as calculated above without specific assumptions about the relative amounts of 1-2 and F1-2 divided by the mol prothrombin. If F1-2-A-S-S-B undergoes co-electrophoresis with prothrombin in unred} \)

\( \text{Sample gels (A) and with F1-2, or I-2 and thrombin after disulfide reduction (B) then (Extent of Reaction)/Prothrombin for all columns labeled B should be greater than in columns labeled A.} \)

\( \text{Calculated from the equation:} \)

\[ F-1-2-A = 0.6 (I-1) \text{REDUCED} - (1-1) \text{UNREDUCED} \]

\( \text{The coefficient, 0.6, is the correction for the difference in the } ^3\text{H sialic acid content of F1-2-A and I-1.} \)
from Intermediate 1 is undoubtedly overestimated. Furthermore, the amount of Intermediate 2 calculated as having come from Fragment 1 is not likely to be quantitatively correct. This being so, the amount of Intermediate 2 calculated as having come from Intermediate 1 is undoubtedly overestimated. Furthermore, if the amount of Intermediate 1 at zero time is actually less than that estimated from the counts present in this region of the gel, e.g. if the counts are due to a radiolabeled contaminant, then the amount of Intermediate 2 calculated as being formed via Intermediate 1 is once again overestimated. It can be seen further from Table I that the division of the gel region containing Intermediate 2 and Fragment 1-2 into separate contributions from each component gives relative amounts of Fragment 1-2 and Intermediate 2 which are more consistent with Intermediate 2 and Fragment 1-2 being formed in equimolar amount as required by Pathway II than with a significant quantity of Intermediate 2 being formed via Intermediate 1. Independent evidence that Intermediate 1 is not contributing significantly to the formation of either Intermediate 2 or thrombin is presented later.

The second requirement for an unambiguous demonstration of the direct formation of Intermediate 2 from prothrombin via the pathway catalyzed by [Xa, phospholipid, Ca\textsuperscript{2+}] is that the rate of Intermediate 2 formation be accelerated by phospholipid. Comparison of the Intermediate 2 present at either 1 min or 2 min in the reaction mixture containing phospholipid (Table I) with the Intermediate 2 present in a reaction mixture without phospholipid (Table I in Ref. 31) indicates a greater than 10-fold increase in the rate of formation of Intermediate 2 in the presence of phospholipid. It appears clear, therefore, that the Intermediate 2 which is observed is formed in the phospholipid-catalyzed reaction pathway.

Data are also shown in Table I for the product distribution after 6 min of reaction. These data demonstrate the inadequacy of iPrP-F in inhibiting thrombin formation completely under these activation conditions and the necessity for very early time point data in the phospholipid-catalyzed system. This latter requirement is evident from the observation that at 6 min, the rate of Intermediate 2 formation has been dramatically decreased. This marked reduction in activation rate is the result of competition between prothrombin and Fragment 1-2 plus Fragment 1 for the lipid surface (20). It has been demonstrated that Fragment 1 and prothrombin compete with equal affinity for the phospholipid surface.1

Relative Rates of Activation of Prothrombin and Intermediate 2 by [Xa, phospholipid, Ca\textsuperscript{2+}]—If Pathway II is to qualify fully as a sufficient route for prothrombin conversion to thrombin by [Xa, phospholipid, Ca\textsuperscript{2+}], it must be possible to show that the rate of conversion of Intermediate 2 to thrombin can account for the greater than 30-fold increase in the activation rate which is observed when phospholipid is added to [Xa, Ca\textsuperscript{2+}].

The ability of Intermediate 2 to satisfy this condition was examined directly by measuring the rate of thrombin formation by [Xa, phospholipid, Ca\textsuperscript{2+}] from Intermediate 2 (Fig. 3). The particular data shown in Fig. 3 are for a concentration of Intermediate 2 approximately equimolar to the phospholipid used in the experiments of Fig. 2 and Table I. Clearly, the necessary condition is not met by Intermediate 2 alone; that is, the rate of conversion of Intermediate 2 to thrombin is less than the rate of conversion of prothrombin to thrombin by [Xa, phospholipid, Ca\textsuperscript{2+}] alone. Upon the addition of Fragment 1-2 to the reaction mixture, the situation is immediately changed and Intermediate 2, in the presence of Fragment 1-2, is converted to thrombin much more rapidly than prothrombin.

It is important as a control experiment to determine whether or not Intermediate 2, in the presence of Fragment 1 and Fragment 2, can be activated to thrombin more rapidly than prothrombin. It can be seen from Fig. 3 that Fragment 1 and Fragment 2 together cannot substitute for Fragment 1-2, i.e. are not sufficient to restore the rapid Intermediate 2 activation and it is clear that the intact non-thrombin-forming half of the prothrombin molecule is required for surface-catalyzed prothrombin activation.

It should be noted that phospholipid can slightly accelerate Intermediate 2 activation in the absence of Fragment 1-2 (Fig. 3). However, under such circumstances, the addition of Intermediate 2 to [Xa, phospholipid, Ca\textsuperscript{2+}] also results in the flocculation of the lipid. No such lipid flocculation occurs if Fragment
samples are, from left to right, Prothrombin, 20 pg; Intermediate 2, 780 pg; and Intermediate 2, 15 pg. The two components seen in the gel on the far right which are not identified by samples in the other gels are nearest the top, "Intermediate 1", i.e. (Intermediate 2 plus Fragment 2) (37), and nearest the bottom, a trace of Fragment 1.

1-2 or Fragment 2 is added to the [X, phospholipid, Ca++] prior to the addition of Intermediate 2.

For comparison, thrombin formation from prothrombin and Intermediate 2 by [X, Ca++] is also shown in Fig. 3.

Noncovalent Association between Intermediate 2 and Fragment 1-2—Association between Intermediate 2 and Fragment 1-2 was investigated by comparing the behavior of Intermediate 2, Fragment 1-2, and Intermediate 2 + Fragment 1-2 with prothrombin by acrylamide gel electrophoresis at pH 7.5 (Fig. 4). It can be seen that Intermediate 2, both because of its relatively low, negative charge and its aggregation, collects at the top of the electrophoresis gel. In contrast, Fragment 1-2, migrates two-thirds of the way between the top of the gel and the marker dye front. The mixture of Fragment 1-2 and Intermediate 2 migrates to a position in the gel which is indistinguishable from the position to which prothrombin migrates, consistent with a tight association between Intermediate 2 and Fragment 1-2.

It must be noted that in the gel containing both Fragment 1-2 and Intermediate 2 that some of the free Fragment 1-2 can be seen because this component is present in a slight excess. The trace of Fragment 1 can be seen below Fragment 1-2 as a result of the thrombin contamination of the Intermediate 2 and the cleavage of the Fragment 1-2. The smeared trailing edge of the prothrombin band is the result of the combination of some of the Intermediate 2 with the Fragment 2, which is also formed by thrombin cleavage of Fragment 1-2. The Fragment 2 combines with Intermediate 2 to form a species with electrophoretic characteristics indistinguishable from those of Intermediate 1 (37). In electrophoresis experiments identical with these, but with a mixture of Intermediate 1 and Fragment 1, no evidence for the association between these components is found consistent with published experiments on the requirement for the covalent attachment of Fragment 1 to Intermediate 1 for phospholipid catalysis of thrombin formation (19).

Thrombin Formation by [X, phospholipid, Ca++] from Prothrombin, Prothrombin plus Intermediate 1, and Prothrombin plus Intermediate 1 and Fragment 1—The difficulty of completely eliminating the formation of Intermediate 1 during prothrombin activation, even when iPrzP-F is included at high concentration, makes it necessary to independently investigate the effect of Intermediate 1 on prothrombin activation by [X, phospholipid, Ca++].

For this purpose, the rate of thrombin formation by [X, phospholipid, Ca++] from prothrombin alone, from prothrombin plus Intermediate 1, and from prothrombin plus Intermediate 1 and Fragment 1 was determined (Fig. 5). The rate of thrombin formation from prothrombin was also determined at twice the molar concentration of prothrombin or Intermediate 1 in the single substrate activation mixture and equal, therefore, to the total molar substrate concentration in the activation mixture that contained both prothrombin and Intermediate 1. The middle curve of Fig. 5 shows thrombin formation from prothrombin alone and from prothrombin plus Intermediate 1 as a function of time. Data points from both experiments fall on the same curve, demonstrating that the addition of Intermediate 1 to prothrombin neither increases nor decreases the rate of thrombin formation as if the Intermediate 1 were not participating in the reaction at all. Because the rate of thrombin formation is doubled when the prothrombin concentration is doubled (top curve, Fig. 5), this result cannot be due to the enzyme being saturated under the conditions of the experiment. Because previous work (31, 38) has demonstrated that in the absence of phospholipid, prothrombin and Intermediate 1 are converted to thrombin at the same rate, the simplest interpretation of this experiment is that Intermediate 1 is a poor, i.e. unrecognized, substrate for [X, phospholipid, Ca++] by virtue of its inability to bind to the phospholipid surface (19). Examination of the bottom curve of Fig. 5, indicates
that the addition of Fragment 1 to a prothrombin and Intermediate 1 activation mixture inhibits the rate of thrombin formation, demonstrating, first, that Intermediate 1 and Fragment 1 do not combine to give a species with kinetic properties of prothrombin and second, that as previously reported (20), Fragment 1 can compete with prothrombin for the phospholipid surface and thus decrease the prothrombin activation rate.

It is clear from these experiments that the Intermediate 1 which is formed during prothrombin activation does not contribute significantly to the thrombin formation process. Moreover, because Intermediate 1 is not bound to the lipid surface (19, 20, 30) it can also be inferred from these experiments that the Intermediate 2 which is observed during the early stages of \([X,\text{ phospholipid, Ca}^2+]\)-catalyzed prothrombin activation cannot be formed in a significant quantity via Intermediate 1. For example, in Fig. 1C, it can be observed that the Intermediate 1, which forms in the activation mixture, appears to accumulate and is present in the greatest amount after all of the prothrombin has disappeared from the reaction mixture. That such an accumulation of Intermediate 1 should occur is also clear from the results of the foregoing experiment.

**Attempt to Detect Intermediate of Pathway III during Prothrombin Activation by \([X,\text{ phospholipid, Ca}^2+]\)—**Even though the evidence presented above indicates that Pathway II exists and, from the large amount of Intermediate 2 which is observed, must be the route by which the bulk of the thrombin which is formed by \([X,\text{ phospholipid, Ca}^2+]\) arises, an alternative to Pathway II has been proposed which also adequately (30) accounts for the formation of Fragment 1-2 during prothrombin activation although clearly not for Intermediate 2. This activation path (Pathway III) was found to contribute negligibly, if at all, to thrombin formation by \([X,\text{ Ca}^2+]\) (31), however, the addition of phospholipid to the activator and the large activation rate increase which results from it, demands that an attempt be made to determine whether any evidence for this pathway can be found.

If Pathway III exists, (Equations 3 and 4) (31) a two-chain activation intermediate with the same molecular weight as prothrombin is predicted. Upon the reduction of the disulfide bonds of this intermediate (Intermediate A/B or Fragment 1-2-A-S-S-B), a unique product consisting of Fragment 1-2 covalently linked to the A chain of thrombin (Fragment 1-2-A) will be produced. This Pathway III product, with a calculated molecular weight of 41,000 to 43,000, should migrate to a position in acrylamide gels between prothrombin and Fragment 1-2 during sodium dodecyl sulfate gel electrophoresis (31).

Examination of the radiolabel profiles of Fig. 2, A and B, shows no radioactive products between prothrombin and Intermediate 2-Fragment 1-2 region except that previously identified as Intermediate 1. The possibility, however, that the radioactive material designated Intermediate 1 in the gels of Fig. 2, A and B, is actually in partFragment 1-2-A.S-S.B might possess a slightly lower gel electrophoretic mobility than prothrombin and consequently be found between the top of the gel and the prothrombin band. Examination of Fig. 2C and Table I (top row) indicates material in this region, which, although not completely resolved from the Fragment 1-2-A.S-S.B conversion to Fragment 1-2-A and the B chain of thrombin. However, the amount of material disappearing from this region (Table I, top entry) does not correlate with the increase seen in the Intermediate 1 region. Furthermore, this material disappears from the zero time gels suggesting that what is being observed is in reality only a tailing of the prothrombin band. In view of these considerations, it must be concluded that no consistent evidence for the existence of Fragment 1-2-A.S-S-B, i.e., Pathway III, can be found, although the limits of the experimental procedures clearly are being strained.

One additional consideration must be made in order to conclude the search for the proposed intermediate of Pathway III. Specifically, it is also possible that Fragment 1-2-A.S-S-B migrates in the same region as does prothrombin when its disulfide bridges are intact and in the Fragment 1-2-Intermediate 2 region after reduction. If the steady state concentration of Fragment 1-2-A.S-S-B is sufficiently large to contribute detectably to the prothrombin band prior to disulfide reduction and to the Fragment 1-2-Intermediate 2 region after reduction, a net transfer of radioactivity between these regions should be measurable after disulfide reduction and electrophoretic separation. Such a net transfer would result in an increase in the ratio of Intermediate 2 plus Fragment 1-2 ("Extent of Reaction," Table I) to prothrombin after reduction. As can be seen from Table I, no reproducible change in this ratio occurs and thus no evidence for any such net transfer between the gel regions can be demonstrated.

**DISCUSSION**

On the basis of the observations made here, prothrombin activation by \([X,\text{ phospholipid, Ca}^2+]\) is concluded to occur principally, if not exclusively, via Pathway II; that is
The direct cleavage of prothrombin to form Intermediate 2 and Fragment 1-2, which had been shown previously to occur when prothrombin is activated by $[X_a, Ca^{2+}]$ (31) is also found to occur when prothrombin is activated by $[X_a, phospholipid, Ca^{2+}]$. Because the rate of activation of prothrombin is increased more than 40-fold by phospholipid (19, 20), the demonstration that the conversion of Intermediate 2 to thrombin also is accelerated to an extent that its conversion rate is greater than or equal to that of prothrombin was a crucial test of the adequacy of Pathway II. This necessary condition was found to be met, if and only if, Fragment 1-2 was present in the reaction mixture, thus demonstrating for the first time a requirement for the complete and intact activation fragment region of prothrombin in order to fulfill a kinetic condition for the prothrombin activation mechanism.

The original question of how Intermediate 2 might be kept on the phospholipid surface, adjacent to Factor $X_a$, for the cleavage of bond $\Phi$, appears to be answered by the noncovalent association between Intermediate 2 and Fragment 1-2. An attempt to demonstrate the existence of Pathway III produced no consistent evidence for this alternative activation mechanism, and thus if it exists as a parallel route which along with Pathway II functions in prothrombin activation, Pathway III is clearly the minor path.

The thrombin-catalyzed cleavage of prothrombin to give Intermediate 1 and Fragment 1 results in the inhibition of prothrombin activation by $[X_a, phospholipid, Ca^{2+}]$ as a result of the competition between Fragment 1 and prothrombin for the lipid surface (20). This observation, the requirement for intact Fragment 1-2 for phospholipid-catalyzed Intermediate 2 activation and the inability of Intermediate 1 to satisfy the necessary requirements for being on the phospholipid-catalyzed activation pathway (19, 20, 38) make the proposals which include Intermediate 1 on the prothrombin activation pathway (1, 39-41) untenable. It must be noted that it has been shown that Intermediate 1 can be activated rapidly to thrombin, however, there is an absolute requirement for Factor $V_a$ for such a rapid Intermediate 1 activation (13, 20).

An interesting picture involving both protein-protein and protein-lipid interactions is beginning to emerge from the data reported here and the results of work carried out in a number of laboratories (4-8, 15, 20, 26, 37, 42) on the prothrombin activation system. It is clear that the rapid formation of thrombin, which depends upon having all of the components of the system present (2, 8-15), directly reflects these noncovalent interactions. First, all of the protein components, prothrombin (8, 16, 18-20), Factor $X_a$ (5-8, 15), and Factor V (4-9, 17, 26) bind to the phospholipid surface with a consequent increase in local concentration. Second, the demonstration that a specific region of the prothrombin polypeptide chain (10) (and that the light chain of Factor $X_a$) is responsible for the binding of these two components to the lipid surface also implies a preferred orientation for the reactants when they are in the adsorbed state. Third, the previously demonstrated interaction between Factor $V_a$ and prothrombin (43) and Factor V and Factor $X_a$ (5, 8) results in the further collection of the reaction components into the vicinity of the Factor $V_a$ molecule. The noncovalent interaction between Fragment 1-2 and Intermediate 2 which is necessary for rapid prothrombin activation and the demonstration (37) that the ability of Factor $V_a$ to accelerate prothrombin activation is specifically dependent on the Fragment 2 region, provides additional examples of the intricate protein-protein interactions that are involved in this symgen activation system. Both the number and the diversity of the types of interactions which occur and which appear to be necessary for the optimum function of the system make it attractive for the further investigation of both the chemistry and physiological consequences of such macromolecular organization.

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