Activation of Human Prothrombin by Human Prothrombinase

INFLUENCE OF FACTOR Va ON THE REACTION MECHANISM

Sriram Krishnaswamy, William R. Church, Michael E. Nesheim, and Kenneth G. Mann

From the Department of Biochemistry, University of Vermont, Burlington, Vermont 05405 and the Departments of Medicine and Biochemistry, Queen’s University, Kingston, Ontario, K7L-3N6

The kinetics of the activation of human prothrombin catalyzed by human prothrombinase was studied using the fluorescent α-thrombin inhibitor dimethylaminine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA). Prothrombinase proteolytically activates prothrombin to α-thrombin by cleavages at Arg273-Thr274 (bond A) and Arg322-Ile323 (bond B). The differential fluorescence properties of DAPA complexed with the intermediates and products of human prothrombin activation were exploited to study the kinetics of the individual bond cleavages in the zymogen. When the catalyst was composed of prothrombinase (human factor Xa, human factor Va, synthetic phospholipid vesicles, and calcium ion), and bond velocity studies of α-thrombin formation indicated that the kinetic constants for the cleavage of bonds A or B were similar to the constants that were obtained for the overall reaction (bonds A + B). The progress of the reaction was also monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The results indicated that the activation of human prothrombin catalyzed by prothrombinase proceeded exclusively via the formation of meizothrombin (bond B-cleaved) as an intermediate. The association of the cofactor, human factor Va, with the enzyme, human factor Xa, on the phospholipid surface.

The activation of human prothrombin to the serine protease α-thrombin is accomplished by the proteolytic cleavage of two peptide bonds in the zymogen (2–6). Prothrombin is converted to α-thrombin by the serine protease factor Xa (2–4). Although factor Xa possesses the catalytic machinery to produce these cleavages in the zymogen, the rate of prothrombin activation catalyzed by factor Xa alone is negligible when compared to the rate observed when the fully assembled prothrombinase complex is the catalyst (7–9). The prothrombinase complex is composed of the protease factor Xa and the protein cofactor factor Va, assembled on a cellular (10–13) or a phospholipid surface (7, 14) in the presence of calcium ion and probably constitutes the physiologically relevant catalyst for prothrombin activation.

The available data indicate that the bovine prothrombinase complex is composed of factor Xa and factor Va complexed in a 1:1 stoichiometry on a membrane surface containing negatively charged phospholipids in the presence of calcium ions and catalyzes the activation of prothrombin by an ordered sequential kinetic mechanism (15). Very little direct information is available regarding the human prothrombinase complex reconstituted in vitro using purified components isolated from human plasma. The present study was undertaken to evaluate rigorously the kinetic properties of human prothrombin activation catalyzed by human prothrombinase under relatively well-defined conditions.

Factor Xa catalyzes the activation of human prothrombin by proteolytic cleavages at Arg273-Thr274 and at Arg322-Ile323 (6). The polypeptide chain composed of residues 1 through 273 (Fragment 1-2) has a molecular weight of 34,500 and is released as an activation peptide. Residues 274–322 comprise the A chain of α-thrombin which is disulfide-linked to the B chain of thrombin (residues 323–581). As two cleavages are required in the prothrombin molecule to produce thrombin, the overall activation of prothrombin can be represented by two kinetic pathways illustrated in Fig. 1. A single cleavage at Arg273-Thr274 (Reaction 1) yields Fragment 1-2 and an intermediate species known as prethrombin 2. Prethrombin 2 is composed of the A and B chains of thrombin with the bond at Arg322-Ile323 intact. Further proteolysis at this bond (Reaction 3) converts prethrombin 2 to α-thrombin. In contrast, a single cleavage in the prothrombin molecule at Arg273-Thr274 (Reaction 2) produces a species known as meizothrombin. Meizothrombin is a catalytically active intermediate (16) composed of fragment 1-2, thrombin A chain and the B chain of thrombin linked by a disulfide bond. Further proteolysis of meizothrombin by factor Xa (cleavage at Arg322-Ile323, Reaction 4) yields the products Fragment 1-2 and the two-chain form of α-thrombin.

In addition to the proteolytic cleavages in the prothrombin molecule catalyzed by factor Xa, thrombin formed during the course of the reaction is capable of catalyzing two cleavages.
Kinetics of Human Prothrombin Activation

**Fig. 1.** Schematic illustration of the pathways for the activation of human prothrombin. Cleavage of prothrombin at Arg575, Thr219 (Reaction 1) yields Fragment 1 and prethrombin 2. Further cleavage of prethrombin 2 at Arg576-Ser578 (Reaction 3) yields a disulfide-linked two-chain form of α-thrombin. Cleavage of prothrombin in the opposite order (cleavage at Arg576-Ser578, Reaction 4) yields meizothrombin. Meizothrombin is composed of the Fragment 1-2 chain and thrombin-B chain that are covalently linked by a disulfide bond. Further cleavage at Arg575, Thr219 produces Fragment 1-2 and α-thrombin. The arrows labeled a and b indicate bonds that are susceptible to cleavage by the feedback action of thrombin. a = Arg575-Ser578; b = Arg576-Thr219.

indicated by arrows a and b in Fig. 1 (6). The feedback action of thrombin results in the cleavage at Arg576-Ser578 in the Fragment 1-2 region to yield Fragment 1 (residues 1-155) and Fragment 2 (residues 156-273). A second feedback reaction catalyzed by thrombin results in a cleavage at Arg576-Thr219 and produces a species of prethrombin 2 or α-thrombin with 13 residues deleted from its amino terminus. The available data indicate that these two cleavages are catalyzed by α-thrombin and not factor Xa in the presence or absence of factor Va (17). The analysis of prothrombin activation catalyzed by prethrombinase or by factor Xa alone is therefore complicated by the side-reactions that result from the feedback action of newly formed meizothrombin or thrombin. The use of the fluorescent, reversible thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) eliminates the thrombin-catalyzed feedback cleavages (18). Due to the fluorescence enhancement that accompanies the formation of the thrombin-DAPA complex, this inhibitor provides a method for continuously monitoring the progress of the conversion of prothrombin to thrombin. In addition, DAPA has been demonstrated to exhibit differential fluorescence properties when complexed with the different intermediates of prothrombin activation (19). The differential fluorescence properties of DAPA complexed with meizothrombin, prothrombin 2, and α-thrombin allow for the evaluation of the kinetics of cleavage of the individual bonds catalyzed by prethrombinase by the independent analysis of the conversion of meizothrombin or prethrombin 2 to thrombin.

The fully assembled prothrombinase complex catalyzes the activation of prothrombin to thrombin at a rate that is approximately 5 orders of magnitude greater than the rate observed when factor Xa alone is used as the catalyst (7, 9). The rate enhancement that accompanies complex formation has been rationalized on the basis of the co-concentration of enzyme and substrate on the phospholipid surface and the increased catalytic efficiency of the enzyme conferred by virtue of its association with the cofactor (20, 21). The experiments described in this paper were undertaken in order to provide a quantitative description of the kinetics of human prothrombin activation catalyzed by prothrombinase and to examine the consequences of complex formation on the kinetic mechanism of the reaction catalyzed by factor Xa.

**EXPERIMENTAL PROCEDURES**

Materials—Tris base, Echis carinatus venom, L-α-phosphatidylcholine (hen egg), and L-α-phosphatidylserine (bovine brain) were obtained from Sigma. Benzamine-Sepharose was from Pharmacia F-L Biochemicals. Phospholipid vesicles (PCPS) composed of 75% (w/w) phosphatidylethanolamine and 25% (w/w) phosphatidylcholine were prepared as previously described (22, 23). DAPA was synthesized as outlined by Nesheim et al. (9). Human factor V was purified by immunoaffinity chromatography as previously described (9, 24). Aliquots of factor V (0.1-0.5 mg/ml) were activated to factor Va by the addition of bovine thrombin by the methods of Nesheim et al. (9). Factor Va stock solutions were maintained on ice and were used within 3 h of preparation. Bovine α-thrombin was prepared according to the procedure of Lundblad et al. (25). The human proteins prothrombin, prethrombin 2, Fragment 1-2, and factor X were purified by previously described procedures (4, 16, 27). Human prothrombin was labeled with [125I]Iodo-Gen method (28). Factor X was activated to factor Xa according to the procedure of Nestorova and Rasmussen (29), using the purified Factor X activator from Russell's viper venom. When activation was complete, as judged by clotting assays, the reaction was chilled to 4 °C and applied to a 1.5 × 15-cm column of benzamine-Sepharose equilibrated in 20 mM Tris, pH 7.4, 0.15 M NaCl at a flow rate of 60 ml/hr-1. The column was washed with the same buffer to remove unbound proteins, and bound factor Xa was eluted with 20 mM Tris, pH 7.4, 0.15 M NaCl containing 4 mM benzamidine. Fractions containing factor Xa activity were pooled and precipitated by the addition of solid (NH₄)₂SO₄ to 80% saturation. The precipitated protein was collected by centrifugation (55,000 × g, 20 min), resuspended in 50% (v/v) glycerol and stored at -20 °C. The prothrombin activator from the venom of E. carinatus was partially purified by the procedures described by Nesheim and Mann (26). The molecular weights and extinction coefficients (26), respectively, used to calculate protein concentrations were human Factor V, 330,000, 0.36 (9); human Factor X, 65,300, 1.16 (30); human Factor Xa, 46,000, 1.16 (30); human prothrombin, 72,000, 1.47 (4); human prothrombin 2, 57,000, 1.95 (4); and human Fragment 1-2, 34,000, 1.47 (4).

Measurement of the Kinetics of Thrombin Formation Using DAPA—The formation of thrombin using prothrombin, prethrombin 2 plus Fragment 2-1, 2, or meizothrombin as substrates was continuously monitored by the change in the fluorescence intensity of DAPA present in the reaction mixtures. The use of DAPA also allowed an assessment of feedback reactions catalyzed by newly formed product. Fluorescence intensity measurements were performed with an SLM 8000 photon-counting fluorescence spectrophotometer. Reaction temperatures were maintained at 25 °C using a refrigerated circulating water bath. The excitation wavelength was 280 nm (band pass, 8 nm), and scattered light was minimized with a long-pass filter (Schott KV 408) in the emission beam. All buffers were filtered using 0.45-μm filters to further reduce scattered light artifacts. When the factor Va concentration was varied, reaction mixtures (2.0 ml) in continuously stirred cuvettes contained 20 mM Tris, pH 7.4, 0.15 M NaCl, 2 mM CaCl₂, 20 mM benzamidine. Fractions containing factor Xa activity were pooled and precipitated by the addition of solid (NH₄)₂SO₄ to 80% saturation. The precipitated protein was collected by centrifugation (55,000 × g, 20 min), resuspended in 50% (v/v) glycerol and stored at -20 °C. The prothrombin activator from the venom of E. carinatus was partially purified by the procedures described by Nesheim and Mann (26). The molecular weights and extinction coefficients (26), respectively, used to calculate protein concentrations were human Factor V, 330,000, 0.36 (9); human Factor X, 65,300, 1.16 (30); human Factor Xa, 46,000, 1.16 (30); human prothrombin, 72,000, 1.47 (4); human prothrombin 2, 57,000, 1.95 (4); and human Fragment 1-2, 34,000, 1.47 (4).

The abbreviations used are: DAPA, dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide; dansyl, 1-dimethylaminonaphthalene 5-sulfonic acid; Gla, γ-carboxyglutamic acid NaDodSO₄-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PCPS, vesicles composed of phosphatidylcholine-phosphatidylserine.
\[ E_0 = (i \cdot [T]_0 + [E_0] + K_d) - \sqrt{i \cdot [T]_0 + [E_0] + K_d^2} - 4 \cdot i \cdot [T]_0 \cdot [E_0] \]

where \([E_0]\) is the fixed concentration of enzyme (factor Xa), \([T]_0\) is the total concentration of tetratant (factor Va), \([E_0]\) is the concentration of enzyme complexed as prothrombinase, \(i\) is the number of moles of enzyme (factor Xa) combining per mole of tetrant (factor Va), and \(K_d\) is the dissociation constant that describes the interaction between enzyme (factor Xa) and tetrant (factor Va). A detailed description of the derivation of this expression is available in the literature (33). If \(R_e\) is the catalytic rate observed when \([E_0] = 0\) (all factor Xa is uncomplexed with factor Va) and \(R_{max}\) is the rate observed when \([E_0] = [E_1]\), then the concentration of bound Xa ([E]) at any time \(t\) can be related to the observed catalytic rate \(R_{obs}\) by Equation 1:

\[ R_{obs} = [E_0] \cdot \frac{R_{max} - R_e}{R_{max}} \]

When \(R_e\) is small in comparison to \(R_{max}\), \(R_{max} - R_e\) can be replaced by \(R_{max}\), Equations 1 and 2 can be combined to yield Equation 3:

\[ R_{obs} = \frac{[E_0] \cdot R_{max}}{[E_0] + R_{max} - R_e} \]

This relates the observed catalytic rate to the concentration of factor Xa complexed in prothrombinase.

The stoichiometry and the dissociation constant for the factor Xa-factor Va interaction were determined by fitting steady-state rates for prothrombin activation obtained at varying concentrations of factor Xa using two fixed concentrations of factor Va to Equation 3 using a nonlinear least-squares computer program (CET Research Group), written in Basic and utilizing the Marquardt algorithm.

RESULTS

Measurement of the Stoichiometry and Dissociation Constant for the Factor Xa-Factor Va Interaction—The dependence of the initial rate of prothrombin activation on the concentration of the cofactor (factor Va) in the presence of two fixed concentrations of factor Xa is illustrated in Fig. 2. In both cases, initial rate increased until saturation was
obtained with increasing concentrations of factor Va, as would be predicted if an interaction between factor Xa and phospholipid-bound factor Va produced a more active enzyme species. The significant shift in the titration curve observed when the fixed concentration of enzyme (factor Xa) is increased by a factor of 2 suggests strongly that the dissociation constant for the interaction between factor Xa and factor Va on the PCPS surface is approached by the fixed concentration of enzyme. The lines illustrated in the figure were drawn according to Equation 3 described under "Data Analysis" and correspond to a $K_o$ of $0.43 \times 10^{-6} \text{ M} \pm 0.29 \times 10^{-6} \text{ M}$ and a stoichiometry of $1.12 \pm 0.11 \text{ mol of factor Va bound per mol of factor Xa at saturation.}

The Michaelis constants obtained for the conversion of prothrombin to thrombin in the absence of cofactor increases as function of increasing concentrations of the substrate prothrombin are replotted in double-reciprocal form in Fig. 3A. The data illustrated in Fig. 3B or a mixture of prothrombin 2 and Fragment 1-2 (Fig. 3C) as substrates. The data obtained in all three cases are fairly similar, and the kinetic constants obtained for the overall conversion of prothrombin to thrombin and for the cleavages of the individual bonds are summarized in Table I. The Michaelis constants obtained for the cleavage of the individual bonds (meizothrombin and prothrombin 2 as substrates) are slightly lower than those obtained for the overall reaction using prothrombin as the substrate. However, the catalytic efficiency of prothrombinase ($k_{cat}/K_m$) for the overall reaction is essentially identical to the catalytic efficiency for the cleavage of the individual bonds in the substrate. These data indicate that the cleavage of the individual bonds in the zymogen is kinetically indistinguishable from the overall conversion of prothrombin to thrombin, hence precluding the identification of a single rate-limiting step in the reaction or the exclusion of one of the two pathways (see Fig. 1) for the activation of prothrombin on a purely kinetic basis.

Analysis of Prothrombin Activation Catalyzed by Prothrombinase in the Presence of DAPA by NaDdSO₄-PAGE—Due to the lack of kinetic evidence for a preferred pathway for prothrombin activation catalyzed by prothrombinase, samples were removed from an on-going reaction mixture of prothrombin activation catalyzed by prothrombinase and subjected to NaDdSO₄-PAGE analysis before and after disulfide bond reduction with 2-mercaptoethanol. The results obtained are illustrated in Fig. 4A (before reduction) and B (after disulfide bond reduction), and the proteins were visualized by staining with Coomassie Brilliant Blue. The various intermediates were identified by comparison to standard prothrombin fragments separately analyzed by NaDdSO₄-PAGE (data not shown). The species identified in Fig. 4A are prothrombin (substrate) and the terminal products of the reaction, thrombin and Fragment 1-2. Notably absent are prethrombin 1 and the species Fragment 1 and Fragment 2, indicating the effectiveness of DAPA in inhibiting the feedback reactions catalyzed by thrombin. In addition, prothrombin is essentially quantitatively consumed to produce α-thrombin during the course of the reaction, indicating the quantitative conversion of substrate to product. The pattern observed after disulfide bond reduction (Fig. 4B) is similar in that the immediately identifiable species correspond to prothrombin, Fragment 1-2, and the A and B chains of thrombin. In addition to these, a species is observed only after reduction which migrates between prothrombin and Fragment 1-2 and has been identified as the Fragment 1-2-A chain. This species is not detected prior to reduction with 2-mercaptoethanol, which is consistent with the hypothesis that Fragment 1-2-A chain arises from the presence of meizothrombin as an intermediate in the overall reaction. This hypothesis is also supported by the observation that Fragment 1-2-A chain is detectable in a transient manner during the early portion of the reaction, exactly as would be expected for an intermediate. Meizothrombin present as an intermediate in the early portion of the reaction would co-migrate with prothrombin before reduction of the disulfide bonds. Reduction with 2-mercaptoethanol converts meizothrombin to Fragment 1-2-A chain and the B chain of thrombin. No prethrombin 2 or prothrombin 2-des-1-13 was detected by this method of analysis and would have been readily resolved from thrombin following reduction (see Fig. 4B). The data observed following NaDdSO₄-PAGE

**Figure 2.** Dependence of initial rate of prothrombin activation on the concentration of cofactor. Reaction mixtures containing 1.4 μM prothrombin, 3 μM DAPA, 30 μM PCPS, and varying concentrations of factor Va in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2.0 mM CaCl₂ were initiated by the addition of factor Xa to a final concentration of 1.02 × 10⁻⁹ M (●) or 2.04 × 10⁻⁹ M (○). The lines are drawn according to Equation 3 described under "Data Analysis" and correspond to a $K_o$ of $0.43 \times 10^{-9} \text{ M} \pm 0.29 \times 10^{-9} \text{ M}$ and a stoichiometry of $1.12 \pm 0.11 \text{ mol of factor Va bound per mol of factor Xa at saturation.}
and the lack of detectable levels of prethrombin 2 indicate that the overall activation of prothrombin catalyzed by fully assembled prothrombinase proceeds exclusively via meizothrombin.

The gels in Fig. 4, A and B, were subjected to densitometry analysis in order to quantify the levels of the identified species. As discussed under "Experimental Procedures," meizothrombin levels were assigned on the basis of the staining intensity of the material identified as Fragment 1-2-A chain and from the difference in the staining intensity at the position of prothrombin before and after reduction. The results obtained by this analysis are illustrated in Fig. 5 where the relative concentration of each species is plotted versus time. The concentration of prothrombin decreased essentially monotonically as a function of time. A short lag was evident in the densitometry data for the disappearance of prothrombin and agrees well with the lag observed in progress curves of prothrombin activation using DAPA (see below and Fig. 6, trace A). The time course for the appearance of thrombin was sigmoidal and did not correspond directly to the curve representing the depletion of substrate. The relative amount of meizothrombin rose to a maximum level of approximately 40% in the first 90 s of the reaction and then decreased to undetectable levels within 200 s of the reaction. These profiles are indicative of an ordered sequential conversion of human prothrombin to thrombin solely via meizothrombin catalyzed by human prothrombinase (15, 34).

**Cofactor Dependence of the Cleavages in Prothrombin Catalyzed by Factor Xa—**Earlier studies of the activation of bovine prothrombin (26) indicated that the cleavage of the two bonds in prothrombin catalyzed by factor Xa were stimulated to different extents by the addition of the cofactor, factor Va. In addition, studies of the activation of bovine and human prothrombin catalyzed by factor Xa alone indicated that the conversion of prothrombin to thrombin exclusively produced prethrombin 2 as an intermediate (2). These data, in addition to the ordered conversion of prothrombin to thrombin determined when prothrombinase was used as the catalyst, indicated that the cofactor, factor Va, influenced the mechanism of the reaction catalyzed by factor Xa.

The data in Table I illustrate the influence of saturating levels of cofactor on the rate of the factor Xa-catalyzed cleavage of the individual bonds in prothrombin. As implied

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**Table I**

<table>
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<th>Substrate</th>
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<th>$K_a$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_a$</th>
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<td>Prothrombin A+B</td>
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<td>Prethrombin 2 plus</td>
<td>0.46 ± 0.05</td>
<td>13.5 ± 0.5</td>
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<tr>
<td>Fragment 1-2</td>
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</table>

* Bond cleaved indicates the bond(s) cleaved during the conversion of the substrate to $\alpha$-thrombin.

The kinetic constants and standard errors were obtained by nonlinear least-squares regression analysis of the data illustrated in Fig. 3.
by the results illustrated in Fig. 2, the overall conversion of prothrombin to thrombin is stimulated approximately 2500-fold by the inclusion of saturating levels of factor Va in the reaction mixture. The conversion of prethrombin 2 to thrombin (cleavage at Arg^273-Ile^274) is also stimulated approximately 2500-fold in the presence of saturating levels of factor Va. The conversion of meizothrombin to thrombin (cleavage at Arg^277-Thr^278) is, however, stimulated only 4.3-fold in the presence of factor Va. As the catalytic rates observed at saturating levels of factor Va are similar, the most significant difference in the rates of utilization of the three substrates is evident in the absence of the cofactor. Under these conditions, the rate of conversion of meizothrombin to thrombin (cleavage at Arg^273-Thr^274) proceeds 500-fold more rapidly than the rate of cleavage at Arg^322-Ile^323 (conversion of prethrombin 2 to thrombin) or the overall conversion of prothrombin to thrombin. The data indicate that, although there is no clearly identifiable rate-limiting step when the catalyst is composed of fully assembled prothrombinase, the cleavage at Arg^273-Ile^274 would be clearly rate-limiting in the absence of the cofactor. One prediction made by this interpretation is that the depletion of factor Va from the catalyst would alter the order of bond cleavage such that prothrombin would be converted to prethrombin 2 plus Fragment 1-2 relatively rapidly and then be protosylated further to thrombin at a much slower rate.

Fluorescence Emission Spectra for DAPA Complexed with Thrombin, Meizothrombin, and Prethrombin 2—DAPA displays differential fluorescence properties when complexed with thrombin, prethrombin 2, or meizothrombin (19). These differences in the fluorescence properties of DAPA have been exploited in order to determine the pathway of activation of bovine prothrombin. Fig. 6 illustrates uncorrected fluorescence emission spectra of human thrombin, prethrombin 2 plus Fragment 1-2, or meizothrombin complexed with DAPA at identical concentrations. The meizothrombin-DAPA complex is characterized by the greatest quantum yield at this excitation wavelength (280 nm). The quantum yield of the meizothrombin-DAPA complex, determined by integration over the emission spectrum, is approximately 2-fold greater than that observed for the thrombin-DAPA complex. This value is somewhat higher than the quantum yield difference observed using bovine proteins (15, 18). The prethrombin 2-DAPA complex is characterized by the lowest fluorescence intensity, with a quantum yield that is 4.3-fold lower than that observed for the thrombin-DAPA complex. In addition to the significant differences in intensity of these species, the emission maxima of the meizothrombin-DAPA and the prethrombin 2-DAPA complexes are significantly blue-shifted in relation to the emission maximum obtained with thrombin, indicating detectable differences in the microenvironment surrounding the fluorophore in the three complexes.

Progress Curves for the Activation of Prothrombin Catalyzed by Factor Xa or Prothrombinase—Fig. 7 illustrates continuous traces for the activation of prothrombin monitored by the fluorophore DAPA. Trace A was obtained after the reaction mixture composed of prothrombin, DAPA, and PCPS was initiated by the addition of 5 nM factor Va and 1 nM factor Xa (A) or 1 × 10^{-7} M factor Xa (B). Data were collected at intervals of 1 s with 10 readings averaged per datum with a time constant of 10 ms. The excitation wavelength was 280 nm (band pass, 4 nm) and the emission wavelength was 320 nm (band pass, 2 nm).
that indicated that meizothrombin would accumulate to signif-
ificant levels during the early phase of this reaction (see above). The appearance of the transient maximum is consid-
erably more dramatic than the maximum observed during kinetic studies of the activation of bovine prothrombin (15), partly because of the selection of the emission wavelength (520 nm) to maximize the difference in fluorescence intensity between the thrombin-DAPA and the meizothrombin-DAPA complexes. Trace B was obtained when an identical reaction mixture was initiated by the addition of 1 x 10^{-7} M factor Va alone, using the same instrumental settings used to obtain trace A. The progress curve obtained when factor Va was deleted from the catalyst was characterized by a burst phase during the initial 6 min of the reaction followed by a much slower increase in fluorescence intensity. The limiting fluo-
rescence intensities measured at 60 min following initiation were 1.19 for trace A and 1.21 for trace B and remained essentially unchanged when remeasured at 80 min. These data indicated that the terminal products of both reactions were identical, as detected by the formation of fluorescent adducts with DAPA. Furthermore, a readdition of an identical concentra-
tion of factor Va to reaction mixture B after 600 s following the first initiation did not result in a second burst of fluorescence intensity. Collectively, the data are consistent with the interpretation that prothrombin activation catalyzed by factor Va alone involves the initial, relatively rapid for-
mation of a less fluorescent species that is converted to thrombin at an extremely slow rate. Given the low fluo-
rescence intensity of the prethrombin 2-DAPA complex (see Fig. 6), the burst in the progress curve for prothrombin activation catalyzed by factor Va alone provides support for the predic-
tions made by the data illustrated in Table II.

Analysis of Prothrombin Activation by NaDodSO4-PAGE and Autoradiography—The activation of $^{125}$I-labeled pro-
thrombin was monitored by NaDodSO4-PAGE followed by autoradiography in order to evaluate the contribution of the two pathways toward prothrombin activation inferred from the traces illustrated in Fig. 7. The use of radiolabeled tracer was necessary in these experiments because of the relatively high concentrations of factor Va utilized to measure prothrombin activation in the absence of factor Va. The activation of prothrombin catalyzed by prothrombinase (factor Va and factor Va) is illustrated in Fig. 8, A (before disulfide reduction) and B (after reduction). As observed after Coom-
masie Blue staining (Fig. 4), only three species corresponding to prothrombin, thrombin, and Fragment 1-2 are visible prior to disulfide reduction. After reduction with 2-mercaptoethanol, the bands observed correspond to prothrombin, Fragment 1-2, thrombin-A chain; Fragment 1-2, thrombin-A chain and thrombin-B chain. In addition, a band identified as corresponding to Fragment 1-2-A chain appears transiently during the early portion of the reaction, exactly as is observed when the gels are stained with Coom-
masie Blue (see Fig. 4B). As several intermediates of pro-
thrombin activation stain poorly with Coommasie Blue (15), the absence of detectable levels of prethrombin 2 or prethrom-
bin-2-des-1-13 in these overexposed autoradiograms provides additional evidence for the conclusion that prothrombin ac-
tivation catalyzed by prothrombinase proceeds exclusively via meizothrombin as the intermediate species. The data illustrated in this figure also demonstrate that $^{125}$I-labeled pro-
thrombin is indistinguishable from unlabeled prothrombin in its ability to function as a substrate for prothrombinase.

The autoradiograms in Fig. 9, A and B, illustrate the fate of $^{125}$I-labeled prothrombin after the initiation of the activa-
tion process by the addition of 1 x 10^{-7} M factor Va alone. The experimental conditions used in this experiment were identical to those used to obtain trace B in Fig. 7. Prior to disulfide bond reduction, the identifiable species are pro-
thrombin and a band migrating at a position characteristic of either prethrombin 2 or Fragment 1-2. Following reduction by 2-mercaptoethanol (Fig. 9B), three bands are visible, cor-
responding to prothrombin, prethrombin 2, and Fragment 1-
2. The latter two species are resolved due to an alteration in the mobility of Fragment 1-2 in this gel system after treat-
ment with 2-mercaptoethanol. The relative amounts of pre-
thrombin 2 and Fragment 1-2 increased systematically during the time course of the experiment, with traces of thrombin appearing after 30 min. The data illustrated in Fig. 9 indicate

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<th>Substrate $^a$</th>
<th>Bond cleaved $^b$</th>
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<td></td>
<td>Factor Va $^d$</td>
<td>+ Factor Va $^d$</td>
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<td>mol factor IIa/min/mol factor Xa $^d$</td>
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<td>Prothrombin</td>
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$^a$ The reaction mixtures (2 ml) contained 1.4 μM substrate, 3 μM DAPA, 30 μM PCPS in 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 2.0 mM CaCl$_2$.

$^b$ The bands cleaved during the conversion of the substrate to pro-
thrombin are A = Arg$^{173}$-Thr$^{174}$ and B = Arg$^{193}$-Ser$^{194}$.

$^c$ Reaction mixtures containing prothrombin or prethrombin 2 plus Fragment 1-2 were initiated with 1 x 10^{-7} M factor Xa and mixtures containing meizothrombin were initiated with 2 x 10^{-9} M Xa.

$^d$ Reaction mixtures were initiated by the addition of 5 x 10^{-5} M factor Va followed by 1 x 10^{-8} M factor Xa.

$^e$ The rates ± S.E. represent the mean ± S.E. of three determinations.
DAPA, aliquots were withdrawn at the indicated times and processed by that, in the presence of factor Xa alone, prothrombin is 2.0 mM CaCl₂ with 10⁷ cpm ¹²⁵I-prothrombin added as a tracer. The raphy.

Then cleaved at Arg322-Ile323 at a much slower rate to yield analyzed by NaDodSO₄-PAGE, and followed by autoradiography.

The ability of the cofactor, factor Va, to influence differentially prothrombin exclusively via the formation of meizothrombin as reaction proceeded at a significantly lower rate (several orders of magnitude slower than in the presence of factor Va) via a pathway that produced prethrombin 2 (3, 8). The latter results have also been reported for the activation of human prothrombin with factor Xa alone as the catalyst (36). In addition, kinetic studies of the cleavage of the individual bonds in bovine prothrombin catalyzed by prothrombinase indicated that the kinetic constants for these two reactions were indistinguishable from each other and from those obtained for the overall conversion of prothrombin to thrombin (26). In agreement with the present results, further studies of the cofactor dependence of the individual bond cleavages in bovine prothrombin indicated that the conversion of meizothrombin to thrombin was modestly, if at all, dependent on the concentration of factor Va, whereas the conversion of prethrombin 2 plus Fragment 1-2 was highly dependent on the concentration of the cofactor.

Human prothrombin differs from the analogous protein isolated from bovine plasma in that it possesses an additional cleavage site at Arg²⁸⁶-Thr²⁸⁷ which has been identified as a site susceptible to cleavage by the feedback action of thrombin (5). During the course of the present work, sufficient levels of DAPA were used to prevent the feedback action of thrombin or meizothrombin during prothrombin activation, and no evidence was obtained for the formation of prethrombin 2 with 13 residues deleted from its amino terminus or Fragment 1-2 plus 13 residues. Recent results indicate that bovine factor Xa may be capable of cleaving human prothrombin at Arg²⁸⁶-Thr²⁸⁷ (35). When insufficient levels of DAPA were present during prothrombin activation catalyzed by factor Xa alone (Fig. 9), in addition to the formation of Fragment 1 and Fragment 2 (a result of the feedback cleavage at Arg³⁵⁶-Ser³⁵⁷), a distinct doublet was observed at the position of prethrombin 2. These data support the earlier conclusion that the cleavage of human prothrombin at Arg³⁵⁶-Thr³⁵⁷ results from the action of thrombin and not factor Xa.

Studies of the kinetics of activation of Gla-deficient variants of bovine prothrombin indicated that a decrease in the level of carboxylation from 10 (normal) to 7 Gla resulted in a decrease in the competence of the substrate (37). Furthermore, NaDodSO₄-PAGE analysis of the activation of the Gla-deficient prothrombins in that study revealed the presence of prethrombin 2 as an intermediate, whereas normal (10 Gla) prothrombin did not (37). It is not known whether Gla-deficient variants of human prothrombin behave in an analogous fashion. However, collectively, the data indicate that the reaction pathway is influenced by the cofactor and by the lipid-binding ability of the substrate.

The kinetic studies described in this paper provide a mechanistic basis for the apparent change in the order of the reaction when the cofactor is absent from the catalyst. These
data also provide an explanation for the results of other studies (38) that demonstrated that increasing amounts of prethrombin 2 were detected in the presence of factor Va and increasing concentrations of factor Xa during studies of prothrombin activation. These results can be rationalized on the basis of an increasing contribution due to the catalytic activity of free factor Xa in the presence of factor Va saturated with factor Xa, hence resulting in the detection of meizothrombin and increasing concentrations of prethrombin 2 as intermediates in the reaction. Collectively, the measured catalytic efficiency for the cleavage of the individual bonds catalyzed by prothrombin exclusively via meizothrombin is a consequence of the association of factor Xa with factor Va on the phospholipid surface.

REFERENCES