Prothrombinase cleaves prothrombin at Arg\(^{271}\) and Arg\(^{320}\) to produce thrombin. The kinetics of cleavage of five recombinant prothrombins were measured: wild-type prothrombin (WT-II), R155A/R284A/R271A prothrombin (rMZ-II), R155A/R284A/R320A prothrombin (rP2-II), S525C prothrombin labeled with fluorescein (WT-II-F\(^*\)), and R155A/R284A/R271A/S525C prothrombin labeled with fluorescein (rMZ-II-F\(^*\)), rMZ-II and rP2-II are cleaved only at Arg\(^{270}\) and Arg\(^{320}\), respectively, to yield the intermediates meizothrombin and prethrombin-2, respectively. WT-II-F\(^*\) and rMZ-II-F\(^*\) were labeled at Cys\(^{325}\) with fluorescein; cleavage was monitored by enhanced fluorescence. Activation kinetics of WT-II, rMZ-II, and rP2-II indicated that the catalytic efficiency of cleavage at Arg\(^{320}\) was increased by 30,000-fold by the cofactor factor Va, as was the conversion of prothrombin to thrombin. However, factor Va increased cleavage at Arg\(^{271}\) only by 34-fold. Although WT-II competitively inhibited cleavage of WT-II-F\(^*\), rMZ-II or rP2-II did not inhibit completely even at saturating concentrations. However, rMZ-II and rP2-II together inhibited WT-II-F\(^*\) cleavage competitively. Both WT-II and rMZ-II competitively inhibited rMZ-II-F\(^*\) cleavage, whereas rP2-II did not. A model of prothrombin activation that includes two equilibrating forms of prothrombinase, each recognizing one of the cleavage sites, is quantitatively consistent with all of the experimental observations. Therefore, we conclude that the kinetics of prothrombin activation can be described by a “ping-pong”-like mechanism.

The coagulation cascade culminates in the activation of prothrombin to thrombin. This reaction is carried out by the multicomponent complex prothrombinase, which comprises the serine protease factor Xa, the activated protein cofactor factor Va, calcium ions, and an appropriate cell membrane or phospholipid surface (1–5). Factor Xa alone can slowly generate thrombin in an extremely inefficient reaction; however, the rate of this reaction is enhanced by 5 orders of magnitude by incorporation of factor Va, Ca\(^{2+}\), and the procoagulant surface (5).

Two bond cleavages are required for thrombin formation, one at Arg\(^{271}\) and one at Arg\(^{320}\). Therefore, two activation pathways are possible during prothrombin activation, yielding the intermediates fragment 1.2-prethrombin-Z (F1.2:Pre2)\(^1\) and meizothrombin. Cleavage at Arg\(^{271}\) first produces the intermediate F1.2:Pre2, whereas cleavage at Arg\(^{320}\) first produces the intermediate meizothrombin. In the absence of factor Va, the accumulation of the intermediate F1.2:Pre2 is observed (6–8). In the presence of fully assembled prothrombinase, however, accumulation of the intermediate meizothrombin is observed (9), suggesting that factor Va directs the reaction toward the meizothrombin pathway.

The first objective of this study was to determine the effects of factor Va on cleavage at Arg\(^{271}\) and Arg\(^{320}\) in prothrombin. Three recombinant prothrombin derivatives were prepared for this purpose (see Fig. 1). These were WT-II, which can be cleaved at both Arg\(^{271}\) and Arg\(^{320}\) to produce thrombin (10); the derivative rMZ-II, which can be cleaved only at Arg\(^{320}\) to produce the intermediate meizothrombin (10), and the derivative rP2-II, which can be cleaved only at Arg\(^{271}\) to produce the intermediate F1.2:Pre2. These mutants, in addition to meizothrombin and F1.2:Pre2, allowed us to isolate each individual step of the overall reaction and to determine the catalytic efficiency of all bond cleavages involved in prothrombin activation.

Two additional recombinant prothrombin derivatives were used to carry out competition assays between each of the three recombinant prothrombin derivatives (see Fig. 1). The derivative WT-II-F\(^*\) is an active-site mutant of prothrombin in which the active-site serine was mutated to cysteine, which was subsequently labeled with fluorescein (11). The derivative rMZ-II-F\(^*\) is the rMZ-II derivative with the active-site serine-to-cysteine mutation labeled with fluorescein. Both of these mutants displayed an increase in fluorescence intensity upon activation and therefore allowed us to monitor their activation specifically in the presence of WT-II, rMZ-II, and rP2-II as competitors. As will be shown, the competition studies suggest that prothrombinase exists in two equilibrating forms, one catalyzing cleavage at Arg\(^{271}\) and the other at Arg\(^{320}\).

**EXPERIMENTAL PROCEDURES**

**Materials**

DNA restriction and modification enzymes were obtained from New England Biolabs Inc. (Mississauga, Ontario, Canada) or Invitrogen (Burlington, Ontario). Recombinant DNA polymerase from *Pyrococcus* sp. (Pfu) with proofreading activity, newborn calf serum, Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (1:1), Opti-MEM, pen-
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The DNA construction and mutagenesis—The cDNAs for rMZ-II (10) and for WT-II* (11) had been prepared previously in our laboratory. The cDNA for rMZ-II* was prepared as described previously for WT-II*+, except that the starting template was that for rMZ-II instead of WT-II, and the DNA polymerase used was Pfu. For the production of the rP-II cDNA, pBlueScript SK+ containing full-length rP-II cDNA was digested with HindIII, SacI, and PstI. The HindIII/SacI fragment spanning nucleotides 1076–1076 and the SacI/PstI fragment spanning nucleotides 1583 and 1583 of the T7 promoter primer (Stratagen) and 5′-CGGGCCGCTACCA-3′ with the T7 primer promoter (Stratagen) and 5′-TTGCAGGCTGACCCCTTTCC-3′ with the T3 promoter primer (Stratagen). The SacI/PstI fragment spanning nucleotides 1076–1583 was excised from the DNA sequence with the appropriate restriction endonuclease along with that for the newly introduced site. Digested fragments were ligated at their new restriction sites and subcloned into pBlueScript SK+. The presence of mutations and correct PCR amplifications were verified by DNA sequence analysis using the T3 and T7 promoter primers. The mutated fragments were ligated into the CDN vector pM2. The resulting cDNA was subcloned into the pNUT vector and prepared for expression as described previously (11). Cell culture, transfection, and selection was carried out as described previously (11).

Recombinant Protein Purification—Recombinant prothrombins were purified as described previously (11). Briefly, stored media were thawed at 4 °C and loaded onto XAD-2 (2.5 × 15 cm) and Q-Sepharose (4.0 cm × 8 cm) columns in tandem at either 4 or 21 °C. The XAD-2 column was washed with buffer medium. Absorbed prothrombinase in the Q-Sepharose was eluted from the Q-Sepharose with 0.02 M Tris-HCl and 0.5 mM NaCl (pH 7.4). Protein-containing fractions were identified using a Bio-Rad assay and pooled. Fluorescent labeling of mutants containing free cysteine residues was performed directly on this pooled fraction by adding a 30-fold molar excess of 5-iocoaactamidofluoresein to a 20 mM stock solution of 5-iocoaactamidofluoresein in N,N-dimethylformamide and incubating the sample for 2 h at room temperature in the dark. Prothrombin pools were subjected to barium citrate adsorption by the addition of sodium citrate to a final concentration of 0.025 M, followed by the addition of 1.0 M BaCl2 solution, with stirring, to a final concentration of 0.08 M. The solution was stirred at 4 °C for 1 h and centrifuged. The precipitating pellet was washed and solubilized as described previously (11). The sample was then dialyzed against 0.02 M Tris-HCl and 0.15 M NaCl (pH 7.4) and subjected to anion-exchange chromatography on a fast protein liquid chromatography Mono Q HR 5/5 column (Amersham Biosciences) at 4 °C. The protein was eluted with a 0–30 mM CaCl2 gradient in 0.02 M Tris-HCl and 0.15 mM NaCl (pH 7.4) (total volume of 30 ml; flow rate of 0.5 m/min). This was performed to separate protein that was fully γ-carboxylated (10). The first peak was pooled, and protein concentrations and labeling efficiencies (where applicable) were determined by absorbance readings at 280 and 495 nm, respectively.

Activation of Prothrombin Derivatives in the Presence of Factor Va—Substrates were activated at various concentrations in the presence of 20 nM rMZ-II and 5 mM DAPA in 0.2 M Tris-HCl, 0.5 mM CaCl2, and 0.01% Tween 80. Reactions were started at room temperature by the addition of 20 nM factor Va. In the cases of WT-II, rMZ-II, and F1.2:Pre2, reactions were allowed to proceed for 10 min, at which time an aliquot was removed and diluted into a 96-well plate that had been pretreated with 0.02 M Tris-HCl, 0.15 mM NaCl, and 1% Tween 80. Wells contained a sufficient volume of S2228 (H-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride) so that the final concentration was 400 µM. Absorbance at 405 nm was read over time in a SpectraMax Plus spectrophotometer (Molecular Devices) at room temperature. Initial rates of S2228 hydrolysis were determined and compared with a thrombin standard curve. In the case of rZ-II, aliquots of the diluted protein eluate were removed from activation reactions and diluted into 2 volumes of 0.2 M acetic acid. Aliquots were concentrated, and reduced samples were subjected to SDS-PAGE on 5–15% polyacrylamide minigels according to Neville (20). Densitometry was carried out to determine the amount of prethrombin-2 produced at each time point. In the case of meizothrombin activation, reactions were carried out in a 96-well plate that had been pretreated with 0.02 M Tris-HCl, 0.15% NaCl, and 1% Tween 80 and monitored in a Spectra Max Gemini fluorescence plate reader (Molecular Devices) at excitation and emission wavelengths of 280 and 545 nm, respectively, with a 515-nm emission cutoff filter in the emission beam. Initial rates of activation for all substrates were calculated and plotted versus the substrate concentration. The data are plotted as the mean ± S.D. at least three experiments. The kinetic constants were determined by nonlinear regression of the data to the Michaelis-Menten equation using the NONLIN module of SYSTAT (SPSS Inc., Chicago, IL).
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SDS-PAGE on 5–15% polyacrylamide minigels. Densitometry was carried out to determine the amount of prothrombin-2 produced at each point. Initial rates of activation for all substrates were calculated and plotted versus the concentration of substrate. The data are plotted as the means ± S.D. of at least three experiments. The $K_m$ and $k_{cat}$ values were determined by nonlinear regression.

**Time Courses of Prothrombin, Meizothrombin, and Thrombin during Prothrombin Activation—**Unlabeled prothrombin (1 μM) was activated in the presence of a trace amount of $^{125}$I-prothrombin (150,000 cpm) with 50 μM PCPS, 20 nM factor Va, 0.035 mM factor Xa, and 5 μM CaCl$_2$ in 0.02 M Tris-HCl, 0.15 M NaCl, and 0.01% Tween 80. The reaction was started at room temperature by the addition of factor Xa. Aliquots were removed from the reaction, diluted into 2 volumes of 0.2 M acetic acid, and concentrated. Reduced samples were subjected to SDS-PAGE on a 5–15% polyacrylamide gel. The gels were fixed in 50% methanol, 20% ethanol, and 6% trichloroacetic acid for 2 h and then stained with Coomassie Blue and destained. Bands were excised and counted in an Amersham Biosciences Mini gamma 1275 γ-counter. The experiment was performed twice, and three gels were analyzed. The data are plotted as the means ± S.D. of three results.

**Competition Experiments and Mathematical Modeling of Prothrombin Activation—**Studies of prothrombin activation indicated that the substrates rMZ-II and rP2-II only partially inhibited prothrombin activation (see below). This phenomenon could not be rationalized by any conceivable equilibrium model that involved only one form of prothrombinase because, in such a model, competing substrate at a sufficiently high concentration would occupy all of the enzyme, thereby completely inhibiting prothrombin activation. Therefore, the following model was constructed to account for partial inhibition by rMZ-II and rP2-II. The model includes two equilibrating forms of prothrombinase, designated $E$ and $E'$, as indicated in Equation 1.

$$k_1 \frac{E}{E'} \rightleftharpoons k_{-1} \frac{E'}{E} \quad (E.1)$$

One of these forms ($E$) catalyzes cleavage at Arg$^{220}$. Thus, it catalyzes conversion of prothrombin (P) to meizothrombin (M), F1.2:Pre2 (P) to thrombin (T), and rMZ-II (rMZ) to rMZ-IIa (rMIIa) and rMIIa. The other form ($E'$) catalyzes cleavage at Arg$^{225}$ and thereby catalyzes conversion of prothrombin to F1.2:Pre2, meizothrombin to thrombin, and rP2-II (rP2) to rP2-IIa (rP2a). In this model, once a particular form of the enzyme catalyzes its corresponding bond cleavage, it releases the product and reverts to the other form of the enzyme. In this sense, the model is similar to the classical “ping-pong” mechanism of enzyme kinetics. The proposition that $E$ and $E'$ can equilibrate is included in the model to account for the fact that rMZ-II, rP2-II, meizothrombin, and F1.2:Pre2 can be completely activated. This could not happen if the two forms of the enzyme could not interconvert spontaneously.

Each form of the enzyme is presumed to interact with its substrate (S) in an equilibrium binding interaction such that $[E][S] = K[E+S]$ and $[E'][S] = K[E'+S]$. The enzyme-substrate complexes then turn over to product at rate $r$, where $r = k_2[S/E]$ or $r = k_2[S/E']$. The term $k$ is the turnover number. Each reaction rate can therefore be expressed in terms of the free enzyme forms according to $r = (k/E)[E][S]$ or $r = (k/E')[E'][S]$. Each specific reaction is allowed its own $k$ and $β$ values. These are shown in Equations 2 and 3.

$$(P, P_2, rMZ) + E \rightleftharpoons (ES) \rightleftharpoons \frac{k_1}{k_{-1}} \frac{E}{E'} \quad (E.2)$$

$$(P, M, rP2) + E \rightleftharpoons (E'S) \rightleftharpoons \frac{k_1}{k_{-1}} \frac{E}{E'} \quad (E.3)$$

The rate of thrombin formation ($r$) is the sum of the rates of conversion of the intermediates meizothrombin and F1.2:Pre2 to thrombin (Equation 4).

$$r = (k_5/K_a)[P][M] + (k_5/K_a)[E'][P] \quad (E.4)$$

To express the rate of the reaction in terms of the prothrombin concentration, steady states with respect to the levels of the intermediates are assumed, such that their rates of formation from prothrombin and subsequent conversion to thrombin are equal. Thus, for meizothrombin, $(k_5/K_a)[P][E] = (k_5/K_a)[E'][M]$; and for prothrombin-2, $(k_5/K_a)[E'][P] = (k_5/K_a)[E][P]$. The rate of prothrombin activation then can be expressed in terms of the prothrombin concentration according to Equation 5.

$$r = (k_5/K_a)[P][M] + (k_5/K_a)[E'][P] \quad (E.5)$$

To further develop the rate equation, the conservation of enzyme is considered. Because some experiments were carried out in the presence of rMZ-II and rP2-II as competing substrates, the interactions of $E$ and $E'$ with them are also accounted for in the conservation equation. The total concentration of the enzyme ([E]$_p$) is given in Equation 6 and again in Equation 7, where all [E] or [E'] forms have been expressed in terms of [E], [E'], [S], and the appropriate $K$ values. rMZ-II and rP2-II are assumed to interact with $E$ and $E'$ with dissociation constants $K_a$ and $K_r$.

$$[E]$ = $[E] + [E'] + [E'][M] + [E']rMZ + [E']$ + [E']rP2 \quad (E.6)$$

$$[E]' = [E] + [E'][P] + [E'][E']/K_a [E']$$

$$[E]' = [E] + [E'][P] + [E']/rMZ/K_a [E'] + [E']/rP2/K_r$$ \quad (E.7)

The steady states in meizothrombin and F1.2:Pre2 are again invoked to eliminate terms containing them from the conservation equation. Thus, $[M][K] = (k_5/K_a)[E][E']$ and $[E'][P] = (k_5/K_a)/[E'][P]$. The conservation of enzyme is then given according to Equation 8.

$$[E]' = [E](1 + (1 + k_5/K_a)[P] + [E'][M] + [E']rMZ + [E']rP2) + [E]'$$

Division of Equation 5 by Equation 8 and then division of the numerator and denominator of the resulting equation by [E] yield Equation 9, which gives the rate per total concentration of enzyme.

$$r[E]_{E'} = \frac{(k_5/K_a) + (k_5/K_a)[E'][E']/P}{1 + (1 + k_5/K_a)[P] + [rMZ][E'] + [rP2][K_r][E']]} \quad (E.9)$$

The ratio $[E']/[E]$ in Equation 8 can be eliminated by assuming a steady state in $E$ (and therefore $E'$). Thus, the rates of formation and removal of $E$ are presumed equal. This is expressed in Equation 10.

$$K_a [E'][P] + K_a [E']rMZ + k_5[E] = \frac{k_5[E']}{K_a}$$

Division of Equation 5 by Equation 8 and then division of the numerator and denominator of the resulting equation by [E] yield Equation 9.

$$[E']/[E] = \frac{(k_5/K_a)[rMZ] + k_5[E]}{(k_5/K_a)[rP2] + k_5[E]} \quad (E.11)$$

Equation 11 is then solved for the ratio $[E']/[E]$, which is given in Equation 12.

$$[E']/[E] = \frac{(k_5/K_a)[rMZ] + k_5[E]}{(k_5/K_a)[rP2] + k_5[E]} \quad (E.12)$$

The right-hand side of Equation 12 is substituted for $[E']/[E]$ in Equation 9. When this is done, Equation 13 is the result, with the terms $k_{cat}$, $α_1$, $α_2$, $α_3$, $α_4$, $b_5$, $b_6$, $b_7$, $b_8$, $c$, and $β$ given in Equations 14–23.

$$r[E]_{E'} = \frac{k_{cat}[P] + b_1[P][rMZ] + b_1[P][rP2]}{K_a + [P] + (α_2 + α_3)[P][rMZ] + (b_2 + b_3)[P][rP2] + c[rMZ][rP2]} \quad (E.13)$$

$$k_{cat} = k(-k_1/K_a)/(k_1/K_a + k_3/K_3) \quad (E.14)$$

$$K_a = (k_1 + k_3/K_a)β \quad (E.15)$$

$$α_1 = k_5/K_a/K_a/β \quad (E.16)$$

$$α_2 = K_a(k_5/K_a + k_5/K_a)β \quad (E.17)$$

$$α_3 = k_5/K_a(k_5/K_a + k_5/K_a)β \quad (E.18)$$

$$b_5 = k_5/K_a(K_a + k_5/K_a)β \quad (E.19)$$
Equation 24 and 30 show that the activation kinetics of rMZ-II and rP2-II, respectively, are given in Equation 30, with

\[ \frac{r}{[E_f]} = \frac{k_{cat}[rMZ]}{K_m + [rMZ][rP2]} \]

(Eq. 24)

\[ K_m = K_a(K_1 + h_2)/K_1 \]

(Eq. 25)

\[ g_1 = k_{cat}h_2K_a/K_1 + h_2 \]

(Eq. 26)

\[ g_2 = (K_2/K_1)(h_1 + h_2)/K_1 + h_2 \]

(Eq. 27)

\[ g_3 = (h_1 + h_2)/K_1 + h_2 \]

(Eq. 28)

Similarly, the rate of activation of rP2-II with rMZ-II as a competing substrate is given in Equation 30, with

\[ \frac{r}{[E_f]} = \frac{k_{cat}[rP2]}{K_m + [rP2][rMZ]} \]

(Eq. 30)

\[ k_{cat} = k_{cat}h_1/K_1 + h_2 \]

(Eq. 31)

\[ K_m = K_a(K_1 + h_1)/K_1 + h_2 \]

(Eq. 32)

\[ h_1 = k_{cat}h_2K_a/K_1 + h_2 \]

(Eq. 33)

\[ h_2 = (K_2/K_1)(h_1 + h_2)/K_1 + h_2 \]

(Eq. 34)

\[ h_3 = (h_1 + h_2)/K_1 + h_2 \]

(Eq. 35)

Equations 24 and 30 show that the activation kinetics of rMZ-II and rP2-II, each in the absence of the other, will conform to the Michaelis-Menten equation

\[ \frac{r}{[E_f]} = \frac{k_{cat}[S]}{K_m + [S]} \]

with \( k_{cat} \) and \( K_m \) values given in Equations 25, 26, 31, and 32. The forms of Equations 24 and 30 also predict that rMZ-II and rP2-II will only partially inhibit the activation of each other, just as they only partially inhibit the activation of prothrombin. In addition, the activation rate of either of the substrates in the presence of the competitor will depend on the concentration of the substrate, as is predicted to occur in prothrombin activation.

The rate equations for activation of meizothrombin and F1.2:Pre2 to thrombin are found also by analogous reasoning. The rate equation for meizothrombin activation is given in Equation 36, with \( k_{cat} \) and \( K_m \) given in Equations 37 and 38.

\[ \frac{r}{[E_f]} = \frac{k_{cat}[M]}{K_m + [M]} \]

(Eq. 36)

\[ k_{cat} = k_{cat}h_1/K_1 + h_2 \]

(Eq. 37)

\[ K_m = K_a(K_1 + h_1)/K_1 + h_2 \]

(Eq. 38)

The rate equation for activation of F1.2:Pre2 to thrombin is given in Equation 39, with \( k_{cat} \) and \( K_m \) values given in Equations 40 and 41.

\[ \frac{r}{[E_f]} = \frac{k_{cat}[P2]}{K_m + [P2]} \]

(Eq. 39)

\[ k_{cat} = k_{cat}h_1/K_1 + h_2 \]

(Eq. 40)

\[ K_m = K_a(K_1 + h_1)/K_1 + h_2 \]

(Eq. 41)
The increase in catalytic efficiency of each cleavage upon the addition of factor Va was also calculated. Efficiency of cleavage for the overall conversion of prothrombin to thrombin was enhanced by 39,700-fold by factor Va. Cleavage at Arg^{271} was enhanced by 20,000-fold with rMZ-II as the substrate) or by 27,200-fold (with F1.2:Pre2 as the substrate), whereas cleavage at Arg^{271} was enhanced only by 453-fold (with rP2-II as the substrate) or by 34-fold (with meizothrombin as the substrate). Therefore, cleavage at Arg^{271} is not nearly as dependent upon factor Va as cleavage at Arg^{270}.

**Activation of 125I-Prothrombin in the Presence of Factor Va**—Prothrombin that had been labeled with 125I was activated in the presence of factor Va and subjected to SDS-PAGE. The time courses of prothrombin, meizothrombin, and thrombin during the reaction are shown in Fig. 3. Fig. 3 illustrates that the decline in the concentration of prothrombin during the reaction was nearly first-order at this prothrombin concentration. The intermediate meizothrombin first increased to a maximum at 6 min, followed by a steady decline. The intermediate F1.2:Pre2 was not observed. The thrombin concentration in the first 300 s is shown in the inset and appeared to rise immediately upon initiation of the reaction, without the lag that is characteristic of a mechanism with an obligatory intermediate. Because this lag did not occur, the time course of thrombin formation suggests that some direct conversion of prothrombin to thrombin occurred without equilibration of free intermediates with the prothrombinase complex, thereby indicating a channeling phenomenon (19). In addition, these data indicate that the catalytic efficiency for conversion of prothrombin to meizothrombin is much larger than that for conversion of prothrombin to F1.2:Pre2. This can be appreciated by comparing the initial rates of meizothrombin and thrombin accumulation. The initial rate of meizothrombin accumulation was less than or equal to the initial rate of meizothrombin formation from prothrombin. Because F1.2:Pre2 did not accumulate, the initial rate of thrombin formation was greater than or equal to the rate of F1.2:Pre2 formation from prothrombin. Because the initial rate of meizothrombin accumulation was much greater than that of thrombin accumulation, the catalytic efficiency of cleavage at Arg^{270} in prothrombin must be considerably greater than that of cleavage at Arg^{271}. This is in contrast to the nearly identical catalytic efficiencies measured with rMZ-II and rP2-II.

**Activation of WT-II-F* and rMZ-II-F* in the Presence of WT-II, rMZ-II, and rP2-II**—The prothrombin derivative WT-II-F* is an active-site mutant of prothrombin in which the active-site serine was mutated to a cysteine, which was subsequently labeled with fluorescein. The prothrombin derivative rMZ-II-F* is the rMZ-II mutant with the same active-site mutation. Both mutants displayed an increase in fluorescence intensity upon activation and therefore allowed us to monitor their activation specifically in the presence of WT-II, rMZ-II, and rP2-II as competitors. Fig. 4 illustrates the inhibition of WT-II-F* activation observed in the presence of WT-II, rMZ-II, or rP2-II at various concentrations. Simple competitive inhibition was observed when WT-II-F* was activated in the presence of WT-II. The substrates rMZ-II and rP2-II did not, however, exhibit simple competitive inhibition when activated in the presence of WT-II-F*. In both cases, inhibition was not complete, even at saturating levels of competitor. In addition, the extent of inhibition was dependent upon the starting concentration of WT-II-F*. However, when both rMZ-II and rP2-II were added together as competitors, inhibition was competitive. As illustrated in Fig. 5, cleavage of rMZ-II-F* was inhibited competitively by both WT-II and rMZ-II. However, inhibition by rP2-II was not complete, and the extent of inhibition was dependent upon the starting concentration of rMZ-II-F*.
These data are consistent with a ping-pong-like model of kinetics in which prothrombinase exists in two equilibrating forms, each specific for a unique site in prothrombin. This model is mathematically outlined under “Experimental Procedures.” In Figs. 4 and 5, the lines on the graphs correspond to global fits of the data by nonlinear regression to the appropriate rate equations. The data of Fig. 4B (with rP2-II as the inhibitor) were fit to Equation 13 with \( [rMZ-II] \) as the inhibitor and \( [rP2-II] \) as the inhibitor. The values obtained upon nonlinear regression to the appropriate rate equations are as follows:

\[
\begin{align*}
\frac{k_{cat}}{K_m} & = \frac{k_{cat}}{K_m} \\
\frac{k_{cat}}{K_m} & = \frac{k_{cat}}{K_m} \\
\frac{k_{cat}}{K_m} & = \frac{k_{cat}}{K_m} \\
\frac{k_{cat}}{K_m} & = \frac{k_{cat}}{K_m} \\
\end{align*}
\]

The k\(_{cat}/K_m\) values for prothrombin activation are given by the ratio of Equations 14 and 15, \( \frac{k_{cat}}{K_m} = \frac{k_{cat}}{K_m} \) for prothrombin as indicated by the above equations, would be expected to equal the sum for the prothrombin derivatives, which is 0.513. However, the experimentally determined value (0.262) is only about one-half of the expected value. Thus, the intrinsic catalytic efficiency of cleavage at one or possibly both of the bonds in prothrombin is not equal to that for cleavage at the same bonds in the prothrombin derivatives.

That this is so is evident in the data of Fig. 3. As shown, meizothrombin was clearly produced and transiently accumulated, but F1.2:Pre2 did not. If the k\(_{cat}/K_m\) values for both cleavages were approximately equal, as they are in the derivatives, one would expect F1.2:Pre2 to accumulate with a time course similar to that of meizothrombin. Even channeling through the F1.2:Pre2 pathway would not account for the lack of this intermediate because if channeling were to occur so that F1.2:Pre2 did not accumulate, the initial rate of thrombin formation would equal or exceed the rate of meizothrombin formation, which clearly is not the case. Thus, the difference in catalytic efficiencies of cleavages at Arg\(^{271}\) and Arg\(^{320}\) in prothrombin compared with the derivatives appears to lie with cleavage at Arg\(^{271}\). If one assumes that cleavage at Arg\(^{271}\) in prothrombin proceeds with the same efficiency as it does with the derivatives, one can calculate the intrinsic catalytic efficiency of cleavage at Arg\(^{271}\) in prothrombin relative to that in the derivatives. Using the above equations to eliminate \( k_{cat}/k_{cat} + k_{cat} \) and \( k_{cat}/k_{cat} \) yields \( \frac{k_{cat}}{K_m} = \frac{k_{cat}}{K_m} \) (where the subscript d designates the derivative).

Similarly, according to Equations 25, 26, 40, and 41, the k\(_{cat}/K_m\) ratios for cleavage at the Arg\(^{320}\) bond in F1.2:Pre2 and rMZ-II are \( \frac{k_{cat}}{k_{cat} + k_{cat}} \). The average k\(_{cat}/K_m\) values for the respective cleavages at the two bonds in the prothrombin derivatives are thus \( \frac{k_{cat}}{k_{cat}} \) for cleavage at Arg\(^{271}\) and \( \frac{k_{cat}}{k_{cat}} \) for cleavage at Arg\(^{320}\). However, the experimentally determined value (0.262) is only about one-half of the expected value. Thus, the intrinsic catalytic efficiency of cleavage at one or possibly both of the bonds in prothrombin is not equal to that for cleavage at the same bonds in the prothrombin derivatives.

Comparison of the Catalytic Efficiencies of Cleavage at the Arg\(^{271}\) and Arg\(^{320}\) Bonds in Prothrombin With Those in Meizothrombin, Prothrombin-2, rMZ-II, and rP2-II—According to Equations 31, 32, 37, and 38, the k\(_{cat}/K_m\) ratios (Table I) for cleavage at the Arg\(^{271}\) bond in meizothrombin and rP2-II are given by \( \frac{k_{cat}}{k_{cat} + k_{cat}} \) and \( k_{cat}/k_{cat} \) for rMZ-II are as follows:

\[
\begin{align*}
\frac{k_{cat}}{K_m} & = \frac{k_{cat}}{K_m} \\
\frac{k_{cat}}{K_m} & = \frac{k_{cat}}{K_m} \\
\frac{k_{cat}}{K_m} & = \frac{k_{cat}}{K_m} \\
\frac{k_{cat}}{K_m} & = \frac{k_{cat}}{K_m} \\
\end{align*}
\]
0.252/0.262 = 0.938, i.e. the intrinsic catalytic efficiency of cleavage at Arg271 in prothrombin is only 3.8% of that of cleavage at Arg271 in meizothrombin and rP2-II. Why this should be the case is not revealed by these studies. These studies suggest, however, that in the activation of prothrombin occurs first to form meizothrombin. This is an event that, in effect, creates the second cleavage site at Arg 271. This site is then cleaved with an efficiency roughly equal to that of the intrinsic catalytic efficiency of prothrombin. The relevant equations are as follows:

\[ k_{cat(271)} = k_{cat}(320) \cdot \frac{k_{cat(271)}}{k_{cat(320)}} \]

Estimation of the Rate Constants for Bond Cleavage and for Equilibration of the Two Forms of Prothrombinase—Analysis of the \( k_{cat}/K_m \) ratios above indicates that the intrinsic catalytic efficiency (k/K) of cleavage at Arg271 in prothrombin is only 3.8% of that of cleavage at Arg271 in prothrombin and at Arg271 or Arg320 in the prothrombin derivatives. If the further simplifying assumptions are made that the low catalytic efficiency is due to a low turnover (low k) rather than weak binding (high K) and that all other k and K values are the same, then the rate constant for bond cleavage (k) and the rate constants for the E and E’ interconversions (k_1 and k_-1) can be calculated. These can be calculated from the \( k_{cat} \) values for cleavage at Arg320 (average \( k_{cat} \) for cleavage of rMZ-II and F1.2.Pree2) and Arg271 (average \( k_{cat} \) for cleavage of rP2-II and meizothrombin) and for cleavage of prothrombin. The relevant equations are as follows:

\[ k_{cat(320)} = k_{cat(271)} \cdot \frac{k_{cat(271)}}{k_{cat(320)}} \]

0.252/0.262 = 0.938, i.e. the intrinsic catalytic efficiency of cleavage at Arg271 in prothrombin is only 3.8% of that of cleavage at Arg271 in meizothrombin and rP2-II. Why this should be the case is not revealed by these studies. These studies suggest, however, that in the activation of prothrombin occurs first to form meizothrombin. This is an event that, in effect, creates the second cleavage site at Arg 271. This site is then cleaved with an efficiency roughly equal to that of the intrinsic catalytic efficiency of prothrombin. The relevant equations are as follows:

\[ k_{cat(271)} = k_{cat(320)} \cdot \frac{k_{cat(271)}}{k_{cat(320)}} \]

Estimation of the Rate Constants for Bond Cleavage and for Equilibration of the Two Forms of Prothrombinase—Analysis of the \( k_{cat}/K_m \) ratios above indicates that the intrinsic catalytic efficiency (k/K) of cleavage at Arg271 in prothrombin is only 3.8% of that of cleavage at Arg271 in prothrombin and at Arg271 or Arg320 in the prothrombin derivatives. If the further simplifying assumptions are made that the low catalytic efficiency is due to a low turnover (low k) rather than weak binding (high K) and that all other k and K values are the same, then the rate constant for bond cleavage (k) and the rate constants for the E and E’ interconversions (k_1 and k_-1) can be calculated. These can be calculated from the \( k_{cat} \) values for cleavage at Arg320 (average \( k_{cat} \) for cleavage of rMZ-II and F1.2.Pree2) and Arg271 (average \( k_{cat} \) for cleavage of rP2-II and meizothrombin) and for
Prothrombin Activation by Two Forms of Prothrombinase

Fig. 5. Activation of rMZ-II-F* in the presence of WT-II, rMZ-II, and rP2-II. rMZ-II-F* at 100 nM (●), 200 nM (○), 300 nM (▲), 400 nM (◇), 500 nM (■), 600 nM (□), and 700 nM (▲) was activated in the presence of WT-II (WT-II; A), rP2-II (B), and rMZ-II (C) at various concentrations. Reactions were carried out in the presence of 50 μM PCPS, 20 nM factor Va, and 5 μM DAPA in 0.02 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, and 0.01% Tween 80. Reactions were treated as described in the legend to Fig. 4. Initial rates of rMZ-II-F* activation were calculated and plotted versus the concentration of inhibitor. The lines correspond to global fits of the data to the appropriate rate equations as outlined under “Experimental Procedures.” For competition of rP2-II against rMZ-II-F*, see Equation 24.

II and [rP2-II] in the numerator and therefore describes simple competitive inhibition of prothrombin activation by rMZ-II and rP2-II.

DISCUSSION

Two recombinant prothrombins labeled with fluorescein were utilized in these studies. Both mutants displayed an increase in fluorescence intensity upon activation. Their activation was monitored specifically in the presence of WT-II, rMZ-II, and rP2-II as competitors. Although WT-II was a competitive inhibitor of WT-II-F*, rMZ-II or rP2-II did not inhibit cleavage of WT-II-F* completely, and the extent of inhibition was dependent upon the starting concentration of WT-II-F*. When both rMZ-II and rP2-II were added together, however, the inhibition observed was competitive. In the case of rMZ-II-F*, both WT-II and rMZ-II behaved as competitive inhibitors, whereas rP2-II did not. Numerous models of prothrombin activation kinetics were investigated to rationalize these data. No model that included only a single form of prothrombinase was consistent with the data. The data instead were consistent with a ping-pong-like model of kinetics in which prothrombinase exists in two equilibrating forms, each specific for a unique site in prothrombin (Fig. 6). The form of the enzyme that cleaves at Arg²⁰⁰ has been designated E, and the form that cleaves at Arg²⁷¹ has been designated E’. This model is like the classical ping-pong model in that two enzyme forms are involved, and the enzyme is converted to its alternate form after a catalytic event. However, the model differs from the ping-pong model in that the two forms of the enzyme spontaneously interconvert in the absence of substrate. This is so because if cleavage had to occur to convert E to E’ or vice versa, activation reactions containing rP2-II or rMZ-II alone, with the substrate in excess over the enzyme, would not go to completion, as they did in these studies.

Ping-pong-type kinetics have been identified in several other mammalian systems. Some examples include cholesterol oxidase, which is a monomeric flavoenzyme that catalyzes the oxidation and isomerization of cholesterol to cholest-4-en-3-one. Two forms of the enzyme have been identified, and the kinetics of the reaction have been shown to follow a ping-pong mechanism (22). Lipopamide dehydrogenase is a flavoprotein that catalyzes the reversible oxidation of hydrolipoamide by NAD⁺, also through a ping-pong kinetic mechanism (23). NADPH-cytochrome P450 oxidoreductase is a membrane-bound protein that is associated with the endoplasmic reticulum and nuclear envelope of most eukaryotic cells and that transfers electrons from NADPH to FAD and then to any number of cytochromes P450 through a nonclassical (two-site) ping-pong mechanism (24).

In the case of prothrombinase, other published data suggest the existence of two forms of the enzyme. The actual cleavage sites in prothrombin are spatially distinct, being separated by as much as 36 Å (25), with one site requiring extensive reorganization before factor Xa can dock (26). Thus, separate forms of the enzyme may be required to cleave these two sites. The ability of factor Xα to cleave the two sites is affected differently by both factor Va (21) and PCPS (27). Rapid kinetics, stopped-flow studies by Walker and Krishnaswamy (27) suggest that two distinct types of enzyme-substrate interactions are required for the two cleavage sites involved in prothrombin activation. Additionally, factor Xα has been shown to behave differently both as an enzyme (1–5) and as a target for antithrombin/heparin (11, 28–36) when it is complexed with some or all of the prothrombinase components. For example, the half-life of factor Xα in the presence of antithrombin and heparin is increased by >100-fold when factor Xα is incorporated into the prothrombinase complex in the presence of prothrombin. Although this protection is profound, it is not complete. Conceivably, this protection could be due to the dynamics of the equilibrium between E and E’, with one form being more susceptible to inhibition than the other.

In the absence of factor Va, cleavage at Arg²⁷¹ was ~50-fold more efficient than cleavage at Arg²⁰⁰. Thus, cleavage at Arg²⁰⁰ appears to be the rate-limiting step. Additionally, the catalytic efficiency of cleavage at Arg²⁷¹ in meizothrombin was 10-fold
greater than that in rP2-II. These data rationalize the accumulation of the intermediate F1.2:Pre2 and the lack of detectable meizothrombin accumulation when prothrombin is activated in the absence of factor Va.

In the presence of factor Va, the estimated values for the catalytic efficiencies of cleavage at Arg	extsuperscript{320} and Arg	extsuperscript{271} in prothrombin are 0.0961 × 10	extsuperscript{9} and 2.52 × 10	extsuperscript{9} s	extsuperscript{-1}, respectively. The value for cleavage at Arg	extsuperscript{320} in prothrombin, in which the bond at Arg	extsuperscript{271} is intact, is very similar to that for the cleavage at Arg	extsuperscript{320} has been cleaved. In contrast, the catalytic efficiency of cleavage at Arg	extsuperscript{271} is not intact. Thus, the catalytic efficiency of cleavage at Arg	extsuperscript{320} does not depend on whether the bond at Arg	extsuperscript{271} has been cleaved. In contrast, the catalytic efficiency of cleavage at Arg	extsuperscript{271} in meizothrombin (2.3 × 10	extsuperscript{9} s	extsuperscript{-1}), in which the bond at Arg	extsuperscript{320} has been cleaved, is 24-fold greater than the estimated value for cleavage at the same bond in prothrombin, in which the bond at Arg	extsuperscript{320} is intact. This suggests that, in prothrombin, with factor Va as a component of prothrombinase, the bond at Arg	extsuperscript{271} is not readily available for cleavage until after the bond at Arg	extsuperscript{320} is cleaved. Curiously, the Arg	extsuperscript{271} bond in rP2-II is cleaved with high efficiency (2.91 × 10	extsuperscript{9} s	extsuperscript{-1}). However, this mutant has an alanine rather than an arginine residue at position 320. Perhaps it interacts with prothrombinase in a way that had the bond at position 320 been cleavable, it would have been cleaved. Although this bond was not cleaved because it could not be, perhaps the interaction with prothrombinase was sufficient to produce high efficiency cleavage at Arg	extsuperscript{271}. A similar phenomenon exists in the absence of factor Va. In this case, the catalytic efficiency of cleavage at Arg	extsuperscript{271} in rMZ-II (1.5 × 10	extsuperscript{4} s	extsuperscript{-1}), in which the bond at Arg	extsuperscript{271} is intact, is very similar to the value for cleavage at this bond in F1.2:Pre2 (7.3 × 10	extsuperscript{3} s	extsuperscript{-1}), in which the bond at Arg	extsuperscript{271} is not intact. In contrast, the catalytic efficiency of cleavage at Arg	extsuperscript{271} in meizothrombin (6.76 × 10	extsuperscript{5} s	extsuperscript{-1}), in which the bond at Arg	extsuperscript{271} is not intact, is 11-fold greater than that of cleavage at this bond in rP2-II (0.64 × 10	extsuperscript{5} s	extsuperscript{-1}), in which the bond at Arg	extsuperscript{271} is intact. Thus, either with or without factor Va, the catalytic efficiency of cleavage at Arg	extsuperscript{271} appears to be minimally affected by the state of cleavage at Arg	extsuperscript{271}. In contrast, the catalytic efficiency of cleavage at Arg	extsuperscript{271} is strongly influenced by the state of cleavage at Arg	extsuperscript{320}, such that it increases by about an order of magnitude if the bond at Arg	extsuperscript{320} is cleaved. In the presence of factor Va, the catalytic efficiency of cleavage at Arg	extsuperscript{271} is nearly identical to that at Arg	extsuperscript{320} once cleavage at Arg	extsuperscript{320} has occurred. This has also been inferred by Krishnaswamy et al. (21).

Factor Va enhances the catalytic efficiency of prothrombin activation by a factor of 39,700 compared with that obtained with factor Xa, PCPS, and Ca	extsuperscript{2+} only. This effect is not expressed equivalently for the two bond cleavages. The magnitude of the effect on cleavage at Arg	extsuperscript{320} ranges from 20,600- to 27,200-fold, depending on whether rMZ-II or F1.2:Pre2, respectively, is the substrate. In contrast, the magnitude of the effect on cleavage at Arg	extsuperscript{271} ranges from only 34- to 453-fold, depending on whether meizothrombin or rP2-II, respectively, is the substrate. Taking F1.2:Pre2 and meizothrombin as the natural substrates for prothrombinase, the best estimates for the enhancement of catalytic efficiencies by factor Va are 27,000-fold for cleavage at Arg	extsuperscript{320} and 34-fold at Arg	extsuperscript{271}.

Prothrombin interacts with prothrombinase at exosites that are separate from the active site of factor Xa. These exosites may mediate substrate recognition and/or cleavage (37-41). In addition, x-ray crystallographic studies of prethrombin-2 indicate that the residues preceding the Arg	extsuperscript{220}-Ile	extsuperscript{221} bond require extensive rearrangement to be successfully docked into the active site of factor Xa (26). Such features are not observed for the identical residues preceding the Arg	extsuperscript{274}-Thr	extsuperscript{275} bond in bovine meizothrombin-des-F1 (25). If incorporation of factor Va into the prothrombinase complex is necessary to reorder the residues preceding Arg	extsuperscript{220} in human prothrombin, perhaps factor Va disproportionately enhances cleavage at Arg	extsuperscript{220}, ultimately shifting the equilibrium toward the meizothrombin pathway.

Acknowledgments—We thank Reg Manuel and Angela Ward for excellent technical assistance with mammalian tissue culture and human protein purification and Tom Abbott for assistance with computing and graphics.

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