Further Evidence for Two Functional Forms of Prothrombinase Each Specific for Either of the Two Prothrombin Activation Cleavages*

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Previous work showed that prothrombin derivatives cleavable only at Arg-320 (rMZ) or Arg-271 (rP2) are partial, rather than competitive, inhibitors of prothrombin activation by prothrombinase. A “ping-pong”-like model, which posits two equilibrating forms of prothrombinase, explained the inhibition pattern. The present studies were undertaken to further investigate this putative mechanism. Two models were developed, one allowing for one form of the enzyme and the other allowing for two forms. Both models also allowed channeling and ratcheting. The models were fit to full time courses of prothrombin, meizothrombin, prethrombin-2, and the B-chain. In the absence of ratcheting and channeling, neither model fits the data. In their presence, however, both models fit very well, and thus they could not be distinguished. Therefore, inhibition of rMZ activation by rP2 was studied. Inhibition was partial and the two-form model fits the data with randomly distributed residuals, whereas the one-form model did not. Initial rates of fluorescein-labeled prothrombin cleavage in the presence of various prothrombin derivatives reported by Brufatto and Nesheim (Brufatto, N., and Nesheim, M. E. (2003) J. Biol. Chem. 278, 6755–6764) were also analyzed using the two models. The two-form model fit the partial inhibition data well, whereas the one-form model did not. In addition, prothrombin at varying concentrations was activated, and subsequently, the initial rates were plotted with respect to the initial prothrombin concentration. When compared with the expected initial rates as determined by the simulation of the models, the two-form model fit the observed rates better than the one-form model. The results obtained here further support the existence of two functional forms of prothrombinase.

Prothrombinase is a multicomponent enzymatic complex that catalyzes the conversion of prothrombin to the blood clotting enzyme thrombin (1–4). It is composed of the serine protease factor Xa, the cofactor factor Va, a negatively charged phospholipid surface, and calcium ion.

Prothrombin is activated to thrombin by two factor Xa-catalyzed cleavages, one at Arg-320 and the other at Arg-271 (5, 6). Because two cleavages are required for activation, two intermediates are possible. Cleavage at Arg-320 first produces the intermediate meizothrombin, whereas cleavage at Arg-271 first produces the pair fragment 1.2 and prethrombin-2.

Factor Xa alone is sufficient to activate prothrombin, but the process is remarkably slow. The combination of factor Va, calcium ion, and phospholipid increases the rate of activation by about a factor of 270,000 (7, 8). In the absence of factor Va, the preferred pathway is through the prethrombin-2 intermediate. In the presence of factor Va, however, the preferred pathway is through the meizothrombin intermediate (9–11).

Many studies have been carried out to determine the mechanism by which factor Va enhances the rate of activation. Earlier studies indicated that it facilitates the association of factor Xa with the surface, thereby lowering the $K_m$ value for prothrombin activation (7, 12–14). It also enhances the turnover number for prothrombin activation by a factor of about 3000 (7, 8). Several groups have shown that factor Va interacts with factor Xa (13, 15–17). Interactions between factor Va and prothrombin have also been characterized (17–20). The heavy chain of factor Va interacts with the fragment 2 domain of prothrombin (21, 22). More recently, Anderson et al. (23) inferred an interaction between factor Va and proexosite I of prothrombin. In addition, Beck et al. (24) have shown that amino acid residues 695–699 are very important for optimum prothrombinase function, presumably because they mediate a prothrombin-factor Va interaction. This conclusion, however, is currently at odds with the data of Tosos and Camire (25) who found that truncated mutants of factor Va lacking these residues were fully functional. Thus, the role of these residues in prothrombinase function is not fully resolved. In addition to these, prothrombin activation appears to involve an exosite for prothrombin on factor Xa, which becomes available upon interaction of factor Xa with factor Va (26–29).

Previous studies have shown that prothrombin activation can involve channeling whereby prothrombin is directly converted to thrombin without release of the intermediates (30). This mechanism is not exclusive in that intermediates are clearly released during the reaction. In addition, recent work by Bianchini et al. (31) has shown that ratcheting during prothrombin activation occurs, i.e. following cleavage at Arg-320, the intermediate meizothrombin spontaneously changes con-
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were prepared as described previously (35). Human prothrombin (36), thrombin (37), factor Xa (36), and factor Va (38) were prepared as described previously. The quality of factor Va was assessed by titration with factor Xa and by SDS-PAGE (supplemental Figs. S4 and S5).

Cell Culture and Recombinant Protein Purification—Baby hamster kidney cells transfected with rMZ-cDNA or rP2-cDNA in pNUT vector were cultured in Dulbecco’s modified Eagle’s medium/F-12 nutrient mixture (1:1) supplemented with 5% newborn calf serum and 88 μM methotrexate. Once confluent, the growth medium was replaced by serum-free Opti-MEM I, supplemented with 50 μM ZnCl₂, 10 μg/ml vitamin K₁, and 1% v/v penicillin/streptomycin/Fungizone mixture. The medium was collected at 24-h intervals and stored at 4 °C for no more than 24 h prior to purification.

The collected media were loaded onto XAD-2 (2.5 × 15 cm) and Q-Sepharose columns (1.4 × 8 cm) in tandem at room temperature. Once the media were loaded, the columns were dissected, and Q-Sepharose was washed with 5–10 column volumes of 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4 (TBS), and the prothrombin derivatives were eluted with 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.4. Fractions containing the eluted protein were identified using a Bio-Rad assay and pooled. The pooled fractions were then subjected to barium citrate precipitation by the addition of sodium citrate to a final concentration of 0.025 M and subsequent slow addition of 1.0 M barium chloride solution to a final concentration of 0.08 M. The solution was stirred at 4 °C for 1 h and centrifuged at 10,000 × g for 20 min. The resulting pellet was washed with the supernatant from a parallel precipitation carried out in 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.4. The final pellet was then dissolved in minimal volume of 0.2 M EDTA, pH 8.0. The sample was then dialyzed against TBS at 4 °C overnight and subjected to anion-exchange chromatography by fast protein liquid chromatography on a Mono-Q HR 5/5 column at 4 °C. The protein was eluted using a 0–30 mM CaCl₂ gradient in TBS over 1 h at a flow rate of 0.5 ml/min. The first peak containing fully γ-carboxylated prothrombin derivatives eluted around 12 mM CaCl₂, whereas the non-fully γ-carboxylated protein eluted in a broad second peak. Fractions in the first peak were pooled, precipitated by 80% ammonium sulfate, and stored at −20 °C in 50% glycerol in TBS.

SDS-PAGE Time Course Analysis of Prothrombin Activation by Prothrombinase—Prothrombin (1.4 μM) with 10 μM DAPA, 50 μM PCPS, 20 nm factor Va, and 5 mM CaCl₂ in 0.02 M HEPES, 0.15 M NaCl, pH 7.4, 0.01% Tween 80 (HBST) was activated by adding 0.07 nm factor Xa at time 0. Aliquots were removed at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4–8, 10, 15, and 30 min and added to acetic acid to a final concentration of 0.134 M. These samples were then dried down, redissolved in gel sample buffer, and resolved by 10–14.5% SDS-PAGE. The gels were fixed in 50% methanol, 20% ethanol, and 6% trichloroacetic acid for at least 2 h and were stained with Coomassie Blue and destained. The gels were then dried down using BioDesign GelWrap (BioDesign Inc., New York) and scanned using CanoScan 5000F (Canon, Canada). Densitometry was carried out to determine the concentration of each band present.

Quantification of Bands—To correct for the different amounts of Coomassie staining in each fragment of prothrom-
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SCHEME 1. The one-form model of prothrombin activation by prothrombinase. This model depicts two ways in which prothrombin may bind prothrombinase, which determines the different intermediate pathways. Ratcheting is indicated by the bold arrows, and channeling is indicated by the dashed arrows. E, prothrombinase; P, prothrombin; M, meizothrombin; P2, prethrombin-2; T, thrombin; M1, ratcheted meizothrombin; P21, ratcheted prethrombin-2.

bin and to convert the counts into molar concentrations, the stoichiometry of prothrombin fragments was used. When prothrombin is fully consumed, the relative concentration between fragment 1.2, B-chain, and A-chain would be 1:1:1. Using this relationship, molar concentrations of various fragments were determined using its respective correction coefficient, which were experimentally determined to be 0.849, 0.4468, 0.3695, 0.6265, 0.5492, and 0.0773 for prethrombin-1, fragment 1.2:A-chain, fragment 1.2, prethrombin-2, B-chain, and A-chain, respectively.

Prothrombinase Models—Although prothrombinase is a multicomponent enzyme, for the purposes of modeling, it is considered to be a single enzymatic entity, designated E. In one of the models, only one form of E is considered. This model is designated one-form prothrombinase (OF). In the other model, two interconvertible forms of the enzyme are considered (E1, E2), and the model is designated two-form prothrombinase (TF). Variations of both of these models, which incorporate either “ratcheting” of the intermediates, as demonstrated by Bianchini et al. (31), for meizothrombin or channeling directly to thrombin without release of intermediates, as described by Boskovic et al. (30), or both are considered as well. Thus, the one-form and two-form models each have four variants; one has neither ratcheting nor channeling (OF, TF); one has ratcheting only (OF-R, TF-R); one has channeling only (OF-C, TF-C), and one has both ratcheting and channeling (OF-RC, TF-RC).

One-form Model—This model is depicted in Scheme 1. The enzyme (E) interacts with prothrombin (P) to form either complex (E-P)1, from which meizothrombin (M) is produced, or complex (E-P)2, from which prethrombin-2 (P2) is produced. The formations of (E-P)1 and (E-P)2 are characterized by Km values, respectively, and . These complexes turn over to free E and the intermediates M and P2, with values and , respectively. The intermediates M and P2 interact freely with E to form complexes E-M and E-P2, with values and , respectively. The complexes E-M and E-P2 turn over to yield free E and thrombin, with values and , respectively. Ratcheting occurs when M or P2 spontaneously convert to altered forms of the intermediates, designated M1 and P21, respectively. These events are depicted in Scheme 1 by boldface arrows. The kinetics of these processes are first order, with respective rate constants and . The altered forms interact with free E to form and , with respective values and . Channeling occurs when (E-P)1 or (E-P)2 converts directly to thrombin without release of intermediates. These channeling pathways are depicted in Scheme 1 by the dashed lines. They are first order processes with rate constants and .

The rate equations for the one-form model with ratcheting and channeling are shown in Equations 1–5,

\[
d[P]/dt = -(k_{1} + k_{C1})/K_{m1} + (k_{4} + k_{C2})/K_{m4} - k_{7}[M]
\] (Eq. 1)

\[
d[M]/dt = k_{1}[E]/K_{m1} - k_{7}[E][M]/K_{m4} - k_{7}[M]
\] (Eq. 2)

\[
d[P1]/dt = k_{4}[E]/K_{m4} - k_{7}[E][P2]/K_{m5} - k_{7}[P2]
\] (Eq. 3)

\[
d[P21]/dt = k_{4}[P2]/K_{m6} - k_{5}[E][P21]/K_{m6}
\] (Eq. 5)

The conservation equation of total enzyme is shown in Equation 6,

\[
[E]_{0} = [E] + [(E \cdot P)_{1}] + [(E \cdot P)_{2}] + [E \cdot M] + [E \cdot M1] + [E \cdot P2] + [E \cdot P21]
\] (Eq. 6)

or Equation 7,

\[
[E]_{0} = [E](1 + [P]/K_{m1} + [P]/K_{m4} + [M]/K_{m2} + [M1]/K_{m3} + [P2]/K_{m5} + [P21]/K_{m6})
\] (Eq. 7)

From Equation 7, free [E] is expressed in terms of [E], [P], [M], [M1], [P2], [P21], and K values as in Equation 8.

\[
[E] = [E]_{0}/(1 + [P]/K_{m1} + [P]/K_{m4} + [M]/K_{m2} + [M1]/K_{m3} + [P2]/K_{m5} + [P21]/K_{m6})
\] (Eq. 8)

The rate equations for the variant without channeling are identical to the above except that and are set to 0. The rate equations for the model without ratcheting are identical to the above except that and are set to 0. The rate equations for the variant lacking both channeling and ratcheting are identical to the above equations, except that and are set to 0.

Two-form (Ping-Pong-like) Model—This model is depicted in Scheme 2. It is similar in many respects to the one-form model. However, it includes two forms of the enzyme, E1 and E2. The form E1 catalyzes cleavage at Arg-320, thereby converting prothrombin to meizothrombin or prethrombin-2 to thrombin. The other form, E2, catalyzes cleavage at Arg-271, thereby converting prothrombin to prethrombin-2 or meizothrombin to thrombin. E1 and E2 spontaneously interconvert with first-order rate constants and . In addition, when either form of the enzyme engages its substrate
and catalyzes bond cleavage, it reverts to the other form. In this respect, the enzyme exhibits a classic ping-pong-like mechanism (39). According to this model, $E_1$ interacts with prothrombin (P) to form the complex $E_1$-P, and $E_2$ interacts with P to form the complex $E_2$-P. The dissociation constants for these interactions are $K_{m1}$ and $K_{m2}$, respectively. $E_1$-P converts to meizothrombin (M) and free $E_2$, whereas $E_2$-P converts to prothrombin-2 (P2) and free $E_1$. The first-order rate constants for these steps are $k_1$ and $k_{10}$, respectively. $E_2$ binds M with $K_{m2}$ to form $E_2$-M, and $E_1$ binds P2 with $K_{m3}$ to form $E_1$-P2. $E_2$-M turns over to form thrombin (T) and free $E_1$, and $E_1$-P2 turns over to form T and free $E_2$. The rate constants for these steps are $k_2$ and $k_{15}$, respectively. As in the one-form model, M and P2 can spontaneously “ratchet” to M1 and P21, with first-order rate constants $k_3$ and $k_{16}$, respectively. These steps are depicted in Scheme 2 with boldface arrows. $E_2$ binds to M1 to form $E_2$-M1 and $E_1$ binds to P21 to form $E_1$-P21. The dissociation constants are $K_{m3}$ and $K_{m6}$, respectively. $E_2$-M1 converts to T and $E_1$, and $E_1$-P21 converts to T and $E_2$. The rate constants for these steps are $k_3$ and $k_{16}$, respectively. In addition, $E_1$ can channel directly to T and $E_1$, and $E_2$-P can channel directly to T and $E_2$. These pathways are depicted in Scheme 2 with dashed lines. The corresponding first-order rate constants are $k_{c1}$ and $k_{c2}$, respectively. The rate equations for this model are shown as Equations 9–13.

$$d[P] / dt = -((k_1 + k_{c1})[E_1] / K_{m1} + (k_4 + k_{c2})[E_2] / K_{m4})[P]$$  \hspace{1cm} (Eq. 9)

$$d[M] / dt = k_3[E_1][P] / K_{m1} - k_4[M] - k_5[E_2][M] / K_{m2}$$  \hspace{1cm} (Eq. 10)

$$d[M_1] / dt = k_3[M] - k_4[E_2][M] / K_{m3}$$  \hspace{1cm} (Eq. 11)

$$d[P_2] / dt = k_4[E_2][P] / K_{m4} - k_5[P_2] - k_10[E_1][P_2] / K_{m5}$$  \hspace{1cm} (Eq. 12)

$$d[P_{21}] / dt = k_5[P_2] - k_10[E_1][P_{21}] / K_{m6}$$  \hspace{1cm} (Eq. 13)

In addition, $[E_1]$ and $[E_2]$ are presumed to be in a steady state so that Equation 14 is the result.

$$d[E_1] / dt = 0 = [E_2](k_2/[M] / K_{m2} + k_3[M_1] / K_{m3} + k_4[P] / K_{m4} + k_{10}) - [E_1](k_3[P] / K_{m1} + k_4[P_2] / K_{m4} + k_5[P_{21}] / K_{m6} + k_9)$$  \hspace{1cm} (Eq. 14)

The conservation equation for the enzyme is shown as Equation 15.

$$[E]_0 = [E_1] + [E_1 \cdot P] + [E_1 \cdot P_2] + [E_1 \cdot P_{21}] + [E_2] + [E_2 \cdot P] + [E_2 \cdot M] + [E_2 \cdot M_1]$$  \hspace{1cm} (Eq. 15)

or Equation 16.

$$[E]_0 = [E_1](1 + [P] / K_{m1} + [P_2] / K_{m5} + [P_{21}] / K_{m6}) + [E_2](1 + [P] / K_{m4} + [M] / K_{m2} + [M_1] / K_{m3})$$  \hspace{1cm} (Eq. 16)

With the definitions given below in Equations 17–20, Equations 14 and 16 can be expressed as in Equations 21 and 22, respectively.

$$\alpha_1 = 1 + [P] / K_{m1} + [P_2] / K_{m5} + [P_{21}] / K_{m6}$$  \hspace{1cm} (Eq. 17)

$$\alpha_2 = 1 + [P] / K_{m4} + [M] / K_{m2} + [M_1] / K_{m3}$$  \hspace{1cm} (Eq. 18)

$$\beta_1 = k_3[P] / K_{m1} + k_4[P_2] / K_{m5} + k_5[P_{21}] / K_{m6} + k_9$$  \hspace{1cm} (Eq. 19)

$$\beta_2 = k_4[P] / K_{m4} + k_5[M] / K_{m2} + k_6[M_1] / K_{m3} + k_{10}$$  \hspace{1cm} (Eq. 20)

$$0 = \beta_2[E_2] - \beta_1[E_1]$$  \hspace{1cm} (Eq. 21)

$$[E]_0 = \alpha_1[E_1] + \alpha_2[E_2]$$  \hspace{1cm} (Eq. 22)

These latter two equations can be solved for $[E_1]$ and $[E_2]$. The solutions are shown in Equations 23 and 24, where $D$ (Equation 25) is the determinant of the matrix implicit in Equations 21 and 22.

$$[E_1] = [E_0] \beta_2 / D$$  \hspace{1cm} (Eq. 23)

$$[E_2] = [E_0] \beta_1 / D$$  \hspace{1cm} (Eq. 24)

$$D = \alpha_1 \beta_2 + \alpha_2 \beta_1$$  \hspace{1cm} (Eq. 25)

Concentrations of P, M, M1, P2, and P21 over time were calculated by numerical integration with Berkeley Madonna software (University of California, Berkeley). For the one-form model, Equations 1–5 were integrated, with $[E]$ given by Equation 8. For the two-form model, Equations 9–13 were integrated, with $[E_1]$ and $[E_2]$ given by Equations 23 and 24, respectively. The concentration of the B-chain was calculated according to $[B] = [P_{20}] = [P] - [P_2] - [P_{21}]$, where $[P_{20}]$ is the initial prothrombin concentration. Parameter values were optimized by the software using the Simplex procedure of Nelder and Mead (40) to minimize the sum of the least squares distances between the calculated and observed results (loss). In addition, two conditions were imposed during the fit of models to data to be consistent with the previous experimental data observed by Brufatto and Nesheim (32). These were as follows: 1) the total turnover rate of meizothrombin to thrombin represented by $k_2$ without ratcheting or $k_3$ with ratcheting in Schemes 1 and 2 was limited to at least 150 s⁻¹, and 2) the total
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turnover rate of prethrombin-2 to thrombin represented by \( k_5 \) or \( k_6 \) in Schemes 1 and 2 was limited to at least 114 s\(^{-1}\).

These models are based on the approximation that all enzyme forms are in the steady state. The validity of this approximation was established by examining the stopped-flow, rapid chemical quench data of Walker and Krishnaswamy (Fig. 4 of Ref. 41), by measuring the time course of fluorescent rMZ cleavage by prothrombinase, and by fitting the time course data on prothrombin activation by numerical analysis without including the steady state approximation. According to the Michaelis-Menten model the reaction can be described by Reaction 1,

\[
[E] + [S] \xrightarrow{k_1} [E \cdot S] \xrightarrow{k_2}{_{k_1^{-1}}} [E] + [P]
\]

**REACTION 1**

The concentration of \( E \cdot S \) is given by Equation 26,

\[
[E] \cdot S = \frac{[E]_0[S] - [S]}{K_m + [S]}(1 - \exp(-k_1(K_m + [S])t)) \quad \text{(Eq. 26)}
\]

At “long” times, the time-dependent term is negligible and steady state in \([E] \cdot S\) exists such that \([E] \cdot S = [E]_0[S]/(K_m + [S]).\) The magnitude of time transient is reflected in the exponential term \(\exp(-k_1(K_m + [S])t)\). Walker and Krishnaswamy showed (see Fig. 4 in Ref. 41) that prothrombin at 0.3 \(\mu M\) is converted to thrombin at a rate of 3.1 \(\mu M/s\). Because the reaction rate is less than or equal to \(k_1[E]_0[S]\), a lower limit for the value of \(k_1\) can be calculated to be \(3.3 \times 10^7/s\). Thus, with a \(K_m\) value of 0.3 \(\mu M\) and a prothrombin concentration of 1.4 \(\mu M\), the value of the exponent in the transient term evaluates to 56.1/s. Thus, the maximum half-time for the assembly of \(E \cdot S\) complex is about 0.012 s. Because the reactions described in this work took place over a period of about 600 s, the transient time is very small on this scale, and the steady state approximation is reasonable. In addition, over the time course of cleavage of rMZ with a minimum time interval of 1 s, no transients were observed (see supplemental Fig. S3). In addition, the time course data could be fit by numerical analysis without invoking steady state approximations (see supplemental Figs. S1 and S2 and Table S1).

**Rate Equations for Inhibition of rMZ Activation by rP2—rMZ and rP2 are mutants of prothrombin that can be cleaved only at Arg-320 and Arg-271, respectively (32). The reactions and rate equations that describe the cleavage of rMZ in the presence or absence of rP2 for the one-form and two-form models are as follows.**

**One-form Model**—In this model, rMZ and rP2 are both processed by a single form of the enzyme, \(E\), which they bind with their respective Michaelis constants \(K_m(rMZ)\) and \(K_m(rP2)\). They are subsequently cleaved with rate constants \(k_{cat(rMZ)}\) and \(k_{cat(rP2)}\) respectively, see Reactions 2 and 3 and Equations 27 and 28.

\[
E + rMZ \xrightarrow{k_{m(rMZ)}} \xrightarrow{k_{cat(rMZ)}} E + rMZ \quad \text{REACTION 2}
\]

\[
\frac{d[rMZ]}{dt} = -\frac{k_{cat(rMZ)}[E][rMZ]}{K_m(rMZ)} \quad \text{(Eq. 27)}
\]

\[
[E]_0 = [E]\left(1 + \frac{[rMZ]}{K_m(rMZ)} + \frac{[rP2]}{K_m(rP2)}\right) \quad \text{(Eq. 28)}
\]

Equation 28 can be used to solve for \([E]\) in terms of \([E]_0\), [rMZ], [rP2], and the two \(K_m\) values. The result, when inserted into Equation 27, yields Equation 29.

\[
\frac{d[rMZ]}{dt} = -\frac{k_{cat(rMZ)}[E]_0[rMZ]}{K_m(rMZ)(1 + \frac{[rP2]}{K_m(rP2)}) + [rMZ]} \quad \text{(Eq. 29)}
\]

Equation 29, which describes the kinetics of rMZ cleavage in the presence of rP2, is identical in form to the rate equation for competitive inhibition (42). The apparent \(k_{cat}\) for rMZ cleavage is unaffected by rP2. The apparent \(K_m(rMZ)\) however, increases with increasing rP2, and the rate of rMZ approaches zero as [rP2] levels are raised indefinitely.

**Two-form Model**—In this model rMZ is processed by \(E1\) (cleavage at Arg-320) and rP2 is processed by \(E2\) (cleavage at Arg-271). rMZ binds \(E1\) with dissociation constant \(K_{d1}\), and the \(E1\)-rMZ complex turns over into product with first-order rate constant \(k_1\). Similarly, rP2 binds \(E2\) with dissociation constant \(K_{d2}\), and the \(E2\)-rP2 complex turns over into product with rate constant \(k_2\) (see Reactions 4 and 5).

\[
K_{d1} \quad E1 + rMZ \xrightarrow{k_1} E1 \cdot rMZ \xrightarrow{K_{cat(rMZ)}} E2 + rMZa \quad \text{REACTION 4}
\]

\[
K_{d2} \quad E2 + rP2 \xrightarrow{k_2} E2 \cdot rP2 \xrightarrow{K_{cat(rP2)}} E1 + rP2a \quad \text{REACTION 5}
\]

In addition, \(E1\) and \(E2\) spontaneously interconvert with rate constants \(k_3\) and \(k_4\) as shown in Reaction 6.

\[
K_3 \quad E1 \xrightarrow{k_3} E2 \quad \text{REACTION 6}
\]

The rate of rMZ cleavage is given by Equation 30,

\[
r = k_1[E1 \cdot rMZ] = \frac{k_3[E1][rMZ]}{k_{d1}} \quad \text{(Eq. 30)}
\]

Steady states in \([E1]\) and \([E2]\) are presumed, such that (for \([E1]\)) Equation 31 is the result,
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Equation 37 is of the form shown in Equation 38,

\[
r(\infty) = \frac{C_0 [rMZ]}{C_1 + [rMZ]}
\]  
(Eq. 38)

where \( C_0 \) and \( C_1 \) are defined by Equations 39 and 40, respectively.

\[
C_0 = \frac{k_2}{(k_1 + k_4)} \left( \frac{k_1 + k_4}{k_4} \right) [E]_0 k_{cat(rMZ)}
\]  
(Eq. 39)

\[
C_1 = \frac{k_{m(rMZ)}(k_1 + k_4) K_{d_2}}{k_{m(rP2)}(k_1 + k_4)}
\]  
(Eq. 40)

Equation 38 indicates that residual activity at saturating \( rP2 \) will depend in a characteristic way on \( rMZ \), as shown previously by Brufatto and Nesheim (32).

In this model, partial inhibition requires that the competing substrate be a “good” substrate and turn over to generate the other form of the enzyme. If it does not turn over, it merely binds all of its form of the enzyme (\( E2 \) in the case of \( rP2 \)). Because \( E1 \) and \( E2 \) spontaneously interconvert, the binding of either form by a substrate that does not turn over can lead to total sequestration of the enzyme as in competitive inhibition and thereby eliminate the reactions catalyzed by the other form of the enzyme. Consequently, the inhibition pattern can be very similar to competitive inhibition, in that the residual rate will tend to zero at saturating \( rP2 \). Thus, if the turnover number of \( rP2 \) is “small,” \( C_0 \) of Equation 38 is approximately zero and residual activity of saturating \( rP2 \) will approach zero, as in competitive inhibition.

Analysis of Inhibition of \( rMZ \) Cleavage by \( rP2 \) Using One-form or Two-form Models—Recombinant prothrombin derivative \( rMZ \) (80–560 nm) was activated in the presence of \( rP2 \) at a range of concentrations (0–765 nm). Using a white 96-well plate pretreated with 0.02 M Hepes, 0.15 M NaCl, pH 7.4, 1% Tween 80, the prothrombin derivative(s) were incubated at 25 °C with 5 μM DAPA, 50 μM PCPS, 20 nm factor Va, and 5 mm CaCl2 in HBST, and the reactions were initiated by adding 0.02 nm factor Xa. Activation was monitored by SpectraMax Gemini XS ( Molecular Devices) using the excitation wavelength of 280 nm and the emission wavelength of 545 nm with a 515 nm cutoff filter in the emission beam.

In the absence of \( rP2 \) ([\( rP2 \]) = 0), Equation 35 is simply the Michaelis-Menten equation for \( rMZ \) cleavage (Equation 36). A completely analogous equation (Equation 36) can be derived for \( rP2 \) cleavage in the absence of \( rMZ \) (not shown).

\[
r = \frac{k_{cat(rMZ)}[E]_0 [rMZ]}{K_{m(rMZ)} + [rMZ]} + \frac{k_{cat(rMZ)} [rMZ]}{K_{m(rMZ)} + [rMZ]}
\]  
(Eq. 36)

Equation 35 predicts only partial inhibition of \( rMZ \) cleavage by \( rP2 \) at saturating levels of \( rP2 ([rP2] \to \infty) \). The residual rate is given by Equation 37.

\[
r = \frac{k_{cat(rMZ)}[E]_0 \left( \frac{k_2}{k_4} \cdot \frac{1}{K_{d_2}} \right) [rMZ]}{[rMZ] \left( \frac{k_1 + k_4}{k_1 + k_4} \cdot \frac{1}{K_{m(rMZ)} + K_{m(rP2)}} + \frac{k_{m(rMZ)} K_{d_2}}{K_{m(rP2)}} \right)}
\]  
(Eq. 37)
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model. Equation 29 was used similarly to determine the $k_{\text{cat}(rMZ)}$, $K_m(rMZ)$, and $K_m(rP2)$ for the one-form model.

Initial Rate Equations for the Two-form, Single $K_m$ Model with Ratcheting and Channeling in the Presence or Absence of the Partial Inhibitors rMZ (I$_1$) and rP2 (I$_2$) — The reactions catalyzed by prothrombinase on inhibitors I$_1$ and I$_2$ are shown in Reactions 7 and 8.

$$K_m = \frac{V}{S}$$

$$E1 + I_1 \leftrightarrow E1 \cdot I_1 \rightarrow E2 + I_1 \alpha$$

**REACTION 7**

$$K_m = \frac{V}{S}$$

$$E2 + I_2 \leftrightarrow E2 \cdot I_2 \rightarrow E1 + I_2 \alpha$$

**REACTION 8**

The rate of prothrombin consumption ($r$, expressed as a positive value) is, according to Equation 9, as given by Equation 44,

$$r = ((k_1 + k_{C1})[E1] + (k_2 + k_{C2})[E2])[P]/K_m$$

(Eq. 44)

The existence of a steady state in [E1] (and thus [E2]) is expressed as shown in Equation 45,

$$[E]_0 = (1 + [P]/K_m + [I_1]/K_m)[E1] + (1 + [P]/K_m + [I_2]/K_m)[E2]$$

(Eq. 45)

Equations 45 and 46 are solved for [E1] and [E2], and the results are inserted into Equation 44. The equation for the initial rate of prothrombin consumption, divided by the total enzyme concentration, is thus obtained. The result is shown in Equation 47, with constants as given by Equations 48–58.

$$\frac{r}{[E]_0} = \frac{(k_{\text{cat(app)}}, a_1[I_1] + b_1[I_2])[P] + a_2[P]}{K_m[I_2] + [P] + d_1[P][I_1] + (b_2 + b_3[I_2])[I_2] + c_1[I_1][I_2]}$$

(Eq. 47)

$$k_{\text{cat(app)}} = \frac{k_{10}(k_1 + k_{C1}) + k_9(k_2 + k_{C2})}{(k_9 + k_{10} + k_1 + k_3)}$$

(Eq. 48)

$$K_m[I_2] = \frac{k_m(k_9 + k_{10} + k_1 + k_3)}{K_m(k_9 + k_{10} + k_1 + k_3)}$$

(Eq. 49)

$$a_1 = \frac{k_1(k_4 + k_{C1})}{K_m(k_9 + k_{10} + k_1 + k_3)}$$

(Eq. 50)

$$a_2 = \frac{k_m(k_{11} + k_{10})}{K_m(k_9 + k_{10} + k_1 + k_3)}$$

(Eq. 51)

The terms in [P]$^2$ of Equation 47 were shown by regression analysis of data on the inhibition of prothrombin conversion by rMZ and rP2 to be negligible over the experimental range of prothrombin concentrations. Thus, to a very good approximation, the rate equation is shown as Equation 59,

$$\frac{r}{[E]_0} = \frac{(k_{\text{cat(app)}}, a_1[I_1] + b_1[I_2])[P]}{K_m[I_2] + [P] + (a_2 + a_3[P])[I_1] + (b_2 + b_3[I_2])[I_2] + c_1[I_1][I_2]}$$

(Eq. 59)

In the absence of both I$_1$ and I$_2$, the rate equation is the familiar Michaelis-Menten Equation 60,

$$\frac{r}{[E]_0} = \frac{k_{\text{cat(app)}}[P]}{K_m[I_2] + [P]}$$

(Eq. 60)

In the presence of I$_1$, but in the absence of I$_2$, the rate equation is shown as Equation 61,

$$\frac{r}{[E]_0} = \frac{(k_{\text{cat(app)}}, a_1[I_1])[P]}{K_m[I_2] + [P] + (a_2 + a_3[P])[I_1]}$$

(Eq. 61)

As the level of I$_1$ is raised without limit, the rate approaches a finite value that depends on the concentration of P as shown in Equation 62.

$$\frac{r}{[E]_0} = \frac{a_1[P]}{a_2 + a_3[P]}$$

(Eq. 62)

A completely analogous expression applies in the presence of I$_2$ but in the absence of I$_1$, i.e. both inhibitors show partial inhibition, as long as $a_1$ or $b_1$ is not 0 (i.e. the partial substrates turnover at non-negligible rates). When both I$_1$ and I$_2$ are present and both are raised to very high levels, the rate equation approaches a value of 0. Thus, both inhibitors together exhibit competitive inhibition.

For the one-form model, a conventional Michaelis-Menten equation in the presence of inhibitor(s) was used, as with Equa-
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Initial Rates of Prothrombin Activation—Prothrombin at varying concentrations (0–1.2 μM) was incubated at 25 °C with 20 nM factor Va, 5 mM CaCl₂, 50 μM PCPS, and 10 μM DAPA in HBST. Prothrombin was activated by the addition of 0.07 nM factor Xa, and the reaction was monitored by fluorescence using an LS50B fluorescence spectrophotometer (PerkinElmer Life Sciences). The excitation and emission wavelengths were set at 280 nm (2.5 nm slit) and 545 nm (5 nm slit), respectively, with a 530-nm emission cutoff filter. The reaction rates were calculated by measuring the time to reach 30% of total fluorescence change. Simulated rates were then calculated identically. Times to 30% consumption were simulated with prothrombin concentrations varying from 0.1 to 1.2 μM, using rate constants that gave the best fit to the full time course data at 1.4 μM prothrombin. In the simulations, the fluorescence quantum yield of meizothrombin was taken to be 1.5 times that of thrombin (10, 43).

RESULTS

Time Course of Prothrombin Activation by Prothrombinase—Four time course experiments, as described under “Experimental Procedures,” were analyzed by SDS-PAGE. One of the four gels is presented in Fig. 1. The bands in Fig. 1, and those of the three additional trials, were quantified, and the average concentrations of each species were plotted with respect to time (Fig. 2). The meizothrombin concentration was determined from the F1.2-A band. Prothrombin consumption showed a modest lag and was 50 and 90% complete by 3.5 and 10 min, respectively. Meizothrombin peaked at 0.183 ± 0.025 μM at 4 min and was completely depleted by 15 min. The prethrombin-2 concentration accumulated to a peak of 0.110 ± 0.012 μM at 8 min and slowly declined thereafter. Its level declined to 0.053 ± 0.011 μM by 30 min (not shown). The B-chain showed a steady increase in its concentration and approached a value of around 1.25 μM at 10 min.

Fits of Various Models to the Time Course Data—In an attempt to distinguish whether the one-form or the two-form model better describes prothrombinase function, the full time courses of the concentrations of prothrombin, meizothrombin, prethrombin-2, and the B-chain were predicted by both models and compared with the experimentally observed time courses. Fig. 3 shows the fits of the two models, in the absence of channeling or ratcheting, the total loss for the fits using the OF model (A) and the TF model (B) were 0.7122 and 0.5841, respectively. The data points represent the average concentration of prothrombin (○) and B-chain (□) in accordance with the scale on the left side, as well as meizothrombin (●) and prethrombin-2 (△) in accordance with the scale on the right side (same as shown in Fig. 2). The solid lines are regression lines obtained by nonlinear regression of the model to the experimental data. In the absence of channeling or ratcheting, the total loss for the fits using the OF model (A) and the TF model (B) were 0.7122 and 0.5841, respectively.

The data points represent the average concentration of prothrombin (○) and B-chain (□) in accordance with the scale on the left side, as well as meizothrombin (●) and prethrombin-2 (△) in accordance with the scale on the right side (same as shown in Fig. 2). The solid lines are regression lines obtained by nonlinear regression of the model to the experimental data. In the absence of channeling or ratcheting, the total loss for the fits using the OF model (A) and the TF model (B) were 0.7122 and 0.5841, respectively.
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in the presence of channeling only. There was little change in the fit with TF-C compared with the fit with TF. However, OF-C showed a substantial improvement in the fit, compared with OF, for prothrombin, meizothrombin, and the B-chain profiles. The total losses for the OF-C and the TF-C models were 0.4749 and 0.5088, respectively. Fig. 5 shows the fits of the two models in the presence of ratcheting only. As indicated under “Experimental Procedures,” ratcheting through the meizothrombin intermediate pathway was achieved by limiting \( k_3 \) to less than 10 s\(^{-1}\). Similarly, ratcheting through the prethrombin-2 intermediate pathway was achieved by limiting \( k_5 \) to less than 10 s\(^{-1}\). TF-R showed a significant improvement in the fit of the prethrombin-2 profile relative to the fit with TF. The B-chain and meizothrombin peak profiles also showed improvements. OF-R also showed a significant improvement in the fit of the prethrombin-2 profile relative to the fit with OF. The B-chain and prothrombin profiles also showed improvements. However, the meizothrombin peak showed a substantially poorer fit, in that it peaked earlier and yet was sustained longer than in the OF fit. The total losses for the OF-R and the TF-R models were 0.4868 and 0.2968, respectively. Thus, the incorporation of ratcheting improved the fit of the two-form model more than it improved the fit of the one-form model. Fig. 6 shows the fits of the two models in the presence of both channeling and ratcheting. Incorporation of both ratcheting and channeling to both OF and TF resulted in excellent fits for all four species. However, TF-RC showed a slightly better fit than OF-RC, especially in the time course profile of prethrombin-2. The total losses for the OF-RC and TF-RC models were 0.1666 and 0.1632, respectively.

These results show that neither of the models fit the experimental data very well in the absence of both channeling and ratcheting. If channeling is included, but ratcheting is not, neither model fits the data satisfactorily (Fig. 4). If only ratcheting is included, the two-form model describes the data very well, but the one-form model does not (Fig. 5). Thus, if channeling does not exist, the time course data favors the two-form model. If, however, both channeling and ratcheting exist, neither model can be excluded because they both fit the data indistinguishably well (Fig. 6).

Inhibition of rMZ Activation by rP2—The one-form model predicts that rP2 would be a classical competitive inhibitor of rMZ cleavage, whereas the two-form model predicts only partial inhibition as described by Brufatto and Nesheim (32). Thus, inhibition of rMZ cleavage by rP2 was investigated to determine which model better fits the data. Fig. 7 shows the initial rates of rMZ activation in the presence or absence of rP2 as an inhibitor. These data were fit to both models. They were fit to Equation 43 of the two-form model or Equation 29 of the one-form model. The results with the two-form model are in Fig. 7A. The lines are regression lines, and the residuals are shown in Fig. 7C. The results with the one-form model are shown in Fig. 7B. The lines are regression lines, and the residuals are shown in Fig. 7D. The optimized fit parameters, along with their asymptotic standard errors, for the two-form model are as follows: \( k_{cat(rMZ)} = 87 \pm 3 \text{s}^{-1}, K_m(rMZ) = 0.279 \pm 0.025 \mu\text{M}, K_m(rP2) = 0.291 \pm 0.056 \)
A

B

C

D

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FIGURE 7. Inhibition of rMZ activation by rP2. The prothrombin derivative rMZ at concentrations of 0.08, 0.16, 0.24, 0.32, 0.40, 0.48, and 0.56 μM (from bottom to top, respectively) were incubated with rP2 at varying levels (0–0.765 μM) in the presence of 5 μM DAPA, 50 μM PCPS, 20 nM factor Xa, and 5 mM CaCl₂. The reactions were initiated by adding factor Xa (20 pmol). The solid lines are regression lines obtained by nonlinear regression. A shows the fit to the two-form model (Equation 43), whereas B shows the fit to the one-form model (Equation 29), and C and D are the respective residual profiles. The two-form model showed a random distribution of residuals about zero, whereas the one-form model showed a nonlinear over-under-over pattern about zero.

μM, $a_1 = 0.87 \pm 0.27 \mu M^{-1}$, and $a_2 = 1.12 \pm 0.38 \mu M^{-1}$. The total loss is 91.38. The optimized fit parameters for the one-form model are as follows: $k_{cat(rMZ)} = 84 \pm 3 s^{-1}$, $K_{m(rMZ)} = 0.273 \pm 0.019 \mu M$, and $K_{m(rP2)} = 0.541 \pm 0.037 \mu M$. The total loss is 122.77. The residuals for the two-form model were approximately randomly distributed around 0, whereas those of the one-form model exhibited a nonrandom pattern, characterized by an over-under-over profile with respect to the rP2 concentration. Therefore, these data favor the two-form model as the better predictor of prothrombinase function.

Analysis of Inhibition of Fluorescein-labeled Prothrombin Cleavage by Various Prothrombin Derivatives—Brufatto and Nesheim (32) studied the inhibition of fluorescein-labeled prothrombin derivative cleavage by wild type prothrombin, rMZ, rP2, or equimolar mixtures of rMZ and rP2. They reported that the wild type prothrombin acted as a typical competitive inhibitor. However, in the presence of either rMZ or rP2, the initial rates for the cleavage of fluorescein-labeled prothrombin exhibited a partial inhibition profile. In the presence of both rMZ and rP2, however, the initial rates again exhibited an apparent competitive inhibition profile.

In this study, the rates reported by Brufatto and Nesheim (32) were subjected to the current analysis using Equation 59 for the two-form model and Equation 63 for the one-form model, to determine which model would fit the data better. For the two-form model, when wild type prothrombin was present as the inhibitor, the rate equation was the Michaelis-Menten equation (Equation 60), with two forms of prothrombin treated as the substrate for the prothrombinase complex. Although the two variants of prothrombin are assumed to be identical in its characteristics, two $K_m$ values were introduced to allow for the possible variation in binding the enzyme complex. This resulted in the rate equation that was identical to Equation 29, which is also the equation used for the analysis of the one-form model in the presence of a single inhibitor. The best fit values for the $k_{cat(app)}$, $K_{m(WT-II)}$, and $K_{m(WT-I)}$ for both models are 104.8 ± 6.8 s⁻¹, 434.7 ± 56.4 nm, and 263.6 ± 24.2 nm, respectively, with an overall loss of 166.1.

When rMZ is present as an inhibitor, the $k_{cat(app)}$, $K_{m(rMZ)}$, $a_1$, $a_2$, and $a_3$ values are 127.3 ± 7.9 s⁻¹, 566.4 ± 64.9 nm, 0.4770 ± 0.0640 s⁻¹ nm⁻¹, 7.0850 ± 0.8410, and 0.0109 ± 0.0015 nm⁻¹, respectively, for the two-form model, with an overall loss of 109.4. For the one-form model, the $k_{cat(app)}$, $K_{m(rMZ)}$, and the $K_{m(1)}$ values are 99.1 ± 11.7 s⁻¹, 399.6 ± 100.5 nm, and 158.3 ± 26.6 nm, respectively, with an overall loss of 745.0.

When rP2 is present as an inhibitor, the $k_{cat(app)}$, $K_{m(rP2)}$, $a_1$, $a_2$, and $a_3$ values are 107.0 ± 7.2 s⁻¹, 403.2 ± 58.4 nm, 1.054 ± 0.1840 s⁻¹ nm⁻¹, 5.9670 ± 1.0600, and 0.0174 ± 0.0028 nm⁻¹, respectively, for the two-form model, with an overall loss of 195.1. For the one-form model, the $k_{cat(app)}$, $K_{m(rP2)}$, and the $K_{m(2)}$ values are 79.1 ± 6.0 s⁻¹, 272.0 ± 53.5 nm, and 566.1 ± 133.2 nm, respectively, with an overall loss of 914.5.

When rMZ and rP2 are present at equimolar concentrations, the $k_{cat(app)}$, $K_{m(rMZ)}$, $a_1$, $a_2$, and $a_3$ values are 143.3 ± 10.9 s⁻¹, 692.0 ± 92.6 nm, 0.0026 ± 0.0220 s⁻¹ nm⁻¹, 2.5960 ± 0.4250, and 0.0000(>0) nm⁻¹, respectively, for the two-form model, with an overall loss of 190.4. For the one-form model, Equation 63 was modified to Equation 64,

$$ r = -\frac{k_{cat(app)}[P]}{K_{m(app)}[P] + [P]} $$

(Eq. 64)

where $I_{inh}$ is the concentration of either of the inhibitors at any point, and $K_{m(inh)}$ is defined as $K_{m(1)} \times K_{m(2)} / (K_{m(1)} + K_{m(2)})$. From this analysis, the $k_{cat(app)}$, $K_{m(app)}$, and the $K_{m(inh)}$ values are 143.5 ± 10.6 s⁻¹, 694.2 ± 89.0 nm, and 272.0 ± 19.3 nm, respectively, with an overall loss of 190.5. The errors in all data represent the asymptotic standard error as returned by the regression algorithm.

The above rate constants and equations were used to produce the regression lines for each inhibitor. Fig. 8 shows the four panels representing the four different inhibition conditions. When wild type prothrombin was the inhibitor (Fig. 8A), both the one-form model (dotted lines) and the two-form
model (solid lines) fit the data well, with losses that were relatively close. When rMZ was the inhibitor (Fig. 8B), the two-form model fit the data very well, whereas the one-form model did not. When rP2 was the inhibitor (Fig. 8C), the two-form model again fit the data very well, whereas the one-form model did not. However, when both rMZ and rP2 were present (Fig. 8D), both models fit the data indistinguishably well, with almost identical losses. These losses were also plotted with respect to various inhibition conditions to compare the two models (Fig. 9). It was apparent that the one-form model did not fit the data observed when only one of rMZ or rP2 was present, as indicated by the grossly large losses. These data again show that the two-form model was able to fit the partial inhibition data well, whereas the one-form model did not. Both models, however, fit the data well when the inhibition was competitive (Fig. 8, A and D).

Comparison of Initial Rates of Prothrombin Activation—The initial rates of prothrombin activation calculated for either the OF-RC or the TF-RC models, along with the observed rates, were plotted with respect to the starting prothrombin concentration (Fig. 10). Although neither model fit the data perfectly, the observed rates fit the simulated rates from the TF-RC model much better than the simulated rates from the OF-RC model. This observation along with the inhibition data favor the two-form model for the prothrombinase mechanism during prothrombin activation.

The Effects of the Inclusion of Inhibition of Prothrombinase by Thrombin on Fits of the One- and Two-form Models to the Time Course Data—Others have shown that thrombin binds to and inhibits prothrombinase (29). Thus, the one- and two-form models were modified to include this effect. This was accomplished by adding the term $\frac{[IIa]}{K_{dth}}$ to Equation 8 for the one-form model, and to Equations 17 and 18 for the two-form model. The regression analyses were repeated with $K_{dth}$ as a parameter. For both the one-form model and the two-form model, best fit values for $K_{dth}$ were 2.04 and 1.84 nM, when ratcheting and channeling were included. These values are consistent with those measured experimentally by others (29). In the absence of either ratcheting or channeling, the lack of fit of the models to the data persisted even when thrombin binding to the enzyme was included in all cases, except for the one-form model with channeling but not ratcheting. In this case, the inclusion of thrombin inhibition of prothrombinase improved the fit of the data to the prethrombin-2 time course. It also predicted 60% channeling through meizothrombin and an unrealistically low $K_a$ values for the thrombin-prothrombinase interaction (0.140 nM). Because of this low value, the one-form model with channeling only was not considered viable.

Parameter Values for Prothrombin Activation According to the Two-form Model of Prothrombinase—The above data indicate that the two-form model better describes prothrombinase than the one-form model. To fully characterize the two-form model, values for all the parameters of the model and their standard deviations were determined by nonlinear regression.
of the model to four independent sets of time course data, the average of which is shown in Fig. 2. In addition, a single $K_m$ value was assumed in these fits because the six different $K_m$ values ($\mu$m) depicted in Scheme 2 were found to be quite similar in the fit of the data to the two-form model with ratcheting and channeling (Fig. 6, $K_m$ = 0.604, $K_m$ = 0.438, $K_m$ = 0.447, $K_m$ = 0.302, $K_m$ = 0.255, and $K_m$ = 0.101). This step was taken to reduce the number of parameters to fit simultaneously, thus decreasing the degrees of freedom in model parameters. The binding of thrombin to the two forms of the enzyme was included. The average concentration data shown in Fig. 2 were then fit to the one $K_m$ model of TF-RC (not shown). There was very little difference between the six $K_m$ model (Fig. 6) and the one $K_m$ model of TF-RC, in that the overall losses were 0.1699 and 0.2095, respectively. Table 1 lists the rate constants, $K_m$ and $K_{anh}$ values, determined for the four individual experiments. Table 1 also presents the average and standard deviation for each of the parameters. The rate constants suggest the following: 1) the equilibrium between $E1$ and $E2$ favors $E2$, which is the form that is specific for Arg-271, by a factor of 1.5 ($k_9/k_{10} = 1.5$); 2) the cleavage at Arg-320 of prothrombin by $E1$ occurs at a rate that is 10.4-fold greater than the cleavage at Arg-271 of prothrombin by $E2$ ($k_1/k_9 = 10.4$); 3) regardless of the pathway, the initial cleavage is limiting ($k_1 < k_3$ and $k_4 < k_9$); 4) the rate constant for ratcheting of meizothrombin is 148-fold greater than that for ratcheting of prothrombin-2 ($k_3/k_{10} = 148$); 5) the rate constant for channeling on the prothrombin-2 pathway is 4.0-fold greater than that on the meizothrombin pathway ($k_{C2}/k_{C1} = 4.0$); 6) channeling accounts negligibly for flow in the meizothrombin pathway ($k_{C1}/(k_1 + k_{C1}) = 0.07$), but it accounts for most of the flow through the prothrombin-2 pathway ($k_{C2}/(k_3 + k_{C2}) = 0.76$); 7) the half-life for meizothrombin ratcheting is 8.2 s, whereas the half-life for prethrombin-2 ratcheting is around 20.0 min; 8) all substrates bind the enzyme with $K_d = 0.33 \pm 0.19 \mu$m; and 9) thrombin binds the enzyme with $K_d = 1.54 \pm 0.49 \mu$m.

**DISCUSSION**

Previous work from this laboratory undertaken to characterize the kinetics of the four partial reactions involved in the prothrombin activation showed that substrates that could be cleaved only at one or the other of the two activation sites were only partial inhibitors of prothrombin activation. To explain this, a model involving two interconvertible forms of prothrombinase was proposed (32). This study was undertaken to further investigate the plausibility of this model. The results strongly suggest that more than one form of prothrombinase exists.

As shown by Orcutt and Krishnaswamy (33), about 95% of the flow from prothrombin to thrombin proceeds through the meizothrombin pathway. Consequently, prothrombin-2 is considered relatively insignificant as an intermediate in prothrombin activation on phospholipid vesicles. As shown presently, and as shown previously by, for example, Bukys et al. (Fig. 4A of Ref. 44), prethrombin-2 appears only as a faint band upon SDS-PAGE with Coomassie Blue stain, and it peaks late in the course of the reaction. Although prethrombin-2 is ordinarily difficult to detect by these methods, the gel system employed in this study focuses the bands very sharply, which facilitates detection and quantitation.

We included prethrombin-2 in our model building because
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we could quantify it and thereby use its levels to maximize the restraints upon the models. In this way, they would have to fit not only prothrombin, meizothrombin, and thrombin time courses but also the prethrombin-2 time course to be considered viable.

In a previous study, Brufatto and Nesheim (32) used recombinant prothrombin derivatives that could be cleaved only at Arg-320 (rMZ) or Arg-271 (rP2) as tools to infer the kinetics of cleavage of these two bonds in prothrombin. The catalytic efficiencies of rMZ and rP2 were very similar. The high value for rP2 was recognized to be inconsistent with the kinetics of prothrombin activation, and the authors inferred that kinetics of rP2 cleavage do not reflect cleavage of the same bond in prothrombin. The preparations of rP2 used by Brufatto and Nesheim (32) for unknown reasons were very efficiently cleaved by prothrombinase. Subsequent preparations, however, have been shown to be cleaved with efficiencies that are about 9.2% of the efficiency of cleavage of rMZ (44). They estimated that the efficiency at cleavage of the bond at Arg-271 in prothrombin is only 3.8% that of the cleavage at Arg-320. Orcutt and Krishnaswamy (33) subsequently showed experimentally that the cleavage at Arg-271 is 30-fold less efficient than cleavage at Arg-320 in intact prothrombin. The values found in the current work by modeling are consistent with these in that the catalytic efficiency for cleavage at Arg-271 is only 9.6% of that for cleavage at Arg-320 (Table 1).

The phenomenon of ratcheting whereby the intermediate formed in a reaction changes conformation prior to subsequent processing to product was included in modeling because models would not fit the time course data without it, and it has been demonstrated experimentally by Bianchini et al. (31) for meizothrombin. Whether it occurs with prethrombin-2 has not been demonstrated experimentally, but it was necessary in the modeling to rationalize the prethrombin-2 time course and satisfy the experimental observation that the combination of fragment 1.2;prethrombin-2 is a very good prothrombinase substrate (32). The slow ratcheting kinetics of prethrombin-2 account for its slow conversion to thrombin over the course of the reaction, which implies that prior to ratcheting it is a poor substrate. The predicted half-life for ratcheting is 20 min. Previous work with fragment 1.2;prethrombin-2 involved preparing the intermediate with factor Xa, removing the factor Xa, and subsequently assessing conversion to thrombin. By the time these experiments were performed, ratcheting may well have occurred so that fragment 1.2;prethrombin-2 was a good substrate. Further work will be necessary to determine whether ratcheting occurs and the kinetics of it. It is essential, however, in either model to rationalize the slow kinetics of prethrombin-2 conversion over the course of the experiment and the fact that “aged” prethrombin-2 is an excellent substrate.

The prothrombin consumption time courses reproducibly showed a small but clear lag phase. Such a lag has also been shown in other studies (33). This phenomenon is predicted by the two-form model. The reason is that meizothrombin engages E2 and the complex of E2 and meizothrombin converts with a high catalytic efficiency to thrombin and E1. This in turn engages prothrombin and the complex of E1, and prothrombin converts to meizothrombin. Because meizothrombin is very efficiently converted to thrombin, it promotes prothrombin consumption and thus prothrombin consumption accelerates as meizothrombin accumulates. Although not shown here, this effect is predicted by simulation with the two-form model. In effect, according to the model, meizothrombin promotes prothrombin consumption. Whether this occurs in reality will require experimental verification.

Recent studies by Bukys et al. (44) have shown that a pentapeptide with the sequence mimicking the sequence 695–699 at the carboxyl terminus of the factor Va heavy chain specifically inhibited, at low levels, cleavage at Arg-320 as opposed to cleavage at Arg-271. The inhibition was interpreted as being due to the interference with a factor Va–prothrombin interaction. If this is the case, this interaction is specifically required for cleavage at Arg-320 and implies that another as yet undisclosed interaction is necessary for cleavage at Arg-271. The existence of such a site would argue in favor of two forms of factor Va, each specific for one of the two cleavages.

Orcutt and Krishnaswamy (33) recently reported that a recombinant derivative of prothrombin that can be cleaved only at Arg-271 showed classical competitive inhibition with respect to prothrombin activation. From this, they concluded that only a single form of the prothrombinase complex exists. The essential point of their argument is that all possible substrate derivatives and products bind to prothrombinase in a mutually exclusive manner, which is considered to be inconsistent with the notion that enzyme forms that are specific for either of the cleavage sites. The two-form model proposed by Brufatto and Nesheim (32), however, allows that the two forms interconvert spontaneously (32). This means that any substrate that binds either one of the forms can bind the entire population of enzyme. The partial inhibition pattern requires that the substrates have a relatively high $k_{cat}$ value, otherwise the pattern will be very similar to competitive inhibition. This was shown previously by Brufatto and Nesheim (see Equation 13 in Ref. 32) and is shown again here (see Equations 38 and 43). The substrate used by Orcutt and Krishnaswamy (33) had a very low $k_{cat}$ value, and thus the competitive inhibition pattern observed by them is predicted by the two-form model and therefore does not logically exclude it.

In summary, our data support the model of prothrombinase in which two forms of the enzyme exist, each specific for one of the cleavage sites. The two forms interconvert spontaneously. In addition, when one form enters the catalytic cycle, it exits the cycle as the other form, in a manner akin to the classical ping-pong mechanism (39). The model provides a straightforward and logical explanation for the phenomenon of partial inhibition. We recognize, however, that prothrombin activation is a complex process involving interactions of factor Xa, factor Va, prothrombin, intermediates, and product with phospholipid vesicles. In addition, experiments were carried out in which the vesicle concentration exceeds the enzyme concentration so that the kinetics of exchange of enzymatic components, substrate, intermediates, and products with vesicles may contrib-
ute to the kinetics of processing of the substrate and the intermediates. Thus, conceivably, the phenomenon of partial inhibition might be explained by the dynamics of exchange of components among the vesicles. This might explain the partial inhibition by rMZ or rP2 alone, but the competitive inhibition when used together is difficult to imagine. Thus, we take the two-form model as a simplest explanation for the existence of partial inhibition.

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