Incorporation of Factor VA Into Prothrombinase Is Required for Coordinated Cleavage of Prothrombin by Factor Xa

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Prothrombin is activated to thrombin by two sequential factor Xa-catalyzed cleavages, at Arg<sup>271</sup> followed by cleavage at Arg<sup>320</sup>. Factor Va, along with phospholipid and Ca<sup>2+</sup>, enhances the rate of the process by 300,000-fold, reverses the order of cleavages, and directs the process through the meizothrombin pathway, characterized by initial cleavage at Arg<sup>271</sup>. Previous work indicated reduced rates of prothrombin activation with recombinant mutant factor Va defective in factor Xa binding (E323FY324F and E330MV331I, designated factor Va<sub>FP/MI</sub>). The present studies were undertaken to determine whether loss of activity can be attributed to selective loss of efficiency at one or both of the two prothrombin-activating cleavage sites. Kinetic constants for the overall activation of prothrombin by prothrombinase assembled with saturating concentrations of recombinant mutant factor Va were calculated, prothrombin activation was assessed by SDS-PAGE, and rate constants for both cleavages were analyzed from the time course of the concentration of meizothrombin. Prothrombinase assembled with factor Va<sub>FP/MI</sub> had decreased<br>\( k_{\text{cat}} \) for prothrombin activation with \( K_m \) remaining unaffected. Prothrombinase assembled with saturating concentrations of factor Va<sub>FP/MI</sub> showed significantly lower rate for cleavage of plasma-derived prothrombin at Arg<sup>270</sup> than prothrombinase assembled with saturating concentrations of wild type factor Va. These results were corroborated by analysis of cleavage of recombinant prothrombin mutants rMz-II (R155A/R284A/R271A) and rP2-II (R155A/R284A/R320A), which can be cleaved only at Arg<sup>270</sup> or Arg<sup>271</sup>, respectively. Time courses of these mutants indicated that mutations in the factor Xa binding site of factor Va reduce rates for both bonds. These data indicate that the interaction of factor Xa with the heavy chain of factor Va strongly influences the catalytic activity of the enzyme resulting in increased rates for both prothrombin-activating cleavages.

Blood coagulation is necessary to stop blood from leaking out of the vasculature and involves a number of specific serine proteases that are activated by limited proteolysis. The end result of this process is the conversion of prothrombin to thrombin, which in turn cleaves fibrinogen to produce the insoluble fibrin mesh (1). Prothrombin is activated physiologically by the protease of the prothrombinase complex, which is composed of the non-enzymatic cofactor, factor Va, the enzyme, factor Xa, and the substrate, prothrombin, associated on a cell membrane surface in the presence of Ca<sup>2+</sup> (2, 3). Activation of prothrombin to thrombin is a consequence of two cleavages at Arg<sup>271</sup> and Arg<sup>320</sup> by factor Xa. Depending on the order of peptide bond cleavage, different intermediates are formed (Fig. 1). Initial cleavage at Arg<sup>271</sup> will produce fragment 1-2 and prothrombin-2, whereas initial cleavage at Arg<sup>320</sup> results in the formation of meizothrombin, which has optimal amidolytic activity but diminished clotting activity (4–13). The existence of these two pathways has long been established (4–9), and the kinetics of the rate of cleavage determined (10–13). Although factor Xa alone cleaves prothrombin sequentially at Arg<sup>271</sup> followed by Arg<sup>320</sup> to produce thrombin (Fig. 1, pathway I), the rate of the overall reaction for thrombin formation is slow and incompatible with survival. The prothrombinase complex catalyzes the activation of prothrombin following the second pathway (Arg<sup>320</sup> followed by Arg<sup>271</sup>, Fig. 1, pathway II), resulting in a 300,000-fold increase in the rate of the overall reaction compared with the rate of prothrombin activation observed with factor Xa alone and represents the physiological relevant pathway for prothrombin activation (3, 12, 13). Although both cleavages are phospholipid-dependent, only initial cleavage of prothrombin at Arg<sup>270</sup> is strictly dependent on factor Va. The overall increase in the catalytic efficiency of prothrombinase when compared with the activation of prothrombin by factor Xa alone appears to arise from a 100-fold decrease in the \( K_m \) and a 3,000-fold increase in the \( k_{\text{cat}} \) of the enzyme (10, 14–16).

Human prothrombin is composed of 579 amino acids and circulates in blood at a concentration of 1.4 \( \mu \text{M} \) (17). Although the importance of the contribution of factor Va to the activity of factor Xa for rapid thrombin generation by prothrombinase at the place of vascular injury has been long established, the consequence of the interaction of the cofactor with the components of prothrombinase and the molecular mechanism by which factor Va reverses the order of cleavages and increases the rate of the overall catalysis remain enigmatic and are the subjects of intense investigation. Proteolytic processing of factor V by thrombin at Arg<sup>709</sup>, Arg<sup>1018</sup>, and Arg<sup>1545</sup> is required for the interaction of the cofactor with the components of prothrombinase and produces a factor Va molecule that consists of a heavy chain (\( M_r, 105,000 \)) and a light chain (\( M_r, 74,000 \)) (18, 19). Although both chains of factor Va and Cu<sup>2+</sup> are required for the interaction with factor Xa, only the heavy chain of the cofactor binds prothrombin in a Ca<sup>2+</sup>-independent manner (20–22). Cleavage of factor Va by APC at Arg<sup>569</sup>/Arg<sup>675</sup> results in de-
creased affinity of the molecule for factor Xa and eliminates its interaction with prothrombin (21, 23, 24). Subsequent cleavage of the membrane-bound cofactor at Arg^{306}, completely abolishes the ability of factor Va to interact with factor Xa (23, 24).

The interaction of factor Va with factor Xa and prothrombin has been long established as a requirement for optimum rates of prothrombin activation (3, 4, 6, 10, 11, 14, 25, 26). However, the consequences of these associations on the mechanism of catalysis and the discrete amino acid regions from the molecules that are involved in protein-protein interaction have remained puzzling. Furthermore, earlier findings as well as data using mutant prothrombin molecules have suggested that the rate of cleavage at Arg^{320} largely benefits from incorporation of factor Va into prothrombinase while the rate of cleavage at Arg^{271} appears to be less affected (12, 13). We have recently identified binding sites for factor Xa and prothrombin in the middle portion and the COOH terminus of the heavy chain of factor Va, respectively (27–31). Our findings suggested that factor Va has a crucial effect on prothrombinase activity, orchestrating the spatial arrangement of enzyme and substrate, ultimately directing cleavage and activation of prothrombin by factor Xa at two spatially distinct proteolytic sites (28). The present study was undertaken to identify and understand the molecular mechanisms underlying the contribution factor Va to the two factor Xa-mediated prothrombin-activating cleavages following its incorporation into prothrombinase.

**EXPERIMENTAL PROCEDURES**

**Materials, Reagents, and Proteins—**t-α-Phosphatidylethanolamine (PS) and t-α-phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). Normal reference plasma and the chromogenic substrate Spectrozyme-TH were from American Diagnostica, Inc. (Greenwich, CT). The thromboplastin reagent for the clotting assays was purchased from Organon Teknika Corp. (Durham, NC). The fluorescent thrombin inhibitor dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA), RRV-factor V activator, human factor Xa, human thrombin, a sheep inhibitor dansylarginine-(3-ethyl-1,5-pentanediyl)amide; rMZ-II, prothrombin (prothrombin with the substitutions Arg155→Ala, Arg271→Ala, and Arg284→Ala) were prepared and purified as described previously (13, 33). The cDNA for factor V was purchased from American Type Tissue Collection (ATCC number 40515 pMT2-V, Manassas, VA). The sequence of this cDNA molecule is identical to the cDNA published by Jenny et al. (34). All restriction enzymes were from New England Biolabs (Beverly, MA), and all other molecular biology reagents and media were from Invitrogen or as specified by the vendor.

**Mutagenesis and Transient Expression of Recombinant Factor V Molecules—**All mutant factor V molecules described herein were synthesized by PCR-based methods as described (28, 31). The activity and the integrity of the molecules were verified prior to and after activation by thrombin by clotting assays using factor V-deficient plasma and by SDS-PAGE followed by Western blotting using well characterized monoclonal antibodies (provided by Dr. Ken Mann, Department of Biochemistry, University of Vermont).

**Measurement of Rates of Thrombin Formation in a Prothrombinase Assay—**All factor V species (plasma and recombinant) were activated with thrombin for 15 min at 37 °C followed by the addition of diisopropylfluorophosphate (2 mM, Sigma). The assay verifying the activity of the recombinant molecules was conducted as described by measuring thrombin formation by the change in the absorbance of a chromogenic substrate at 405 nm (Spectrozyme-TH) monitored with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) (31). Absorbance at 405 nm was compared with a standard curve prepared daily using purified thrombin (0–50 nM).

For the functional calculation of the apparent dissociation constants (K_{D}^{app}) between the recombinant factor Va molecules and factor Xa, experiments were performed in the presence of a limited concentration of factor Xa (between 15 and 30 pM) and varying concentrations of factor Va (up to 20 nM). The initial rates of thrombin generation in the various assay mixtures under the conditions employed were linear, and in all experiments no more than 10% of prothrombin was consumed during the initial course of the assay. The initial rate of the formation of thrombin (initial velocity in nM/H) was calculated, and the data were analyzed and plotted assuming a 1:1 stoichiometry between factor Va and factor Xa (one binding site) using the software Prism (GraphPad, San Diego, CA) as described (27, 38). Once the value of the K_{D}^{app} for the bimolecular interaction of each species of factor Va with factor Xa was determined, the amount of recombinant factor Va required to completely saturate factor Xa and provide similar amount of enzyme (prothrombinase) when using various mutant recombinant factor Va.

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1 The abbreviations used are: FS, t-α-phosphatidylethanolamine; APC, activated-protein C; PC, t-α-phosphatidylcholine; PCPS, small unilamellar phospholipid vesicles composed of 75% PC and 25% PS (w/w); DAPA, dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide; rMZ-II, prothrombin with the substitutions Arg^{306}→Ala, Arg^{271}→Ala, and Arg^{284}→Ala; rP2-II, prothrombin with the substitutions Arg^{306}→Ala, Arg^{271}→Ala, and Arg^{284}→Ala; and rP2-II, prothrombin (prothrombin with the substitutions Arg^{306}→Ala, Arg^{271}→Ala, and Arg^{284}→Ala) were prepared and purified as described previously (13, 33). The cDNA for factor V was purchased from American Type Tissue Collection (ATCC number 40515 pMT2-V, Manassas, VA). The sequence of this cDNA molecule is identical to the cDNA published by Jenny et al. (34). All restriction enzymes were from New England Biolabs (Beverly, MA), and all other molecular biology reagents and media were from Invitrogen or as indicated. Human plasma factor V and factor Va were purified and concentrated using methodologies previously described employing the monoclonal antibody αhFV#1 coupled to Sepharose and protein G-Sepharose (30, 35). The cofactor activity of the plasma-derived and recombinant factor Va preparations was measured by a clotting assay using factor V-deficient plasma using an automated coagulation analyzer (START-4, Diagnostica Stago, Parsippany, NJ). Phospholipid vesicles composed of 75% PC and 25% PS (referred to as PCPS vesicles throughout the manuscript) were prepared as previously described (36). The concentration of phospholipid vesicles was determined by phosphorous assay as described earlier and is given as the concentration of inorganic phosphate (37).

FIG. 1. **Schematic of the pathways for the activation of human prothrombin.** Prothrombin contains ten γ-carboxyglutamic acid residues (hatched region), two kringle domains (checker region), and a serine protease domain (black region) (17). Initial cleavage at Arg^{271} by membrane-bound factor Xa in the absence of factor Va, results in the generation of fragment 1 and 2 and prethrombin-2 followed by subsequent cleavage at Arg^{320} resulting in thrombin formation (pathway I). Initial cleavage at Arg^{306} by prothrombinase (factor Va bound to factor Xa on a membrane surface in the presence of Ca^{2+}) results in the production of a catalytically active intermediate (meizothrombin), which has the same molecular weight as prothrombin under non-reducing conditions. Following reduction, the intermediate gives rise to fragment 1-A and the B chain of thrombin. Subsequent cleavage of this intermediate at Arg^{311} produces thrombin and fragment 1-2 (pathway II). Once formed thrombin also cleaves prothrombin at Arg^{306} to form prethrombin-1, and at Arg^{312} to form the A chain of thrombin minus thirteen amino acids (dashed arrow).
molecules (between 90% and 99% saturation) can be calculated as abundantly described in the literature (39, 40).

For the calculation of the kinetic constants of prothrombinase assembled with the various recombinant mutant factor Va molecules as well as with plasma factor Va \( K_m \) and \( V_{max} \), experiments were conducted in the presence of a limited amount of factor Xa (5 pM) in the presence of a fixed and saturating concentration of factor Va (5–30 nM) while varying the amount of the substrate (plasma-derived prothrombin) from 25 nM to 6 \( \mu \)M. Kinetic constants \( (K_m \text{ and } V_{max}) \) were extracted directly from the plotted data.

**Additivity of the Mutational Effects of Substitutions**  

Additivity of the Mutational Effects of Substitutions 323EY324 and 330EV331 on Prothrombinase Function—Prothrombinase is composed of factor Va and factor Xa assembled on a membrane surface in the presence of divalent metal ions. Any perturbation in the inter-

**TABLE I**  

Rate of activation of native plasma-derived prothrombin in the presence of prothrombinase assembled with various recombinant factor Va species

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Prothrombin consumption*</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Xa alone</td>
<td>0.0 ± 0.09</td>
<td>1 -fold</td>
</tr>
<tr>
<td>Prothrombinase with wild type factor Va</td>
<td>15.4 ± 0.9</td>
<td>3.14</td>
</tr>
<tr>
<td>Prothrombinase with factor VaMI</td>
<td>4.9 ± 0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Prothrombinase with factor VaFF/MI</td>
<td>6.7 ± 0.3</td>
<td>16.7</td>
</tr>
<tr>
<td>Prothrombinase with factor VaFF</td>
<td>0.92 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

* The rates of prothrombin consumption were obtained following scanning densitometry of the gels shown in Fig. 2. The final rate of prothrombin consumption in the presence of prothrombinase assembled with the various factor Va species, was extracted following plotting of the data according to the method described under “Experimental Procedures.”
action between the two molecules caused by a mutation may influence or modify the stability of the catalytic site of the enzyme and can be measured by the change in the transition-state stabilization of free energy for prothrombin activation as extensively described (41–45). Thus, the consequence of the mutations in factor Va affecting factor Xa catalytic efficiency and their additivity can be measured relative to the change in transition-state stabilization free energy (\(\Delta G^\ddagger\)) of the enzyme as previously established (41–45).

To assess whether the two sets of mutations affecting the interaction of factor Va with factor Xa (E323F/Y324F, E393M/V393I) energetically interact, four separate free energies associated with the catalytic efficiency of prothrombinase must be measured: \(\Delta G_{\text{cat}}\), the functional free energy in prothrombinase assembled with wild-type factor Va; \(\Delta G_{\text{FP}}\), the functional free energy in prothrombinase assembled with factor Va\(^{\text{FF}}\); \(\Delta G_{\text{FFint}}\), the functional free energy in prothrombinase assembled with factor Va\(^{\text{FF/MI}}\). In general, the perturbation to the function of prothrombinase assembled with wild type factor Va (state A), caused by a mutation in factor Va (state B) affecting the transition state, can be defined in general as follows,

\[
\Delta G_{\text{B}} = \Delta G_{\text{FF}} - \Delta G_{\text{FF/MI}}
\]

and because we are measuring prothrombinase activity assembled in the presence of various factor Va molecules against the same substrate (prothrombin), the changes in transition-state stabilization free energy (\(\Delta G^\ddagger\)) during catalysis caused by a mutation in factor Va (state B) affecting the transition state, can be defined from the following general equation (42–45),

\[
\Delta G^\ddagger_{\text{A-B}} = -RT \ln(k_{\text{cat}}/K_{\text{m}})/(k_{\text{cat}}/K_{\text{m}})_{\text{A}}
\]

with \(R\) being the universal gas constant (2 cal·K\(^{-1}\)·mol\(^{-1}\)), \(T\) the absolute temperature in Kelvin (298 K in all our experiments), \(k_{\text{cat}}\) is the turnover number and \(K_{\text{m}}\) is the Michaelis-Menten constant for prothrombinase assembled with either wild-type or mutant factor Va molecules. In addition, the free energy of the interaction between two mutations in factor Va (A→B and A→C; where A represents the wild type factor Va, B and C represent cofactor molecules with two different mutations, and B/C represent a double mutant factor Va molecule) can be calculated with the following general equation,

\[
\Delta G_{\text{int}} = \Delta G_{\text{A-B}} - \Delta G_{\text{A-C}} - \Delta G_{\text{A-B}} + \Delta G_{\text{B-C}}
\]

The term \(\Delta G_{\text{int}}\) reflects an exchange in free energy between the side chains of the amino acids studied (i.e. 323EY\(^{\ddagger}\) and 324EV\(^{\ddagger}\)). A value of \(\Delta G_{\text{int}}\) >0 or <0 implies that the side chains of the mutated amino acids interact with each other, reducing or enhancing, respectively, the catalytic efficiency of prothrombinase.

**Analysis of Prothrombin Activation by Gel Electrophoresis**—Plasma-derived prothrombin or recombinant mutant prothrombin molecules (1.4 μM) were incubated with PCPS vesicles (20 μM), DAPA (50 μM), and factor Va (10 nM or as indicated in the figure legends) in the presence of wild type factor Va. These combined data demonstrated that the deficiency in cofactor activity of all mutant factor Va molecules appears to be a consequence of delayed thrombin formation because of delayed cleavage at Arg\(^{220}\) (Fig. 2B, lanes 3–5). Similar results were found in the presence of plasma-derived factor Va, fragment 1-2-A, which is a transient fragment during prothrombin activation, and results from initial cleavage of prothrombin at Arg\(^{220}\) (Fig. 1), is rapidly consumed following cleavage at Arg\(^{271}\) to produce fragment 1-2 and thrombin (Fig. 2B, lanes 3–5). The linearity of the relationship between amount of protein loaded and the intensity measured by densitometry was confirmed by loading known quantities of prothrombin and subjecting the stained gels to the above procedure.

**RESULTS**

**Effect of the Interaction of Factor Va with Factor Xa on the Rates of Prothrombin-activating Cleavages**—To test the effect of the interaction of factor Va with factor Xa on the rates of cleavage of prothrombin by prothrombinase, we have used recombinant factor Va molecules mutated at the factor Xa-binding sites and plasma-derived prothrombin. Membrane-bound factor Xa alone was a poor activator of prothrombin (Fig. 2A and Table 1). In the presence of plasma-derived factor Va, fragment 1-2-A, which is a transient fragment during prothrombin activation, and results from initial cleavage of prothrombin at Arg\(^{220}\) (Fig. 1), is rapidly consumed following cleavage at Arg\(^{271}\) to produce fragment 1-2 and thrombin (Fig. 2B, lanes 3–5). Similar results were found in the presence of wild type factor Va. These combined data demonstrate that prothrombin activation is significantly accelerated in the presence of the wild type and/or plasma cofactor molecule (Fig. 2B and C, and Table 1). Analysis of the products of prothrombin activation by gel electrophoresis demonstrated that the deficiency in cofactor activity of all mutant factor Va molecules appears to be a consequence of delayed thrombin formation because, of delayed cleavage at both Arg\(^{220}\)/Arg\(^{271}\) (Fig. 2, D–F). Further, while the rates in prothrombin consumption were decreased by 3.1- and 2.3-fold when using factor Va\(^{\text{FF}}\) and factor Va\(^{\text{M}}\), respectively (Table 1), the rate of thrombin generation was diminished by 17-fold with factor Va\(^{\text{FF/M}}\) as compared with the rates of thrombin formation in the presence of the wild type cofactor molecule (Table 1). The data demonstrate that mutating all four amino acids in the factor Xa binding site of factor Va heavy chain has a dramatic effect on factor Va cofactor activity than disabling either of the two sites individually.

**Kinetic Analyses of the Effect of the Mutations in Factor Va on Prothrombinase Complex Assembly and Function**—The efficiency of prothrombinase in activating prothrombin is remarkable when compared with the efficiency of the catalytic subunit alone, factor Xa, on the same reaction (Refs. 10 and 11; also illustrated in Fig. 2, A–C). To evaluate the effect of the mutations in factor Va on prothrombinase assembly and function,
results were found when using factor Va<sup>FF/MI</sup> at 50 nM (not shown). Prothrombinase assembled with the other two mutant recombinant factor Va molecules (5 nM) were characterized by normal $k_{cat}$ values (1806 and 2040 nM H<sub>11011</sub> IIa formed-min<sup>−1</sup>nM prothrombinase<sup>−1</sup>, Table II). Comparable results were found with these two mutant molecules even when used at 30 nM (not shown). Altogether, these data demonstrate that substitution of either of the two sets of amino acid residues at the extremities of the factor Xa-binding site of factor Va has no significant effect for the overall catalysis at saturating cofactor concentrations and appear to be well tolerated by prothrombinase; however, substitution of both sets, all four amino acids, at the same time has a profound effect on the catalytic efficiency of the enzyme, decreasing the second order rate constant by ~5-fold (Table II). The inability of factor Xa to function optimally because of improper interaction with factor Va can be solely explained by the inability of factor Xa to efficiently convert prothrombin to thrombin because of diminished productive collisions (difference in $k_{cat}$). To quantify the interaction between the mutations in the factor Xa-binding site of factor Va heavy chain and their synergistic or additive effect on prothrombinase function, we have calculated the difference in free energy of the transition-state complex of the enzyme assembled with various mutant cofactor molecules. We have also constructed a thermodynamic cycle for cleavage of prothrombin by prothrombinase (provided in Scheme I). The positive value of $\Delta G_{int}$ reveals that mutations at the extremities of the factor Xa-binding site on factor Va heavy chain are not additive, but their effect is synergistic and disruptive in nature, resulting in the destabilization of the transition-state complex and slower rate of catalysis (41–45). These data establish the fact that the interaction of factor Va with factor Xa through amino acids Glu<sup>323</sup>, Tyr<sup>324</sup>, Glu<sup>330</sup>, and Val<sup>331</sup> is crucial for efficient rates of cleavage of prothrombin by factor Xa.

Cleavage of Mutant Prothrombin Molecules—The kinetic data obtained with plasma-derived prothrombin were confirmed by using mutant recombinant prothrombin molecules (Fig. 5). Factor Xa alone was a poor catalyst for cleavage of both rMZ-II and rP2-II (Fig. 5A). In the presence of plasma and/or wild type factor Va the expected fragments were obtained following cleavage of rMZ-II (Fig. 5, B and C, lanes 1–9) or rP2-II (Fig. 5, B and C, lanes 10–18) (13). Under similar experimental conditions when using prothrombinase assembled with wild-type factor Va, cleavage at Arg<sup>220</sup> in rMZ-II occurred 9-fold faster than cleavage at Arg<sup>271</sup> in rP2-II (Table III). Thus, incorporation of factor Va into prothrombinase is more favorable for cleavage of prothrombin at Arg<sup>220</sup> than for cleavage at Arg<sup>271</sup> (12, 13). Cleavage of rMZ-II and rP2-II in the presence of prothrombinase assembled with factor Va<sup>PP</sup>, factor Va<sup>MI</sup>, and factor Va<sup>FF/MI</sup> was considerably delayed compared with the same cleavages in the presence of the wild type molecule (Fig. 5, panels D–F, lanes 1–18, and Table III). Interestingly, factor Va<sup>PP</sup> and factor Va<sup>MI</sup> had similar deficiency in cofactor activity for cleavage of both rMZ-II and rP2-II when compared with the cleavage of both recombinant prothrombin molecules by prothrombinase assembled with wild-type factor Va. From the data shown in Fig. 5F it is also evident that factor Va<sup>FF/MI</sup> has the largest deficiency in cofactor activity among the three molecules, and this is mostly apparent when using rMZ-II (Table III). These data are confirmatory of our kinetic studies and the findings with plasma-derived prothrombin.

It is well established that increased catalytic efficiency of prothrombinase as compared with the catalytic efficiency of factor Xa alone, is due to the fact that prothrombinase preferentially cleaves prothrombin through pathway II (Fig. 1) (12, 13). Hence, factor Va converts factor Xa from an enzyme that

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**FIG. 3. Determination of the affinity of recombinant factor Va molecules for plasma-derived factor Xa.** Initial rates of thrombin generation were determined as described under “Experimental Procedures.” Prothrombinase assembled with wild-type factor Va is shown by filled squares, prothrombinase assembled with factor Va<sup>FF</sup> is depicted by filled inverse triangles, prothrombinase assembled with factor Va<sup>MI</sup> is depicted by filled triangles, and prothrombinase assembled with factor Va<sup>FF/MI</sup> is depicted by filled diamonds. Titrations were carried out to 20 nM factor Va; however, for graphical purposes the data show the titration for up to 10 nM cofactor. The solid lines represent a nonlinear regression fit of the data as detailed under “Experimental Procedures” using the software Prism and the model for one binding site. The apparent dissociation constant ($K_{D}$) for each species was derived from each titration performed at least in triplicate with four different preparations of recombinant molecules and is listed in Table II.

kinetics experiments were designed to determine the dissociation constant for prothrombinase assembly as well as the $K_m$ and $k_{cat}$ values for prothrombinase function. The apparent dissociation constants ($K_{D}$) for complex formation between membrane-bound factor Xa and the mutant factor Va molecules was inferred from kinetic experiments in which the steady-state rate of thrombin generation in the presence of a fixed concentration of factor Xa, prothrombin, and PCPS was determined as a function of factor Va (Fig. 3). The $K_{D}$ values derived from these experiments are summarized in Table II. Wild type factor Va had a similar $K_{D}$ to plasma-derived factor Va. The data show that, although factor Va<sup>FF</sup> and factor Va<sup>MI</sup> have ~4- and 10-fold lower affinities for factor Xa than wild type factor Va, factor Va<sup>FF/MI</sup> has ~61-fold lower affinity than the wild-type cofactor molecule for the enzyme ($K_{D}$ = 10.9 nM). These data are consistent with our previous findings using synthetic peptides (30, 31).

To elucidate which parameters of prothrombinase are affected by mutations in the regulatory subunit, we determined the kinetic parameters of the enzyme under conditions where all membrane-bound factor Xa was saturated with factor Va. Fig. 4 depicts the initial steady-state rate of prothrombin activation as a function of substrate concentration, and the calculated kinetic constants are provided in Table II. The data show that prothrombinase assembled with recombinant wild type factor Va as well as with recombinant factor Va<sup>FF</sup>, recombinant factor Va<sup>MI</sup>, and recombinant factor Va<sup>FF/MI</sup> were characterized by similar $K_m$ values (~0.15–0.17 µM). Furthermore, although prothrombinase assembled with the wild type cofactor molecule had similar $k_{cat}$ as prothrombinase assembled with plasma factor Va (Table II), prothrombinase assembled with factor Va<sup>FF/MI</sup> (30 nM) was characterized by a $k_{cat}$ value of ~406 nM H<sub>11011</sub> IIa formed-min<sup>−1</sup>nM prothrombinase<sup>−1</sup> (Table II). Similar results were found using factor Va<sup>FF/MI</sup> at 50 nM (not shown).
factor Va; however, substitution of all four amino acids at both sites of prothrombinase to efficiently convert prothrombin to thrombin activation by gel electrophoresis under conditions where factor Xa was saturated with factor VaFF/MI (1 nM factor Xa with 100 nM factor VaFF/MI, corresponding to ~90% saturation of factor Xa). The data demonstrated that prothrombinase assembled with saturating amounts of mutant cofactor molecule activates prothrombin through pathway II with a slower rate than that observed for prothrombinase assembled with the wild type cofactor molecule (data not shown). Cleavage at Arg320 and meizothrombin formation were considerably delayed. Further, meizothrombin did not accumulate, because the intermediate was cleaved immediately as it was formed to produce thrombin. These data verify our findings shown in Fig. 2F and strongly suggest that binding of factor Va heavy chain to factor Xa is required for efficient initial cleavage at Arg320 and rapid meizothrombin formation. Overall, our findings suggest that the interaction of amino acids Glu323, Tyr324, Glu330, and Val331 from the heavy chain of factor Va with factor Xa is required for the positioning of the active site of the enzyme in a position necessary for optimal productive collisions between the enzyme and prothrombin, efficient initial cleavage at Arg320, and competent thrombin formation.

**DISCUSSION**

Our data demonstrate that binding of the heavy chain of factor Va to factor Xa through amino acids Glu323, Tyr324, Glu330, and Val331 is necessary for achieving optimal catalytic rates for cleavage of prothrombin by prothrombinase. Data obtained from the crystal structure of meizothrombin desF1 and prethrombin-2 demonstrate that 1) the two prothrombin-activating cleavages are 36 Å apart and 2) for the molecule to be initially cleaved at Arg320 a rotation of ~150° around the Gly319 and Gly324 hinge points must occur (48–51). As a consequence, and keeping in mind the results presented herein, it is logical to hypothesize that the cofactor, factor Va, rather than being an inert accessory protein, following its incorporation into prothrombinase and its interactions with factor Xa, directs catalysis of the substrate by the enzyme at two spatially distinct sites (i.e. Arg320 and Arg271).

Our data show that a mutation at either one of the two extremities of the factor Xa-binding site on factor Va heavy chain has no major effect on the kinetic parameters of prothrombinase assembled with saturating concentrations of factor Va; however, substitution of all four amino acids at both sites simultaneously results in a damaging effect on the ability of prothrombinase to efficiently convert prothrombin to thrombin through the meizothrombin pathway. These data imply that amino acid residues 330 and 331 together with any remaining factor Xa-binding site from the heavy chain of the cofactor molecule can compensate for substitutions at amino residues 323 and 324 and *vice versa* when factor Va is used in excess. However, substitution of all four amino acid residues at the same time slowly cleaves prothrombin initially at Arg271 to an enzyme that efficiently cleaves prothrombin initially at Arg220. To ascertain the exact activation pathway of prothrombin by prothrombinase assembled with factor VaFF/MI, we followed pro-

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**Table II**

*Kinetic constants for prothrombinase assembly and function in the presence of various factor Va species*

<table>
<thead>
<tr>
<th>Factor Va species</th>
<th>$K_{cat}^{app}$</th>
<th>$V_{max}^{app}$</th>
<th>$K_{m}$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_{m}$</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma factor Va</td>
<td>0.29 ± 0.06</td>
<td>9.72 ± 0.6</td>
<td>0.12 ± 0.03</td>
<td>1944 ± 120</td>
<td>2.7 × 10^8</td>
<td>=0</td>
</tr>
<tr>
<td>Wild type recombinant factor Va</td>
<td>0.18 ± 0.02</td>
<td>9.85 ± 0.4</td>
<td>0.16 ± 0.02</td>
<td>1970 ± 80</td>
<td>2.1 × 10^8</td>
<td>=0</td>
</tr>
<tr>
<td>Factor VaFF</td>
<td>0.7 ± 0.3</td>
<td>9.03 ± 0.2</td>
<td>0.10 ± 0.01</td>
<td>1806 ± 40</td>
<td>3.0 × 10^8</td>
<td>=0</td>
</tr>
<tr>
<td>Factor VaMI</td>
<td>1.8 ± 0.7</td>
<td>10.2 ± 0.24</td>
<td>0.14 ± 0.01</td>
<td>2040 ± 48</td>
<td>4.5 × 10^7</td>
<td>=5</td>
</tr>
<tr>
<td>Factor VaFF/MI</td>
<td>10.9 ± 2.4</td>
<td>2.03 ± 0.07</td>
<td>0.15 ± 0.02</td>
<td>4068 ± 14</td>
<td>9.03 × 10^5</td>
<td>=0</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Determination of kinetic parameters of prothrombinase assembled with various recombinant factor Va species. Initial rates of thrombin generation were determined as described under “Experimental Procedures.” Prothrombinase assembled with two different preparations of wild type factor Va is shown by filled squares (5 nM) and filled circles (15 nM), whereas prothrombinase assembled with plasma-derived factor Va (5 nM) is depicted by filled triangles. The open diamonds represent prothrombinase assembled with factor VaFF (5 nM), whereas the open circles show prothrombinase with factor VaMI (5 nM). Prothrombinase assembled with factor VaFF/MI is depicted by filled inverted triangles (30 nM). The values of the $K_{m}$ and $V_{max}$ extracted from these graphs are listed in Table II.

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**Scheme I.** Thermodynamic cycle for prothrombin activation by prothrombinase following substitutions in factor Va that affect factor Xa binding. $\Delta G_{int}$ is the free energy of interaction between the side chains of $^{323}$EY$^{324}$ and $^{330}$EV$^{331}$ of factor Va heavy chain and was calculated using Equations 2 and 3 described under “Experimental Procedures.”
cannot be rescued by any other residue nor by the factor Xa-

binding site located on the light chain of factor Va resulting in
diminished catalytic rate. Using prothrombinase assembled
with factor VaFF/MI and plasma-derived prothrombin, we have
shown a 16.7-fold decreased rate of prothrombin consumption
compared with the rate of prothrombin consumption obtained
with factor Xa, respectively. A, factor Xa alone no factor Va; B, prothrombinase assembled with plasma-derived factor Va; C, prothrombinase assembled with wild type factor Va; D, prothrombinase assembled with factor VaFF; E, prothrombinase assembled with factor VaMI; F, prothrombinase assembled with factor VaFF/MI. Positions of prothrombin-derived fragments are indicated at right as detailed in the legend to figure 2. For the easy reading of the manuscript, the factor Va species used for the reconstitution of prothrombinase are also shown under each panel. Gels were submitted to scanning densitometry as described under "Experimental Procedures," and the rates of prothrombin consumption are reported in Table III.

![Gel Image](image_url)

**Figure 5. Analysis of the activation of mutant prothrombin molecules by prothrombinase assembled with mutant factor Va molecules.** Mutant prothrombin molecules were incubated in different mixtures with PCPS vesicles, DAPA, and various factor Va molecules (10 nM). The reaction was started by the addition of factor Xa, and the samples were treated as detailed under “Experimental Procedures.” Lanes 1–9 represent samples of the reaction mixture following incubation of prothrombinase with rMZ-II, before (lane 1), or following 0.5 min, 1 min, 2.5 min, 4 min, 6 min, 10 min, 20 min, and 30 min incubation with factor Xa, respectively; lanes 10–18 represent samples of the reaction mixture following incubation of prothrombinase with rP2-II before (lane 10), or following 0.5 min, 1 min, 2.5 min, 4 min, 6 min, 10 min, 20 min, and 30 min incubation with factor Xa, respectively. Positions of prothrombin-derived fragments are indicated at right as detailed in the legend to figure 2. For the easy reading of the manuscript, the factor Va species used for the reconstitution of prothrombinase are also shown under each panel. Gels were submitted to scanning densitometry as described under “Experimental Procedures,” and the rates of prothrombin consumption are reported in Table III.

**Table III**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>rMZ-II (cleavage at Arg²²⁰)</th>
<th>Decrease&lt;sup&gt;a&lt;/sup&gt;</th>
<th>rP2-II (cleavage at Arg²⁷¹)</th>
<th>Decrease&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor Xa alone</td>
<td>0.07 ± 0.11</td>
<td>-fold</td>
<td>0.05 ± 0.08</td>
<td>-fold</td>
</tr>
<tr>
<td>Prothrombinase with wild type</td>
<td>24.6 ± 2.8</td>
<td>2.72 ± 0.23</td>
<td>0.33 ± 0.04</td>
<td>8.2</td>
</tr>
<tr>
<td>Prothrombinase with factor Va&lt;sub&gt;FF&lt;/sub&gt;</td>
<td>2.8 ± 0.2</td>
<td>8.8</td>
<td>0.52 ± 0.04</td>
<td>5.2</td>
</tr>
<tr>
<td>Prothrombinase with factor Va&lt;sub&gt;MI&lt;/sub&gt;</td>
<td>4.6 ± 0.5</td>
<td>5.3</td>
<td>0.31 ± 0.06</td>
<td>8.8</td>
</tr>
<tr>
<td>Prothrombinase with factor Va&lt;sub&gt;FF/MI&lt;/sub&gt;</td>
<td>1.4 ± 0.1</td>
<td>17.6</td>
<td>0.31 ± 0.06</td>
<td>8.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> The -fold decrease is the ratio of the activation rates of recombinant mutant prothrombin molecules in the presence of wild-type factor Va and mutant factor Va species as measured by the disappearance of prothrombin following scanning densitometry of the gels shown in Fig.5. The final rate of prothrombin consumption in the presence of prothrombinase assembled with various factor Va species was extracted following plotting of the data according to the method described under “Experimental Procedures.”
Further, although R3A-TAP has a 4,000-fold decreased interaction with amino acids from the active site of factor Xa and suggest that the side chains of these four amino acids act synergistically to stabilize allosterically the transition state complex of the enzymatic reaction (41–45). However, the change in free energy observed following substitution at the extremities of the factor Xa-binding site on factor Va could also be explained by subtle changes in the overall conformation of the 323–331 amino acid segment due to the two sets of double mutations. Taken as a whole, the results suggest that the interaction of factor Va heavy chain with factor Xa is required for the correct positioning of amino acids in and/or around the active site of factor Xa with respect to the two scissile bonds in prothrombin, resulting in increased frequency of productive collisions between enzyme and substrate required for optimum rates of thrombin formation.

Conclusive evidence for subtle alterations of amino acid residues in and around the active site of factor Xa occurring upon its interaction with factor Va, was provided by studies using recombinant tick anticoagulant protein (TAP) and a recombinant mutant TAP molecule (Arg3→Ala, R3A-TAP) (52–55). TAP, purified from the soft tick Ornithodoros moubata, is a potent inhibitor of factor Xa (K_I 180 pm), and its potency is considerably increased toward prothrombinase (K_I 5.3 pm) (55). It has been established that the four NH_2-terminal amino acid residues of TAP are responsible for its inhibitory activity and interact directly with amino acids from the active site of factor Xa (56). Further, although R3A-TAP has a 4,000-fold decreased K_I toward factor Xa alone, its potency toward prothrombinase is only decreased by 30-fold. The fact that factor Va does not interact with TAP together with results demonstrating decreased inhibitory efficiency of recombinant TAP molecules mutated at residues Asp37 and Asp54, which were thought to interact with a site remote from the active site of the enzyme (3-fold decrease in the K_I) (56), led to the suggestion that prothrombinase exposes cryptic exosites on factor Xa for substrate binding/recognition following incorporation of factor Va into the complex (54, 55). Because these macromolecular recognition sites on factor Xa that are typically providing a pro-thrombin binding site in prothrombinase were assumed to be occupied by R3A-TAP, it was concluded that inhibition of factor Xa by R3A-TAP is favored kinetically when the enzyme is part of the prothrombinase complex (53–56). These data are intriguing and of extreme physiological importance, because theoretical R3A-TAP would be able to discriminate between factor Xa and prothrombinase and could be used as a preventive therapeutic anticoagulant, albeit its slow on rate (53–55). Notwithstanding the potency of R3A-TAP and its potential therapeu-tic importance, the crystal structure of recombinant TAP-factor Xa clearly demonstrated that amino acid residues Asp37 and Asp54 from recombinant TAP, which are part of an amino acid sequence sharing a great deal of homology with prothrombin sequence 305–320 (preceding cleavage at Arg320), interact with amino acids Lys54, Arg222, and Lys224 from factor Xa, which in turn are adjacent to the active site of the enzyme (57). These latter amino acids play a preeminent role in optimum docking of the inhibitor and appear to be rearranged following the interaction of factor Xa with factor Va. These data support the hypothesis that one of the exosites provided by factor Xa upon its interaction with factor Va for prothrombin tethering appears to be composed of amino acids near and/or around the active site of the enzyme promoting optimal docking of the scissile bonds of the substrate in the catalytic groove of the enzyme. In conclusion, our findings, put in the context of the available literature, indicate that factor Va directs catalysis of prothrombin by factor Xa within prothrombinase at two spatially distinct sites.

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