7-22-2005

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

Michael A. Bukys

Melissa A. Blum

Paul Y. Kim

Nicole Brufatto

Michael E. Nesheim

See next page for additional authors

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 29, Issue of July 22, pp. 27393–27401, 2005
Printed in U.S.A.

Incorporation of Factor Va into Prothrombinase Is Required for Coordinated Cleavage of Prothrombin by Factor Xa*

Received for publication, March 29, 2005, and in revised form, May 12, 2005
Published, JBC Papers in Press, May 16, 2005, DOI 10.1074/jbc.M503435200

Michael A. Bukys‡, Melissa A. Blum‡, Paul Y. Kim§, Nicole Brufatto§, Michael E. Nesheim§, and Michael Kalafatis‡¶‖

From the ‡Department of Chemistry, Cleveland State University, Cleveland, Ohio 44115, the §Department of Biochemistry, Queen's University, Kingston, Ontario Canada, and the ¶Department of Molecular Cardiology, The Lerner Research Institute, The Cleveland Clinic Foundation Cleveland, Ohio 44195

Prothrombin is activated to thrombin by two sequential factor Xa-catalyzed cleavages, at Arg^{271} followed by cleavage at Arg^{320}. Factor Va, along with phospholipid and Ca^{2+}, 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^{320}. Previous work indicated reduced rates of prothrombin activation with recombinant mutant factor Va defective in factor Xa binding (E323F/Y324F and E330M/V331I, designated factor $Va^{FF/MI}$). 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 $Va^{FF/MI}$ had decreased k_{cat} for prothrombin activation with K_m remaining unaffected. Prothrombinase assembled with saturating concentrations of factor $Va^{FF/MI}$ showed significantly lower rate for cleavage of plasma-derived prothrombin at Arg^{320} 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^{320} or Arg^{271}, 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 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^{2+} (2, 3). Activation of prothrombin to thrombin is a consequence of two cleavages at Arg^{271} and Arg^{320} by factor Xa. Depending on the order of peptide bond cleavage, different intermediates are formed (Fig. 1). Initial cleavage at Arg^{271} will produce fragment 1·2 and prethrombin-2, whereas initial cleavage at Arg^{320} 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^{271} followed by Arg^{320} 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^{320} followed by Arg^{271}, 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^{320} 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_{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 μM (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^{709}, Arg^{1018}, and Arg^{1545} is required for the interaction of the cofactor with the members 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 Ca^{2+} are required for the interaction with factor Xa, only the heavy chain of the cofactor binds prothrombin in a Ca^{2+}-independent manner (20–22). Cleavage of factor Va by APC at Arg^{506}/Arg^{679} results in de-

* This work was supported by Grant 5R01HL-073343 from the NHLBI, National Institutes if Health (NIH) (to M. K.) and by U. S. Public Health Services, NIH Grant HL-46703-6 (to M. E. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Chemistry, Cleveland State University, 2121 Euclid Ave., Science Bldg. SR370, Cleveland, OH 44115. Tel.: 216-687-2460; Fax: 216-687-9298; E-mail: m.kalafatis@csuohio.edu.

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.

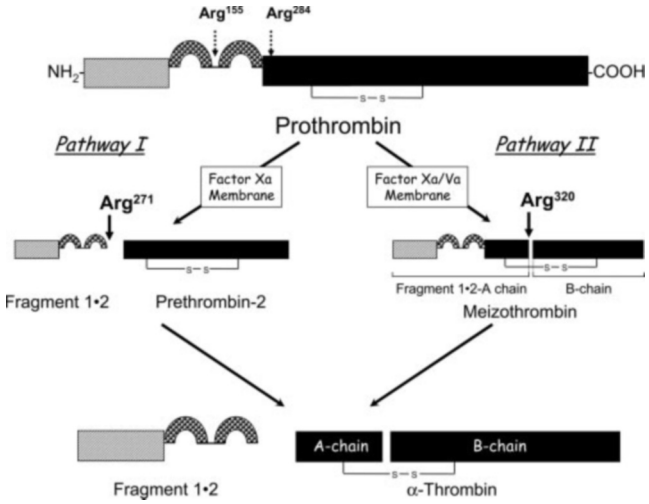


FIG. 1. **Schematic of the pathways for the activation of human prothrombin.** Prothrombin contains ten γ-carboxyglutamic acid residues (*hatched region*), two kringle domains (*checkered region*), and a serine protease domain (*black region*) (17). Initial cleavage at Arg^{271} by membrane-bound factor Xa in the absence of factor Va, results in the generation of fragment 1·2 and prethrombin-2 followed by subsequent cleavage at Arg^{320} resulting in thrombin formation (*pathway I*). Initial cleavage at Arg^{320} 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 1·2-A and the B chain of thrombin. Subsequent cleavage of this intermediate at Arg^{271} produces thrombin and fragment 1·2 (*pathway II*). Once formed thrombin also cleaves prothrombin at Arg^{155} to form prethrombin-1, and at Arg^{284} to form the A chain of thrombin minus thirteen amino acids (*dashed arrows*).

EXPERIMENTAL PROCEDURES

Materials, Reagents, and Proteins—L-α-Phosphatidylserine (PS)[1] and L-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), RVV-factor V activator, human factor Xa, human thrombin, a sheep anti-human factor V polyclonal antibody, and the monoclonal antibody αhFV#1 coupled to Sepharose were from Hematologic Technologies Inc. (Essex Junction, VT). Human prothrombin was purified according to the procedure detailed by Bajaj and Mann (32). A final step was included in the purification procedure that included the use of hydroxyapatite column chromatography (Bio-Rad) to eliminate a proteolytic product from prothrombin that was consistently present in all final prothrombin preparations. Recombinant prothrombin molecules rMZ-II (prothrombin with the substitutions $Arg^{155} \rightarrow$ Ala, $Arg^{284} \rightarrow$ Ala, and $Arg^{271} \rightarrow$ Ala) and rP2-II (prothrombin with the substitutions $Arg^{155} \rightarrow$ Ala, $R^{284} \rightarrow$ Ala, and $R^{320} \rightarrow$ 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 and tissue culture 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 heparin-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). Phospholipids 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).

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 before 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 $nM \cdot IIa \cdot min^{-1}$) 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 Prizm (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

[1] The abbreviations used are: PS, L-α-phosphatidylserine; APC, activated-protein C; PC, L-α-phosphatidylcholine; PCPS, small unilamellar phospholipids 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 $Arg^{155} \rightarrow$ Ala, $Arg^{284} \rightarrow$ Ala, and $Arg^{271} \rightarrow$ Ala; rP2-II, prothrombin with the substitutions $Arg^{155} \rightarrow$ Ala, $Arg^{284} \rightarrow$ Ala, and $Arg^{320} \rightarrow$ Ala; factor Va^{FF}, recombinant human factor Va with the mutations $Glu^{323} \rightarrow$ Phe and $Val^{324} \rightarrow$ Phe; factor Va^{MI}, recombinant human factor Va with the mutations $Glu^{330} \rightarrow$ Met and $Val^{331} \rightarrow$ Ile; factor $Va^{FF/MI}$, quadruple mutant, recombinant human factor Va with the mutations $Glu^{323} \rightarrow$ Phe and $Val^{324} \rightarrow$ Phe and $Glu^{330} \rightarrow$ Met and $Val^{331} \rightarrow$ Ile; TAP, tick anticoagulant protein.

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

Enzyme	Prothrombin consumption[a]	Decrease
	moles consumed·s^{-1}·mole factor Xa^{-1}	*-fold*
Factor Xa alone	0.0 ± 0.09	
Prothrombinase with wild type factor Va	15.4 ± 0.9	
Prothrombinase with factor Va^{FF}	4.9 ± 0.4	3.14
Prothrombinase with factor Va^{MI}	6.7 ± 0.3	2.3
Prothrombinase with factor Va$^{FF/MI}$	0.92 ± 0.08	16.7

[a] 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."

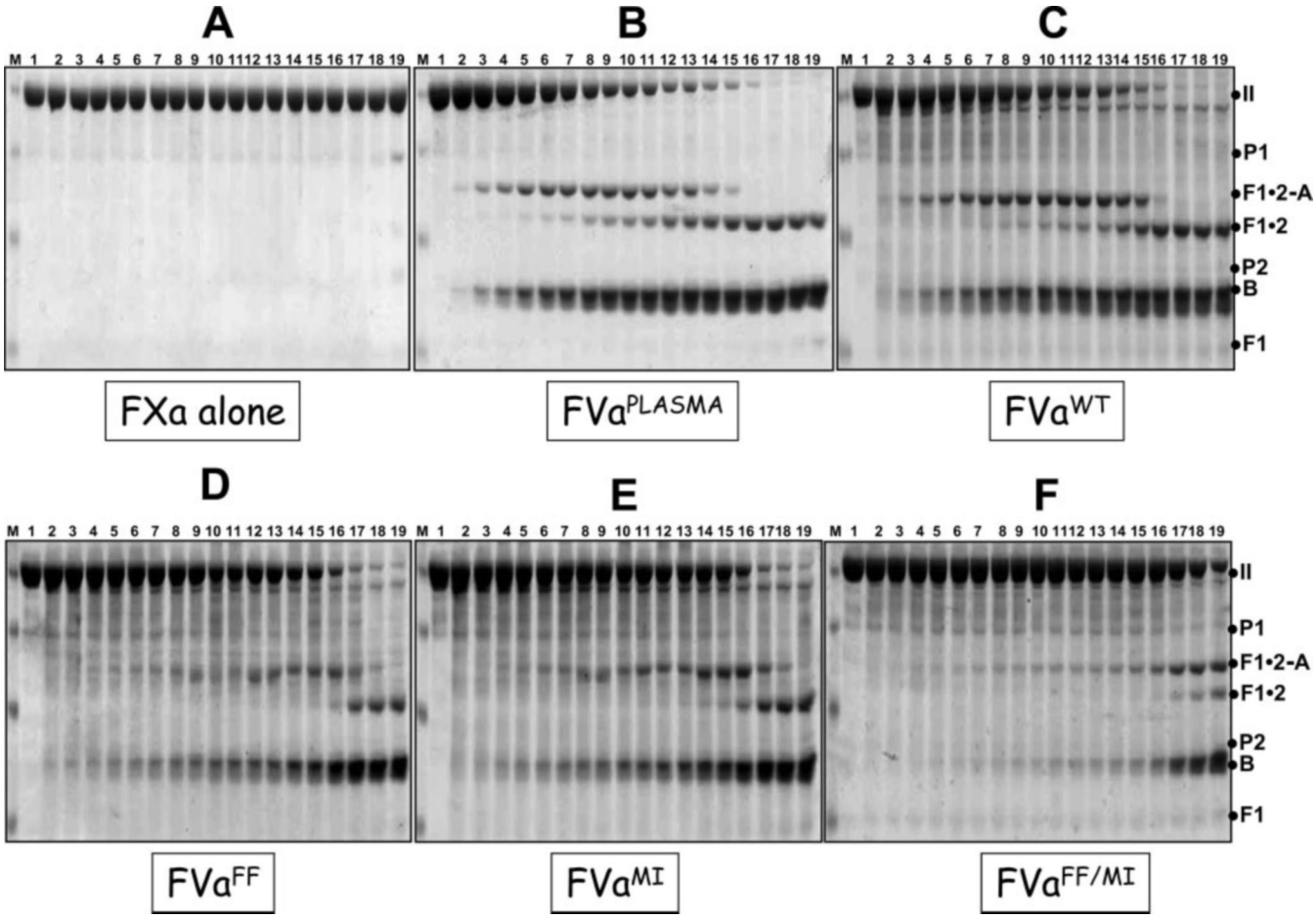


FIG. 2. **Analysis of the activation of plasma-derived prothrombin by prothrombinase assembled with mutant factor Va molecules.** Plasma-derived prothrombin was incubated with PCPS vesicles, DAPA, and various factor Va molecules (10 nM). The reaction was started by the addition of factor Xa. At selected time intervals aliquots of the reactions were withdrawn and treated as described under "Experimental Procedures." *M* represents the lane with the molecular weight markers (from *top* to *bottom*): M_r 98,000, M_r 64,000, M_r 50,000, and M_r 36,000. *Lanes 1–19* represent samples from the reaction mixture before (0 min) the addition of factor Xa and 20 s, 40 s, 60 s, 80 s, 100 s, 120 s, 140 s, 160 s, 180 s, 200 s, 220 s, 240 s, 5 min, 6 min, 10 min, 20 min, 30 min, and 60 min, respectively, following the addition of factor Xa. *A*, factor Xa alone and no factor Va; *B*, prothrombinase assembled with plasma-derived factor Va; *C*, prothrombinase assembled with wild type factor Va; *D*, prothrombinase assembled with factor Va^{FF}; *E*, prothrombinase assembled with factor Va^{MI}; *F*, prothrombinase assembled with factor Va$^{FF/MI}$. For the easy reading of the manuscript the factor Va species used for the reconstitution of prothrombinase are also shown *under each panel*. The identity of each fragment generated following cleavage of prothrombin by prothrombinase was verified in separate experiments by NH_2-terminal sequencing and comparison of the data with the primary structure of prothrombin (17). The prothrombin-derived fragments are numbered consecutively from the NH_2 terminus of prothrombin and are shown as follows: *II*, prothrombin (amino acid residues 1–579); *F1·2-A*, fragment 1·2-A chain (amino acid residues 1–320); *F1·2*, fragment 1·2 (amino acid residues 1–271); *B*, B chain of thrombin (amino acid residues 321–579); *F1*, fragment 1 (amino acid residues 1–155); *P1*, prethrombin-1 (amino acid residues 156–579); *P2*, prethrombin-2 (amino acid residues 272–579) (17). Gels were submitted to scanning densitometry as described under "Experimental Procedures," and the rates of prothrombin consumption are reported in Table I.

molecules (between 90% and >99% saturation) can be calculate 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 μM. Kinetic constants (K_m and k_{cat}) were extracted directly from the plotted data.

Additivity of the Mutational Effects of Substitutions ^{323}EY^{324} → FF and ^{330}EV^{331} → MI 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-

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 ($\Delta\Delta G^{\ddagger}$) 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; E330M/V331I) energetically interact, four separate free energies associated with the catalytic efficiency of prothrombinase must be measured: ΔG_{wt}, the functional free energy in prothrombinase assembled with wild-type factor Va; ΔG_{FF}, the functional free energy in prothrombinase assembled with factor Va^{FF}; ΔG_{MI}, the functional free energy in prothrombinase assembled with factor Va^{MI}; and $\Delta G_{FF/MI}$, the functional free energy in prothrombinase assembled with factor $Va^{FF/MI}$. 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,

$$\Delta\Delta G_B = \Delta G_B - \Delta G_A \quad \text{(Eq. 1)}$$

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 ($\Delta\Delta G^{\ddagger}$) during catalysis caused by a mutation in factor Va (state B) can be calculated from the following general equation,

$$\Delta\Delta G^{\ddagger}_{A\rightarrow B} = -RT \ln[(k_{cat}/K_m)_B/(k_{cat}/K_m)_A] \quad \text{(Eq. 2)}$$

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_{cat} is the turnover number, and K_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 (42–45).

$$\Delta\Delta G_{int} = \Delta\Delta G_{A\rightarrow B/C} - (\Delta\Delta G_{A\rightarrow B} + \Delta\Delta G_{A\rightarrow C}) \quad \text{(Eq. 3)}$$

The term $\Delta\Delta G_{int}$ reflects an exchange in free energy between the side chains of the amino acids studied (*i.e.* ^{323}EY^{324} and ^{330}EV^{331}). A value of $\Delta\Delta G_{int} > 0$ 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 5 mM Ca^{2+} in 20 mM Tris, 0.15 M NaCl, pH 7.4. The reaction was started by the addition of factor Xa (0.5 nM or as indicated in the figure legends) at room temperature. At selected time intervals (also indicated in the figure legends) aliquots from the reaction were removed and immediately diluted into 2 volumes of 0.2 M glacial acetic acid and concentrated using a Centrivap concentrator attached to a Centrivap cold trap (Labconco, Kansas City, MO). The dried samples were dissolved in 0.1 M Tris-base, pH 6.8, 1% SDS (final concentration), 1% β-mercaptoethanol (final concentration), heated for exactly 75 s at 90 °C, mixed again, and subjected to SDS-PAGE using 9.5% gels according to the method of Laemmli (46). Usually, 6 μg of total protein per lane were applied. Protein bands were visualized following staining by Coomassie Brilliant Blue R-250 and destained by diffusion in a methanol/acetic acid/water solution. In several experiments, proteins were transferred to polyvinylidene difluoride membranes according to the method described by Towbin *et al.* (47). Following staining with Coomassie Brillant Blue R and destaining in a solution of acetic acid/methanol/water, the NH_2-terminal sequences of all intermediates were determined using automatic Edman degradation on two separate Applied Biosystems protein sequencing systems (Procise 494 HT and Procise 494 cLC) in the laboratory of Dr. Alex Kurosky and Steve Smith (University of Texas, Medical Branch at Galveston).

Scanning Densitometry of SDS Gels and Calculation of the Rate of Prothrombin Consumption—The stained gels were scanned with a Lexmark printer/scanner, and the final images were captured in PowerPoint. They were subsequently imported as 8-bit grayscale images into Corel Photo Paint. They were analyzed in the National Institutes of Health Image module. Bands were isolated, and the total number of pixels and the average pixel intensities were determined on a scale from 0 to 255. A region of each lane was chosen as background. The average pixel intensity of the background was subtracted from that of each band. The total intensity in each band was then calculated by multiplying the result by the total number of pixels. To compensate for minor loading differences from lane to lane, all intensities were normalized to the sum of intensities of all the bands in each lane. The Coomassie Blue staining intensities of fragment 1·2-A and the B chain, relative to prothrombin, were determined based on the stoichiometric relationship that the sum of prothrombin plus the B chain is constant throughout the reaction in the presence of DAPA. Thus, for a given experiment, the intensity data were subjected to linear regression to the equation, intensity of B chain = total intensity + R·intensity of prothrombin, where R is the intensity per mole of B chain relative to that per mole prothrombin. The relationship was strictly linear for all extents of reaction, as expected. The value of R was typically ~0.65, indicating that, mole per mole, the B chain stained with 65% the intensity of prothrombin. Thus, fragment 1·2-A, which comprises the part of prothrombin not represented by the B chain, was assigned a relative staining intensity of 0.35. The molar concentration of prothrombin as observed on the gels was then calculated by normalizing its staining intensity to the initial prothrombin concentration (usually 1.4 μM). The concentrations of fragments 1·2, and the B chain were calculated similarly, except that their staining intensities were divided by 0.35 and 0.65, respectively. 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. 2*A* and Table I). 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^{320} (Fig. 1), is rapidly consumed following cleavage at Arg^{271} to produce fragment 1·2 and thrombin (Fig. 2*B*, *lanes 3–15*). 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. 2, *B* and *C*, and Table I). 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^{320}/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^{FF} and factor Va^{MI}, respectively (Table I), the rate of thrombin generation was diminished by ~17-fold with factor $Va^{FF/MI}$ as compared with the rates of thrombin formation in the presence of the wild type cofactor molecule (Table I). 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,

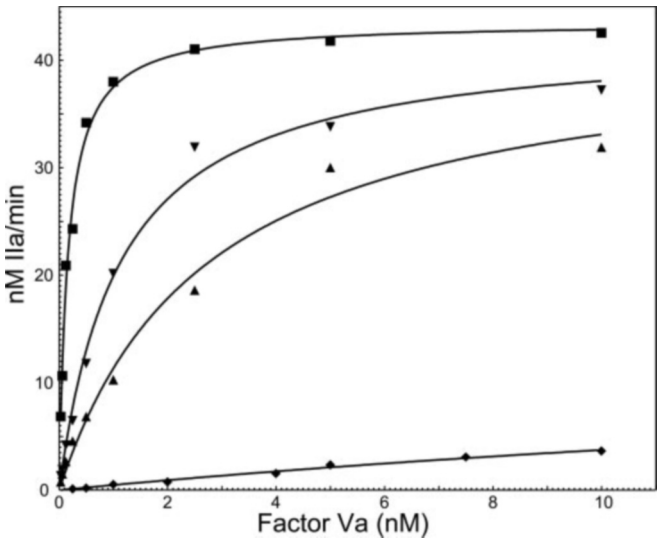


FIG. 3. **Determination of the affinity of recombinant factor Va molecules for plasma-derived factor Xa.** Initial rates of thrombin generation were determined as described under "Experimental Procedures." Prothrombinase assembled with wild-type factor Va is shown by *filled squares*, prothrombinase assembled with factor Va^{FF} is depicted by *filled inverse triangles*, prothrombinase assembled with factor Va^{MI} is depicted by *filled triangles*, and prothrombinase assembled with factor $Va^{FF/MI}$ 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 Prizm and the model for one binding site. The apparent dissociation constant (K_D^{app}) 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^{app}) 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^{app} values derived from these experiments are summarized in Table II. Wild type factor Va had a similar K_D^{app} to plasma-derived factor Va. The data show that, although factor Va^{FF} and factor Va^{MI} have ~4- and 10-fold lower affinities for factor Xa than wild type factor Va, factor $Va^{FF/MI}$ has ~61-fold lower affinity than the wild-type cofactor molecule for the enzyme (K_D^{app} ~ 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^{FF}, recombinant factor Va^{MI}, and recombinant factor $Va^{FF/MI}$ were characterized by similar K_m values (~0.15 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 $Va^{FF/MI}$ (30 nM) was characterized by a k_{cat} value of ~406 nM IIa formed·min^{-1}·nM prothrombinase^{-1} (Table II). Similar results were found when using factor $Va^{FF/MI}$ 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 IIa formed·min^{-1}·nM prothrombinase^{-1}, 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 $\Delta\Delta G_{int}$ 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^{323}, Tyr^{324}, Glu^{330}, and Val^{331} 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. 5*A*). 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^{320} in rMZ-II occurred 9-fold faster than cleavage at Arg^{271} in rP2-II (Table III). Thus, incorporation of factor Va into prothrombinase is more favorable for cleavage of prothrombin at Arg^{320} than for cleavage at Arg^{271} (12, 13). Cleavage of rMZ-II and rP2-II in the presence of prothrombinase assembled with factor Va^{FF}, factor Va^{MI}, and factor $Va^{FF/MI}$ 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^{FF} and factor Va^{MI} 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. 5*F* it is also evident that factor $Va^{FF/MI}$ 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

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

	K_D^{app}	V_{max}^{app}	K_m	k_{cat}	k_{cat}/K_m	Decrease
Factor Va species	*nM*[a]	*nM IIa/min*[b]	*μM*[b]	*min*$^{-1}$[b]	$s^{-1} \cdot M^{-1}$	*-fold*[c]
Plasma factor Va	0.29 ± 0.06	9.72 ± 0.6	0.12 ± 0.03	1944 ± 120	2.7×10^8	
Wild type recombinant factor Va	0.18 ± 0.02	9.85 ± 0.4	0.16 ± 0.02	1970 ± 80	2.1×10^8	
Factor Va^{FF}	0.7 ± 0.3	9.03 ± 0.2	0.10 ± 0.01	1806 ± 40	3.0×10^8	≅0
Factor Va^{MI}	1.8 ± 0.7	10.2 ± 0.24	0.14 ± 0.01	2040 ± 48	2.4×10^8	≅0
Factor Va$^{FF/MI}$	10.9 ± 2.4	2.03 ± 0.07	0.15 ± 0.02	406 ± 14	4.5×10^7	≅5

[a] Apparent dissociation constants of recombinant factor Va for plasma-derived factor Xa (K_D^{app}) were determined as described in the legend to Fig. 3 at limiting factor Xa concentrations (15 pM).
[b] Apparent V_{max}, K_m, and k_{cat} were determined as described in the legend to Fig. 4 at limiting factor Xa concentrations (5 pM) and saturating concentrations of factor Va.
[c] The -fold decrease is the ratio of the second order rate constant (k_{cat}/K_m) of prothrombinase assembled with wild type recombinant factor Va compared to the second order rate constant of prothrombinase assembled with the recombinant mutant factor Va molecules.

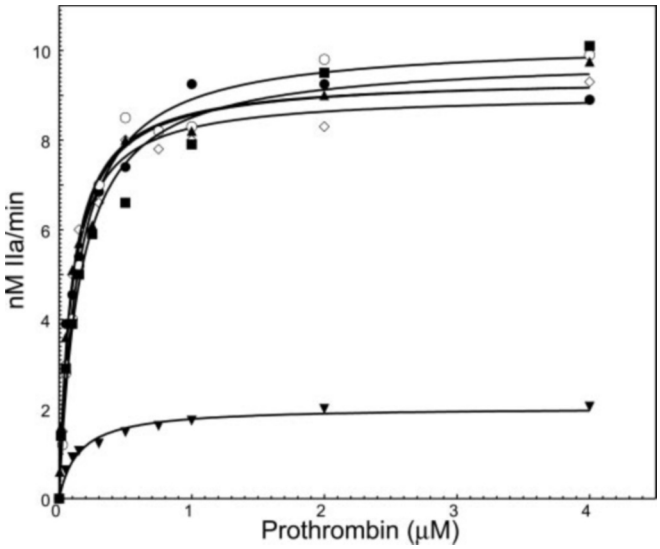
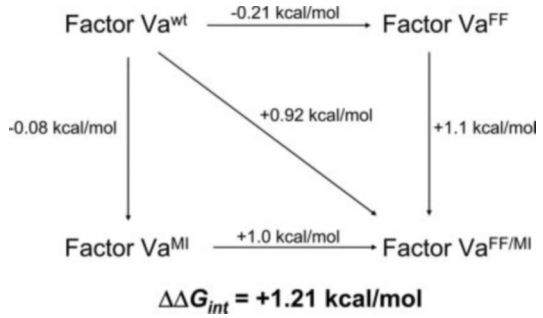


FIG. 4. **Determination of kinetic parameters of prothrombinase assembled with various recombinant factor Va species.** Initial rates of thrombin generation were determined as described under "Experimental Procedures." Prothrombinase assembled with two different preparations of wild type factor Va is 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 Va^{FF} (5 nM), whereas the *open circles* show prothrombinase with factor Va^{MI} (5 nM). Prothrombinase assembled with factor Va$^{FF/MI}$ is depicted by *filled inversed triangles* (30 nM). The values of the K_m and V_{max} extracted from these *graphs* are listed in Table II.



SCHEME I. **Thermodynamic cycle for prothrombin activation by prothrombinase following substitutions in factor Va that affect factor Xa binding.** $\Delta\Delta G_{int}$ 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."

slowly cleaves prothrombin initially at Arg^{271} to an enzyme that efficiently cleaves prothrombin initially at Arg^{320}. To ascertain the exact activation pathway of prothrombin by prothrombinase assembled with factor Va$^{FF/MI}$, we followed prothrombin activation by gel electrophoresis under conditions where factor Xa was saturated with factor Va$^{FF/MI}$ (1 nM factor Xa with 100 nM factor Va$^{FF/MI}$, 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 Arg^{320} 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. 2*F* and strongly suggest that binding of factor Va heavy chain to factor Xa is required for efficient initial cleavage at Arg^{320} and rapid meizothrombin formation. Overall, our findings suggest that the interaction of amino acids Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} 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 Arg^{320}, and competent thrombin formation.

DISCUSSION

Our data demonstrate that binding of the heavy chain of factor Va to factor Xa through amino acids Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} 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 Arg^{320} a rotation of ~150° around the Gly^{319} and Gly^{324} 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 that 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.* Arg^{320} and Arg^{271}).

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 and/or the factor Xa-binding site located on the light chain of the cofactor 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

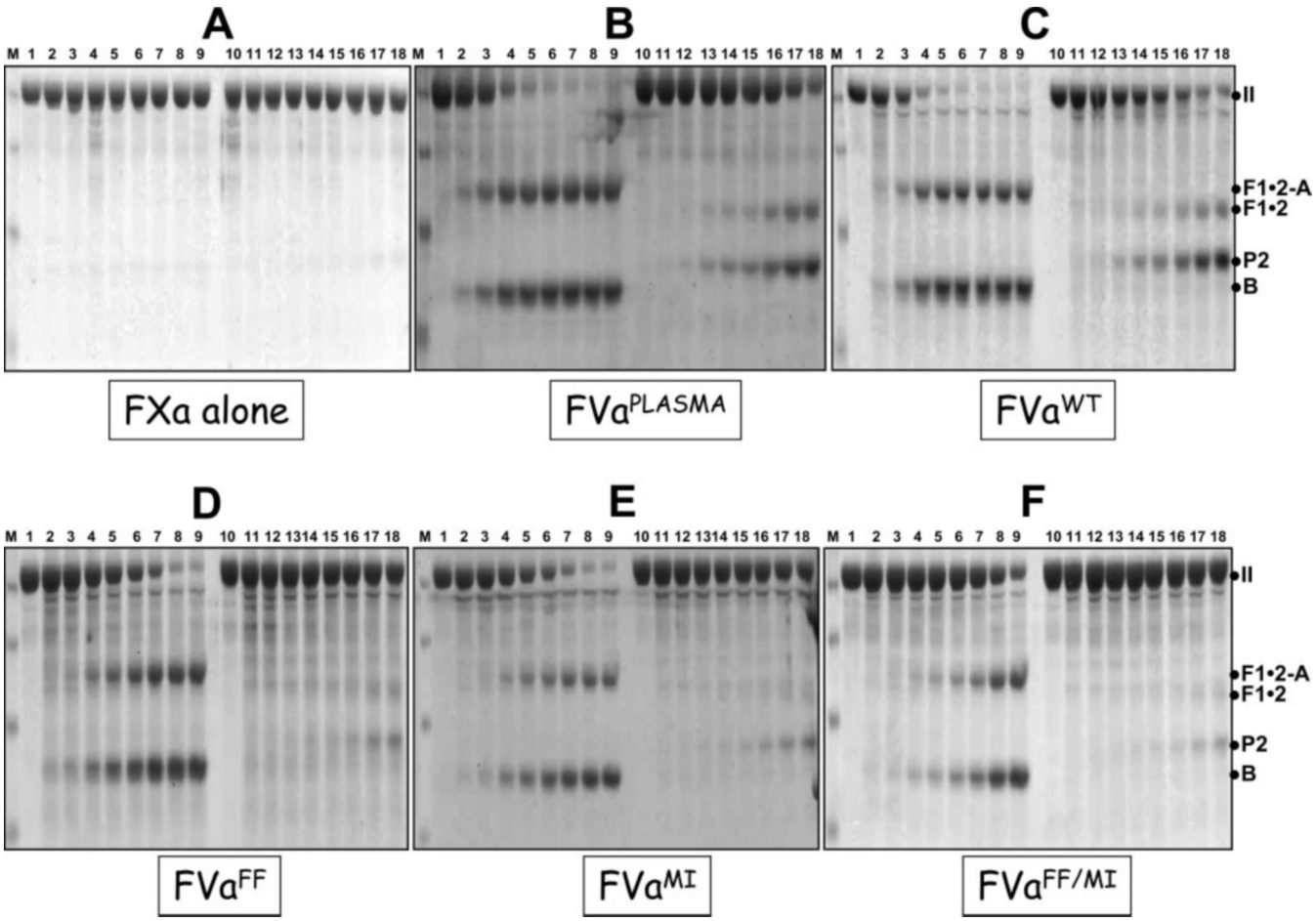


FIG. 5. **Analysis of the activation of mutant prothrombin molecules by prothrombinase assembled with mutant factor Va molecules.** Mutant prothrombin molecules were incubated in different mixtures with PCPS vesicles, DAPA, and various factor Va molecules (10 nM). The reaction was started by the addition of factor Xa, and the samples were treated as detailed inder "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. *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 Va^{FF}; *E*, prothrombinase assembled with factor Va^{MI}; *F*, prothrombinase assembled with factor $Va^{FF/MI}$. 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.

TABLE III
Rate of activation of recombinant prothrombin derivatives in the presence of various recombinant factor Va species

Enzyme	rMZ-II (cleavage at Arg^{320})	Decrease[a]	rP2-II (cleavage at Arg^{271})	Decrease[a]
	moles consumed·s^{-1}·mole factor Xa^{-1}	*-fold*	*moles consumed·s^{-1}·mole factor Xa^{-1}*	*-fold*
Factor Xa alone	0.07 ± 0.11		0.05 ± 0.08	
Prothrombinase with wild type	24.6 ± 2.8		2.72 ± 0.23	
Prothrombinase with factor Va^{FF}	2.8 ± 0.2	8.8	0.33 ± 0.04	8.2
Prothrombinase with factor Va^{MI}	4.6 ± 0.5	5.3	0.52 ± 0.04	5.2
Prothrombinase with factor $Va^{FF/MI}$	1.4 ± 0.1	17.6	0.31 ± 0.06	8.8

[a] 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."

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 $Va^{FF/MI}$ and plasma-derived prothrombin, we have shown a 16.7-fold decreased rate of prothrombin consumption compared with the rate of prothrombin consumption obtained by prothrombinase assembled with the wild type cofactor molecule. A similar decreased rate of prothrombin consumption by prothrombinase assembled with the same mutant cofactor molecule under similar experimental conditions (~17-fold) was observed with rMZ-II (which is only cleaved at Arg^{320}). Collectively, the data suggest that prothrombinase assembled with factor $Va^{FF/MI}$ has a lower k_{cat} because of impaired initial cleavage at Arg^{320}. These data were confirmed by experiments studying prothrombin cleavage by prothrombinase saturated with factor $Va^{FF/MI}$ showing that activation still proceeds through the intermediate meizothrombin, albeit less efficiently because of a considerable decrease in the rate of cleavage at

Arg^{320}. Because factor $Va^{FF/MI}$ is only impaired in its interaction with factor Xa, our findings demonstrate that one of the roles of factor Va in prothrombinase is to adjust (increase) the k_{cat} of the enzyme by relocating the amino acids in and/or around the active site of factor Xa in an optimum position necessary to efficiently cleave at two sites distant by 36 Å (51). The thermodynamic data obtained demonstrate that mutation at residues Glu^{323}, Tyr^{324}, Glu^{330}, and Val^{331} of factor Va heavy chain have a cumulative effect on prothrombin catalysis by 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/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/or 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 ($Arg^3 \rightarrow Ala$, 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 Asp^{47} and Asp^{54}, 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 prothrombin 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 theoretically 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 therapeutic importance, the crystal structure of recombinant TAP-factor Xa clearly demonstrated that amino acid residues Asp^{47} and Asp^{54} 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 Arg^{320}), interact with amino acids Lys^{147}, Arg^{222}, and Lys^{224} 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.

Acknowledgments—We thank Dr. Ken Mann from the University of Vermont for providing the monoclonal antibodies to factor V, Dr. Alex Kurosky and Steve Smith from the University of Texas, Medical Branch at Galveston, for the sequencing of all prothrombin-derived fragments from polyvinylidene difluoride membranes, and Kerri Smith for technical support. We thank Dr. Edward Plow from the Department of Molecular Cardiology at the Cleveland Clinic Foundation for helpful advice and for critical reading of the manuscript.

REFERENCES

1. Kalafatis, M., Egan, J. O., van't Veer, C., Cawthern, K. M., and Mann, K. G. (1997) *Crit. Rev. Eukariotic Gene Expression* **7,** 241–280
2. Mann, K. G., and Kalafatis, M. (2003) *Blood* **101,** 20–30
3. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) *J. Biol. Chem.* **254,** 10952–10962
4. Heldebrant, C. M., Butkowski, R. J., Bajaj, S. P., and Mann, K. G. (1973) *J. Biol. Chem.* **248,** 7149–7163
5. Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 594–605
6. Esmon, C. T., Owen, W. G., and Jackson, C. M. (1974) *J. Biol. Chem.* **249,** 606–611
7. Bajaj, S. P., Butkowski, R. J., and Mann, K. G. (1975) *J. Biol. Chem.* **250,** 2150–2156
8. Downing, M. R., Butkowski, R. J., Clark, M. M., and Mann, K. G. (1975) *J. Biol. Chem.* **250,** 8897–8906
9. Butkowski, R. J., Elion, J., Downing, M. R., and Mann, K. G. (1977) *J. Biol. Chem.* **252,** 4942–4957
10. Rosing, J., Tans, G., Govers-Riemslang, J. W., Zwaal, R. F., and Hemker, H. C. (1980) *J. Biol. Chem.* **255,** 274–283
11. Nesheim, M. E., and Mann, K. G. (1983) *J. Biol. Chem.* **258,** 5386–5391
12. Krishnaswamy, S., Church, W. R., Nesheim, M. E., and Mann, K. G. (1987) *J. Biol. Chem.* **262,** 3291–3299
13. Brufatto, N., and Nesheim, M. E. (2003) *J. Biol. Chem.* **278,** 6755–6764
14. Krishnaswamy, S., (1990) *J. Biol. Chem.* **265,** 3708–3718
15. Krishnaswamy, S., Jones, K. C., and Mann, K. G. (1988) *J. Biol. Chem.* **263,** 3823–3834
16. Boskovic, D. S., Bajzar, L. S., and Nesheim, M. E. (2001) *J. Biol. Chem.* **276,** 28686–28693
17. Degen, S. J. F., and Sun, W. Y. (1998) *Crit. Rev. Eukariotic Gene Expression* **8,** 203–224
18. Nesheim, M. E., Foster, W. B., Hewick, R., and Mann, K. G. (1984) *J. Biol. Chem.* **259,** 3187–3196
19. Suzuki, K., Dahlbäck, B., and Stenflo, J. (1982) *J. Biol. Chem.* **257,** 6556–6564
20. Esmon, C. T., Owen, W. G., Duiguid, D. L., and Jackson, C. M. (1973) *Biochim. Biophys. Acta* **310,** 289–294
21. Guinto, E. R., and Esmon, C. T. (1984) *J. Biol. Chem.* **259,** 13986–13992
22. Kalafatis, M., Xue, J., Lawler, C. M., and Mann, K. G. (1994) *Biochemistry* **33,** 6538–6545
23. Kalafatis, M., Rand, M. D., and Mann, K. G. (1994) *J. Biol. Chem.* **269,** 31869–31880
24. Mann, K. G., Hockin, M. F., Begin, K. J., and Kalafatis, M. (1997) *J. Biol. Chem.* **272,** 20678–20683
25. Anderson, P. J., Nesset, A., Dharmawardana, K. R., and Bock, P. E. (2000) *J. Biol. Chem.* **275,** 16435–16442
26. Chen, L., Yang, L., and Rezaie, A. R. (2003) *J. Biol. Chem.* **278,** 27564–27569
27. Kalafatis, M., Beck, D. O., and Mann, K. G. (2003) *J. Biol. Chem.* **278,** 33550–33561
28. Beck, D. O., Bukys, M. A., Singh, L. S., Szabo, K. A., and Kalafatis, M. (2004) *J. Biol. Chem.* **279,** 3084–3095
29. Kalafatis, M., and Mann, K. G. (2001) *J. Biol. Chem.* **276,** 18614–18623
30. Kalafatis, M., and Beck, D. O. (2002) *Biochemistry* **41,** 12715–12728
31. Singh, L. S., Bukys, M. A., Beck, D. O., and Kalafatis, M. (2003) *J. Biol. Chem.* **278,** 28335–28345
32. Bajaj, S. P., and Mann, K. G. (1973) *J. Biol. Chem.* **248,** 7729–7741
33. Côté, H. C. F., Stevens, W. K., Bajzar, L., Banfield, D. K., Nesheim, M. E., and MacGillivray, T. A. (1994) *J. Biol. Chem.* **269,** 11374–11380
34. Jenny, R. J., Pittman, D. D., Toole, J. J., Kriz, R. W., Aldape, R. A., Hewick, R. M., Kaufman, R. J., and Mann, K. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4846–4850
35. Nesheim, M. E., Katzmann, J. A., Tracy, P. B., and Mann, K. G. (1980) *Methods Enzymol.* **80,** 243–275
36. Barenholz, Y., Gibbs, D., Litmann, B. J., Goll, J., Thompson, T., and Carlson, D. (1977) *Biochemistry* **16,** 2806–2910
37. Gomori, G. (1942) *J. Lab. Clin. Med.* **27,** 955–960
38. Camire, R. M., Kalafatis, M., and Tracy, P. B. (1998) *Biochemistry* **37,** 20527–20534
39. Krishnaswamy, S., Williams, E. B., and Mann, K. G. (1986) *J. Biol. Chem.* **261,** 9684–9693
40. Krishnaswamy, S. (1992) *J. Biol. Chem.* **272,** 23696–23706

41. Ackers, G. K., and Smith, F. R. (1985) *Annu. Rev. Biochem.* **54,** 597–629
42. LiCata, V. J., and Ackers, G. K. (1995) *Biochemistry* **34,** 3133–3139
43. Wells, J. A. (1990) *Biochemistry* **29,** 8509–8517
44. Mildvan, A. S. (2004) *Biochemistry* **43,** 14517–14520
45. Mildvan, A. S., Weber, D. J., and Kuliopulos, A. (1992) *Arch. Biochem. Biophys.* **294,** 327–340
46. Laemmli, U. K. (1970) *Nature* **227,** 680–685
47. Towbin, H., Staehlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
48. Vijayalakshmi, J., Padmanabhan, K. P., Mann, K. G., and Tulinsky, A. (1994) *Protein Sci.* **3,** 2254–2271
49. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) *EMBO J.* **8,** 3467–3475
50. Skrzypczak, E., Rydel, T., Tulinsky, A., Fenton, J. W., and Mann, K. G. (1989) *J. Mol. Biol.* **206,** 755–757
51. Martin, P. D., Malkowski, M. G., Box, J., Esmon, C. T., and Edwards, B. F. (1997) *Structure* **5,** 1681–1693
52. Waxman, L., Smith, D. E., Arcuri, K. E., and Vlasuk, G. P. (1990) *Science* **248,** 593–596
53. Jordan, S. P., Mao, S. S., Lewis, S. D., and Shafer, J. A. (1992) *Biochemistry* **31,** 5374–5380
54. Krishnaswamy, S., Vlasuk, G. P., and Bergum, P. W. (1994) *Biochemistry* **33,** 7897–7907
55. Betz, A., Vlasuk, G. P., Bergum, P. W., and Krishnaswamy, S. (1997) *Biochemistry* **36,** 181–191
56. Dunwiddie, C. T., Neeper, M. P., Nutt, E. M., Waxman, L., Smith, D. E., Hofman, K. J., Lumma, P. K., Garsky, V. M., and Vlasuk, G. P. (1992) *Biochemistry* **31,** 12126–12131
57. Wei, A., Alexander, R. S., Duke, J., Ross, H., Rosenfeld, S. A., and Chang, C. H. (1998) *J. Mol. Biol.* **283,** 147–154