Cooperative Regulation of the Activity of Factor Xa within Prothrombinase by Discrete Amino Acid Regions from Factor Va Heavy Chain†

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ABSTRACT: The prothrombinase complex catalyzes the activation of prothrombin to α-thrombin. We have repetitively shown that amino acid region ^{695}DYDY^{698} from the COOH terminus of the heavy chain of factor Va regulates the rate of cleavage of prothrombin at Arg^{271} by prothrombinase. We have also recently demonstrated that amino acid region ^{334}DY^{335} is required for the optimal activity of prothrombinase. To assess the effect of these six amino acid residues on cofactor activity, we created recombinant factor Va molecules combining mutations at amino acid regions 334–335 and 695−698 as follows: factor V^{3K} (^{334}DY^{335} → KF and ^{695}DYDY^{698} → KFKF), factor V$^{KF/4A}$ (^{334}DY^{335} → KF and ^{695}DYDY^{698} → AAAA), and factor V^{6A} (^{334}DY^{335} → AA and ^{695}DYDY^{698} → AAAA). The recombinant factor V molecules were expressed and purified to homogeneity. Factor Va^{3K}, factor Va$^{K4/4A}$, and factor Va^{6A} had reduced affinity for factor Xa, when compared to the affinity of the wild-type molecule (factor Va^{Wt}) for the enzyme. Prothrombinase assembled with saturating concentrations of factor Va^{3K} had a 6-fold reduced second-order rate constant for prothrombin activation compared to the value obtained with prothrombinase assembled with factor Va^{Wt}, while prothrombinase assembled with saturating concentrations of factor Va$^{KF/4A}$ and factor Va^{6A} had approximately 1.5-fold reduced second-order rate constants. Overall, the data demonstrate that amino acid region 334–335 together with amino acid region 695−698 from factor Va heavy chain are part of a cooperative mechanism within prothrombinase regulating cleavage and activation of prothrombin by factor Xa.

α-Thrombin is required for survival and is formed following activation of its inactive precursor, prothrombin, by the prothrombinase complex (*1*). The prothrombinase complex is composed of the enzyme, factor Xa, and the protein cofactor, factor Va, assembled on a membrane surface in the presence of divalent metal ions (*1, 2*). Even though factor Xa alone can activate prothrombin, the rate of this reaction is not compatible with survival. Incorporation of factor Va into the prothrombinase complex increases the catalytic efficiency of prothrombin activation by 5 orders of magnitude, thus providing the physiological pathway for α-thrombin production. This increase in the efficiency of prothrombinase is the result of a 100-fold decrease in the K_m and a 3000-fold increase in the k_{cat} of prothrombinase compared to those for factor Xa alone. The decrease in the K_m is the result of a tighter interaction of the prothrombinase complex with the membrane surface, which results in higher local concentrations of the enzyme, while the increase in the k_{cat} of the enzyme is credited solely to the productive interaction between factor Va and factor Xa (*3*−*5*). While the exact molecular mechanism by which factor Va exerts its cofactor function is still under investigation, it has been well established that the productive interaction of the heavy chain of factor Va with prothrombin involves anion binding exosite I of prothrombin (*6*−*11*). Human factor V circulates in plasma as a single-chain inactive precursor. Proteolytic cleavage of the procofactor by α-thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545} produces a heterodimer consisting of a heavy chain ($M_r \sim 105000$; A1 and A2 domains) and a light chain ($M_r \sim 74000$; A3, C1, and C2 domains) associated through divalent metal ions (*12*−*15*) (Figure 1). We have demonstrated that a binding site for factor Xa is contained within the heavy chain of the cofactor (*16, 17*), and we have established that the COOH terminus of the factor Va heavy chain (amino acids 680−709) is essential for optimal expression of cofactor activity because it promotes a productive interaction with prothrombin, regulating the rate of cleavage at Arg^{271} by prothrombinase (*18*−*21*). This region of the cofactor is highly acidic in nature and contains several tyrosine residues that have been shown to have potential to be involved in factor V activation by α-thrombin and proper cofactor function (*22*). We have also recently shown that

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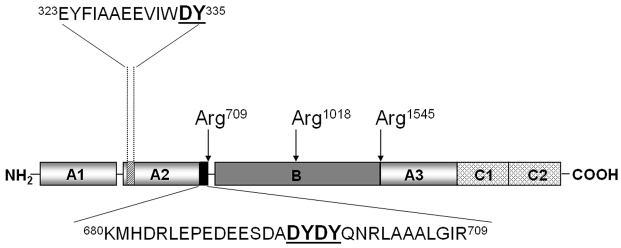


Factor V^{Wt}	**No Mutation**
Factor V^{3K}	**$D^{334} \rightarrow K$, $Y^{335} \rightarrow F$, $D^{695} \rightarrow K$, $Y^{696} \rightarrow F$, $D^{697} \rightarrow K$, $Y^{698} \rightarrow F$**
Factor $V^{KF/4A}$	**$D^{334} \rightarrow K$, $Y^{335} \rightarrow F$, $D^{695} \rightarrow A$, $Y^{696} \rightarrow A$, $D^{697} \rightarrow A$, $Y^{698} \rightarrow A$**
Factor V^{6A}	**$D^{334} \rightarrow A$, $Y^{335} \rightarrow A$, $D^{695} \rightarrow A$, $Y^{696} \rightarrow A$, $D^{697} \rightarrow A$, $Y^{698} \rightarrow A$**

FIGURE 1: Human factor V structure. Factor V is composed of 2196 amino acids with three A domains, a connecting B region, and two C domains. The procofactor is activated following three cleavages by α-thrombin at Arg^{709}, Arg^{1018}, and Arg^{1545}. The mutations within a 13-amino acid region of the A2 domain of the heavy chain of the molecule that is crucial for cofactor activity (amino acid residues 323−335) (*23*) and within the acidic hirudin-like COOH-terminal region of the heavy chain (amino acid residues 680−709) required for the optimal function of prothrombinase (*20, 21*) are indicated together with the designation of the recombinant mutant factor V molecules created and used throughout this work. Residues DY from both regions that are mutated in this study are bold and underlined.

residues from the central portion of the heavy chain of factor Va (amino acid region 334–335) are crucial for cofactor function (*23*). This study was undertaken to understand the importance of these two spatially distinct, important regions of factor Va heavy chain when acting in concert on prothrombinase complex assembly and function.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Diisopropyl fluorophosphate (DFP), *O*-phenylenediamine (OPD) dihydrochloride, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), Trizma (Tris base), and Coomassie Brilliant Blue R-250 were purchased from Sigma (St. Louis, MO). Factor V-deficient plasma was from Research Protein Inc. (Essex Junction, VT). Secondary anti-mouse and anti-sheep IgG coupled to peroxidase were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL). L-α-Phosphatidylserine (PS)[1] and L-α-phosphatidylcholine (PC) were from Avanti Polar Lipids (Alabaster, AL). Chemiluminescent reagent ECL^+ and Heparin-Sepharose were from AmershamPharmacia Biotech Inc. (Piscataway, NJ). Normal reference plasma and chromogenic substrate Spectrozyme-TH were from American Diagnostica Inc. (Greenwich, CT). RecombiPlasTin for the clotting assays was purchased from Instrumentation Laboratory Co. (Lexington, MA). Dansylarginine-*N*-(3-ethyl-1,5-pentanediyl) amide (DAPA), human factor Xa, human α-thrombin, and human prothrombin were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Factor V cDNA was from American Type Tissue Collection (Manassas, VA) (ATCC catalog no. 40515 pMT2-V). All restriction enzymes were from New England Biolabs (Beverly, MA). All molecular biology and tissue culture reagents, specific primers, and medium were from Gibco, Invitrogen Corp. (Grand Island, NY), or as indicated. Human factor V monoclonal antibodies ($\alpha HFV_{HC}17$ and $\alpha HFV_{LC}9$) and monoclonal antibody αHFV1 coupled to Sepharose were provided by K. G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT).

Construction of Recombinant Factor V Molecules. Factor V^{KFKF} and factor V^{4A} were constructed as previously described by our laboratory (*19, 21*). Recombinant factor V molecules with combining mutations, factor V^{3K}, factor $V^{KF/4A}$, and factor V^{6A}, were constructed using Stratagene's QuikChange XL site-directed mutagenesis kit. Factor V^{3K} was constructed using the mutagenic primers 5′-C ATT TGG **A**A**G**T**T**T GCA CCT G-3′ (forward) and 5′-C AGG TGC A**A**A **C**T**T**CCA AAT G-3′ (reverse) (corresponding to $^{334}DY^{335} \rightarrow KF$ mutations), using factor V^{KFKF} as a template (bold underlined letters identify the mutated bases) (*19*). To construct factor $V^{KF/4A}$, these same primers were used and factor V^{4A} (*21*) was used as the template in the PCR. Factor V^{6A} was constructed with the primers 5′-GAG GAA GTC ATT TGG G**C**C **GCC** GCA CCT GTA ATA-3′ (forward) and 5′-TAT TAC AGG TGC **GGC** G**G**C CCA AAT GAC TTC CTC-3′ (reverse), with factor V^{4A} (*21*) as the template in the PCR. The mutations were confirmed by DNA sequencing (DNA Analysis Facility, Cleveland State University).

Transient Transfection, Purification, and Assay of Recombinant Factor V Molecules. Purified wild-type factor V (factor V^{Wt}), factor V^{3K}, factor $V^{KF/4A}$, and factor V^{6A} plasmids were transfected into the COS-7L cells with fugene 6 (Roche Diagnostics), according to the manufacturer's instructions as described extensively by our laboratory (*17, 24*). The recombinant proteins were purified on a 2 mL column of monoclonal antibody αhFV1 coupled to Sepharose as described previously (*25*). The purified proteins were stored at −80 °C in small aliquots to avoid repeated freeze−thaw cycles. The activity and the integrity of the recombinant factor V molecules were confirmed by clotting assays using factor V-deficient plasma and Western blotting with monoclonal and polyclonal antibodies as described previously (*26, 27*). Following activation of factor V, α-thrombin was inhibited by the addition of DFP. It is also important to note that any residual α-thrombin activity that could contribute to our experimental data is eliminated by the addition of DAPA to the reaction mixtures. DAPA is a very potent synthetic inhibitor of α-thrombin and eliminates any feedback activity that would interfere in experiments presented herein. A detailed description of all details relating to our experimental procedures is provided in refs *17*, *24*, *25*, and *28*.

Gel Electrophoresis and Western Blotting. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analyses were carried out using 4 to 12% gradient gels or

[1] Abbreviations: PS, L-α-phosphatidylserine; PC, L-α-phosphatidylcholine; PCPS, small unilamellar phospholipid vesicles composed of 75% PC and 25% PS (w/w); SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; factor Va^{Wt}, recombinant human wild-type factor Va; factor Va^{KF}, recombinant human factor Va with the $D^{334} \rightarrow K$ and $Y^{335} \rightarrow F$ mutations; factor Va^{AA}, recombinant human factor Va with the $D^{334} \rightarrow A$ and $Y^{335} \rightarrow A$ mutations; factor V^{KFKF}, recombinant human factor V with the $^{695}DYDY^{698} \rightarrow KFKF$ mutation; factor V^{4A}, recombinant human factor V with the $^{695}DYDY^{698} \rightarrow AAAA$ mutation; factor Va^{3K}, recombinant human factor Va with the $D^{334} \rightarrow K$, $Y^{335} \rightarrow F$, $D^{695} \rightarrow K$, $Y^{696} \rightarrow F$, $D^{697} \rightarrow K$, and $Y^{698} \rightarrow F$ mutations; factor Va^{6A}, recombinant human factor Va with the $D^{334} \rightarrow A$, $Y^{335} \rightarrow A$, $D^{695} \rightarrow A$, $Y^{696} \rightarrow A$, $D^{697} \rightarrow A$, and $Y^{698} \rightarrow A$ mutations; factor $V^{KF/4A}$, recombinant human factor Va with the $D^{334} \rightarrow K$, $Y^{335} \rightarrow F$, $D^{695} \rightarrow A$, $Y^{696} \rightarrow A$, $D^{697} \rightarrow A$, and $Y^{698} \rightarrow A$ mutations.

9.5% gels following reduction with 2% β-mercaptoethanol, according to the method of Laemmli, and the protein was visualized by being stained with Coomassie Brilliant Blue R-250, followed by destaining in a solution of methanol, acetic acid, and water (*29*). In several experiments, the protein was transferred to polyvinylidene difluoride (PVDF) membranes following the method described by Towbin et al. (*30*). Factor Va heavy and light chains were probed with the appropriate monoclonal and polyclonal antibodies and visualized with chemiluminescence.

Kinetic Titrations of Prothrombinase. The ability of the recombinant factor V molecules to assemble in the prothrombinase complex and bind to the enzyme was assessed in a discontinuous assay described in detail elsewhere (*24*). All recombinant factor V molecules were activated with α-thrombin, and reaction mixtures contained PCPS vesicles (20 μM), DAPA (3 μM), and factor Xa (varying concentrations), in reaction buffer consisting of HEPES, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.01% Tween 20 (pH 7.40). For the practical calculation of the K_D between the factor Va molecules and factor Xa, assays were performed in the presence of a limiting factor Xa concentration (15 pM) and varying concentrations of the recombinant factor Va species (25 pM to 5 nM). For the determination of the kinetic constants of prothrombinase function (K_m and k_{cat}), experiments were conducted with a limiting amount of factor Xa (5 pM) in the presence of a fixed saturating amount of the various recombinant factor Va molecules (10−30 nM) and varying the substrate concentration. The rate of α-thrombin generation was measured using a chromogenic substrate, Spectrozyme TH, which probes for α-thrombin generation. The initial rate of α-thrombin generation was analyzed with Prizm (GraphPad). The change in transition-state stabilization free energy, which measures the effect of the mutations in the cofactor on the catalytic site of the enzyme, was calculated for the double mutants as extensively described previously by our laboratory (*23, 24*).

Studies of the Pathway for Prothrombin Activation by Gel Electrophoresis. Prothrombin was incubated in a reaction mixture containing 20 μM PCPS, 50 μM DAPA, and various concentrations of factor Va^{Wt}, factor Va^{3K}, factor $Va^{KF/4A}$, and factor Va^{6A} (activated with α-thrombin, 10−30 nM) in TBS with Ca^{2+}. A zero point was taken, and the addition of factor Xa (1 nM) marked the start of the reaction. Aliquots of the reaction mixture were removed at selected time points and treated as described previously (*20, 24*). Calculations of the rates of prothrombin consumption were performed as described (*20*).

Molecular Dynamics Simulations. The amino acid changes in the factor Va model (*15*) were introduced using SwissPdbViewer. Amino acid side chain orientation was selected using the rotamer library provided with SwissPdbViewer (*31*). The new model [factor Va^{3K} ($D^{334} \rightarrow K$, $Y^{335} \rightarrow F$, $D^{695} \rightarrow K$, $Y^{696} \rightarrow F$, $D^{697} \rightarrow K$, $Y^{698} \rightarrow F$)] was solvated in a simulation box using the SPC water model using the GROMACS package (*32*). The minimum distance between the periodic images of the molecule was set to 1 nm. The simulation time was set to 3 ns. Simulation details were kept the same as recently described for the study of factor Va molecules with mutations in the D^{334}-Y^{335} region (*23*). The simulation equilibrated after 2 ns, and the last nanosecond was used for analysis. Equilibration of the system was also assessed on the basis of the negligible drift of several parameters such as the potential energy, pressure, and temperature.

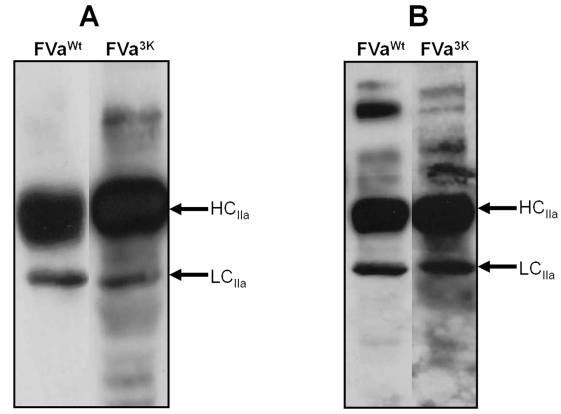


FIGURE 2: Electrophoretic analyses of wild-type factor V and recombinant factor V^{3K}. (A) Factor V^{Wt} and factor V^{3K} were activated with α-thrombin as described in Experimental Procedures and analyzed by SDS−PAGE. Following transfer to a PVDF membrane, immunoreactive fragments were detected with monoclonal antibodies $\alpha HFVa_{HC}17$ (recognizing an epitope on the heavy chain of the cofactor between amino acid residues 307 and 506) and $\alpha HFVa_{HC}9$ (recognizing the light chain). (B) Factor V^{Wt} and factor V^{3K} were activated with α-thrombin and analyzed by SDS−PAGE. Following transfer to a PVDF membrane, immunoreactive fragments were detected with a polyclonal antibody. In both cases, the immunoblot containing the mutant molecule was overexposed. At the right are shown the positions of the heavy and light chains of factor Va.

RESULTS

Transient Expression and Activation of Recombinant Factor V Molecules. To assess the importance of amino acid regions 334–335 and 695−698 from the heavy chain of factor Va, we constructed molecules that combine mutations in these previously identified "hot spots" of the molecule (*21, 23*) (Figure 1). We prepared recombinant factor Va molecules, factor V^{3K} (D334K, Y334F, D695K, Y696F, D697K, and Y698F), factor $V^{KF/4A}$ (D334K, Y335F, D695A, Y696A, D697A, and Y698A), and factor V^{6A} (D334A, Y335A, D695A, Y696A, D697A, and D698A) (Figure 1). Recombinant factor V^{Wt} and the mutant molecules were expressed in mammalian cells and purified to homogeneity as described previously (*25*). Figure 2 illustrates a typical quality control procedure highlighting factor V^{3K}. Following activation by α-thrombin, SDS−PAGE analyses followed by immunoblotting with specific monoclonal antibodies to the heavy and light chain of the cofactor demonstrate that the mutant recombinant protein is intact, homogeneous, and composed of heavy and light chains that migrated according to their expected molecular weights (Figure 2A). In addition, control experiments using a polyclonal antibody to factor V demonstrate that all factor V molecules were fully activated prior to use (Figure 2B). Similar results were obtained with factor $V^{KF/4A}$ and factor V^{6A}.

The recombinant molecules were first assessed for their clotting activity in a two-stage assay. α-Thrombin activation of factor V^{Wt} resulted in a cofactor with clotting activity (2400 units/mg) similar to that of the plasma-derived molecule (2500 units/mg). Under similar experimental conditions, all mutant recombinant factor Va molecules displayed

reduced clotting activities compared to the wild-type cofactor molecule. Factor Va^{6A} had a 4-fold decrease in clotting activity (600 units/mg); factor $Va^{KF/4A}$ displayed a 6.3-fold reduction in clotting activity (380 units/mg), while factor Va^{3K} was most impaired in its clotting activity with a 20-fold decrease (120 units/mg). These data demonstrate that the mutations are detrimental to factor Va clotting activity.

Kinetic Analyses of Recombinant Factor Va Molecules. We examined the ability of the recombinant factor Va molecules to bind factor Xa and assemble in prothrombinase using an assay employing purified reagents and a chromogenic substrate to probe for α-thrombin generation. The assay was performed under conditions of limiting factor Xa concentrations while the concentration of recombinant factor Va molecules was varied. Figure 3 and Table 1 show the results of the kinetic studies. The data demonstrate that under the experimental conditions that were used, factor Va^{Wt} has an affinity for the enzyme, factor Xa, similar to that of its plasma counterpart (Table 1). Conversely, the recombinant factor Va molecules, factor Va^{3K}, factor $Va^{KF/4A}$, and factor Va^{6A}, were impaired in their ability to bind the enzyme, having dissociation constants ~6−8-fold higher than that of the wild-type molecule. These results are surprising because when amino acid regions 334–335 and 695−698 are mutated separately, there is no significant effect on the dissociation constant of the recombinant molecules for plasma-derived factor Xa (Table 1) (*21, 23*). These data suggest that the mutations destabilize the factor Va binding domain(s) for factor Xa.

We further evaluated the effect of the mutations on the K_m and k_{cat} values of prothrombinase. The raw data are displayed in Figure 3B, and the kinetic constants are provided in Figure 4 and Table 1. Because we wanted to prevent the possibility that differences between prothrombinase assembled with factor Va^{Wt} and prothrombinase assembled with the factor Va mutant molecules may be attributed to subtle differences in the K_{Dapp} of factor Va for factor Xa, which would result in a smaller amount of prothrombinase formed, all experiments described below were conducted under conditions where more than 95% of factor Xa was saturated with factor Va. Calculations were performed using the dissociation constants of all recombinant molecules for factor Xa shown in Table 1. Under the experimental conditions that were employed, the mutations had no effect on the K_m of the reaction, while the catalytic efficiencies of prothrombinase assembled with the mutant recombinant cofactor molecules were decreased compared to the catalytic efficiency of prothrombinase assembled with factor Va^{Wt} (Figure 4A). The data show that prothrombinase assembled with factor Va^{3K}, factor $Va^{KF/4A}$, or factor Va^{6A} had a 6-, 1.7-, or 1.3-fold reduced catalytic efficiency, respectively, compared to the value obtained for prothrombinase assembled with factor Va^{Wt}. Conversely, when amino acid region of residues 695−698 alone is mutated to either KFKF or AAAA, factor Va^{KFKF} and factor Va^{4A} have 1.2- and 1.4-fold increased catalytic efficiencies, respectively, compared to the wild-type molecule (Table 1 and Figure 4A). This increased catalytic efficiency can be explained by sustained meizothrombin accumulation as recently demonstrated (*21*). Indeed, when the ability of the recombinant factor Va molecules to be incorporated into prothrombinase and activate prothrombin was investigated by gel electrophoresis,

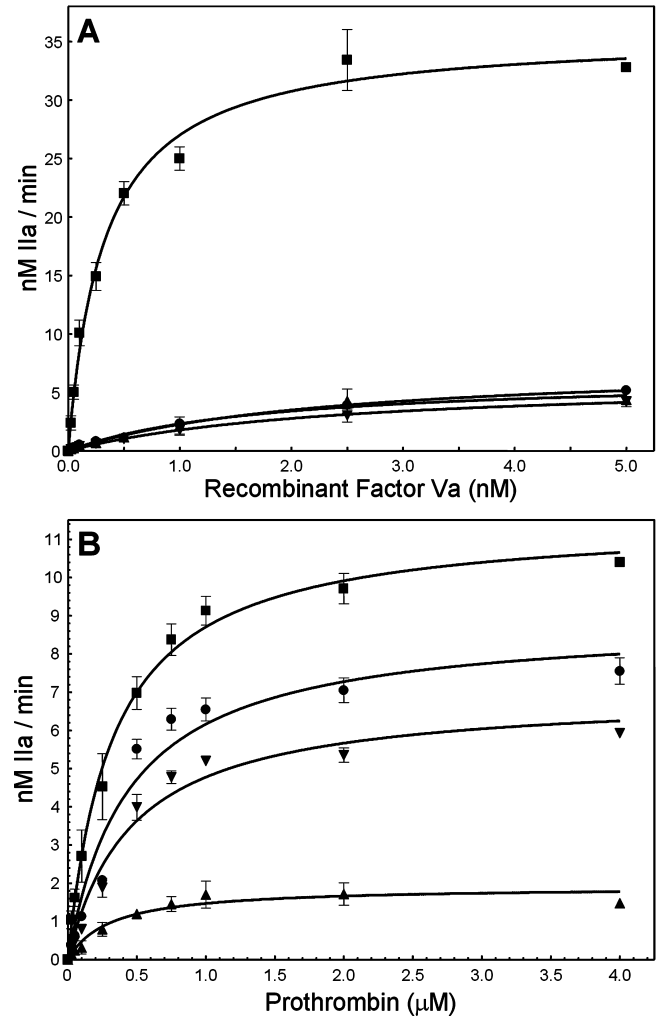


FIGURE 3: (A) Factor Va titrations to determine the affinity of the recombinant factor Va molecules for factor Xa. α-Thrombin generation experiments were carried out as described in Experimental Procedures. The prothrombinase complex was assembled with varying concentrations of recombinant factor Va^{Wt} (■), factor Va^{3K} (▲), factor $Va^{KF/4A}$ (▼), and factor Va^{6A} (●). The solid lines represent a nonlinear regression fit of the data using GraphPad Prizm for a one-binding site model. Titrations were performed in triplicate with at least three different preparations of purified proteins. (B) Prothrombin titrations to determine the kinetic parameters of prothrombinase assembled with various recombinant factor Va species. α-Thrombin generation experiments were carried out as described in Experimental Procedures by varying the substrate concentration (from 25 nM to 4 μM) with 5 pM factor Xa saturated with the factor Va species. The prothrombinase complex was assembled with recombinant factor Va^{Wt} (■), factor Va^{3K} (▲), factor $Va^{KF/4A}$ (▼), and factor Va^{6A} (●). The solid lines represent the nonlinear regression fit of the data using GraphPad Prizm for the Michaelis−Menten equation. The data shown are the averages of five different titrations with different preparations of purified proteins.

persistence of meizothrombin was observed in the case of prothrombin activation by prothrombinase assembled with factor Va^{KFKF} and factor Va^{4A} as assessed by the lingering of fragment 1•2-A which is indicative of initial cleavage at Arg^{320} (not shown). However, prothrombinases assembled with the cofactor molecules bearing the combined mutations have a slower rate of prothrombin consumption compared to the rates of prothrombin consumption by prothrombinase assembled with factor Va^{Wt}, indicating that prothrombinase assembled with factor Va^{3K}, factor $Va^{KF/4A}$, and factor Va^{6A}

Table 1: Functional Properties of Recombinant Factor Va Molecules

factor Va species	K_{Dapp} (nM)[a]	K_m (μM)[b]	k_{cat} (s^{-1})[b]
factor Va^{Plasma}	0.5 ± 0.2[c]	0.3 ± 0.05	40.5 ± 2
factor Va^{Wt}	0.3 ± 0.1	0.3 ± 0.04	38.4 ± 1
factor Va^{KF}[d]	0.6 ± 0.2	0.4 ± 0.06	19.2 ± 0.5
factor Va^{AA}[d]	0.6 ± 0.15	0.6 ± 0.07	14.4 ± 1
factor Va^{4A}	0.4 ± 0.2[c]	0.3 ± 0.06	53.4 ± 2
factor Va^{KFKF}	0.4 ± 0.2	0.3 ± 0.04	46.7 ± 3
factor Va^{3K}	1.8 ± 0.7	0.3 ± 0.10	6.4 ± 1
factor $Va^{KF/4A}$	2.5 ± 0.8	0.5 ± 0.06	23.2 ± 1
factor Va^{6A}	2.3 ± 0.2	0.4 ± 0.08	29.6 ± 2

[a] Apparent dissociation constants of recombinant factor Va for plasma-derived factor Xa (K_{Dapp}) were determined as described in Experimental Procedures at limiting factor Xa concentrations (the raw data are provided in Figure 3A). [b] Apparent K_m and k_{cat} were determined as described in Experimental Procedures at limiting factor Xa concentrations (5 pM) and saturating concentrations of factor Va (the raw data are provided in Figure 3B). [c] From ref *21*. [d] The recombinant molecules were fully characterized in ref *23*.

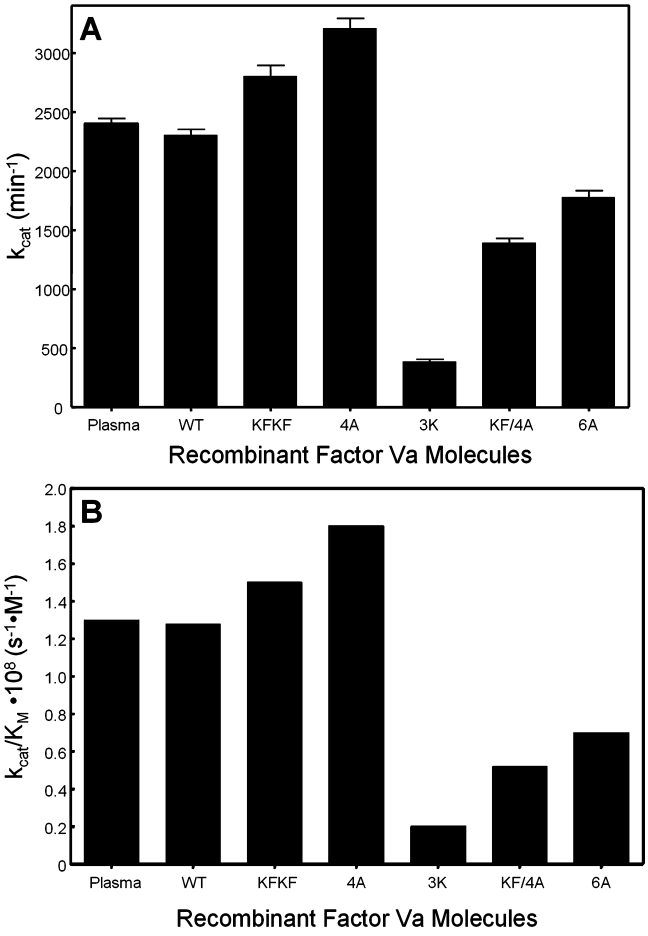
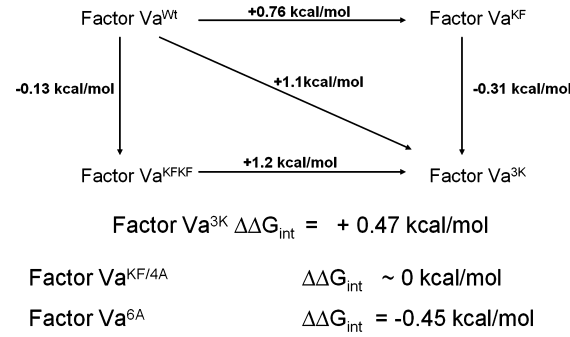


FIGURE 4: Determination of the kinetic parameters of prothrombinase assembled with the various recombinant factor Va species. α-Thrombin generation experiments were carried out as described in Experimental Procedures by varying the substrate concentration (see Figure 3B). The K_m for all cofactor molecules is given in Table 1, while the k_{cat} is shown in panel A (min^{-1}). Panel B provides the second-order rate constants (M^{-1} s^{-1}).

are impaired in their ability to cleave prothrombin at both Arg^{320} and Arg^{271}. The inability of prothrombinase assembled with the three mutant cofactor molecules to function optimally can be explained by the inability of factor Xa to efficiently convert prothrombin to α-thrombin because of diminished productive collisions between the enzyme and prothrombin. Comparison of the second-order rate constant

Scheme 1: Thermodynamic Cycle for Prothrombinase Activity following Substitutions in Factor Va Heavy Chain[a]



[a] $\Delta\Delta G_{int}$ is the free energy of interaction between the side chains of $^{334}DY^{335}$ and $^{695}DYDY^{698}$ of factor Va heavy chain and was calculated with eqs 3 and 4 of ref *23*. The values illustrated on the arrows identify the $\Delta\Delta G^{\ddagger}$ between recombinant species as indicated. Parallel arrows show the difference in catalytic efficiency between prothrombinase assembled with different mutant factor Va molecules. Different forms of prothrombinase are compared for their ability to activate prothrombin ($\Delta\Delta G^{\ddagger}_{WT \rightarrow KF}$ and $\Delta\Delta G^{\ddagger}_{WT \rightarrow AA}$ taken from ref *23*).

between prothrombinase assembled with the recombinant molecules (k_{cat}/K_m) demonstrates that the turnover number for prothrombinase assembled with the mutant molecules is 1.7−6-fold reduced (Figure 4B). Overall, the data demonstrate that amino acid regions 334–335 and 695−698 have important cooperative roles during prothrombin activation by prothrombinase.

Thermodynamic Effect of the Mutations in the Heavy Chain of Factor Va on Prothrombinase Function. Since prothrombinase is an enzyme composed of a catalytic subunit (factor Xa) and a regulatory subunit (factor Va), any perturbation in the interaction between the two proteins or any perturbations in the interaction of prothrombinase with the substrate caused by a mutation in the regulatory subunit may (will) influence the catalytic activity of factor Xa. The kinetic data showed that the factor Va^{Wt} → factor Va^{3K} transition results in a dramatic decrease in the catalytic efficiency of factor Va translated by a positive value for $\Delta\Delta G^{\ddagger}_{Wt \rightarrow 3K}$ of ~1.1 kcal/mol (Scheme 1). To determine if the side chains of the amino acids that are part of the two separate sites on the heavy chain of factor Va under investigation ($^{334}DY^{335}$ and $^{695}DYDY^{698}$) interact with each other to affect the catalytic efficiency of factor Xa, thermodynamic cycles were created and the exchange in free energy was calculated and is shown in Scheme 1. Factor Va^{3K} has a $\Delta\Delta G_{int}$ value of +0.47 kcal/mol, indicating that the DY → KF substitutions at amino acid regions 334–335 and 695−698 indeed have an additive effect. However, the severe detrimental effect of the $^{334}DY^{335}$ → KF substitution in the heavy chain for cofactor activity (*23*) is partially offset by the increased catalytic activity of meizothrombin that lingers following activation of prothrombin by prothrombinase assembled with factor Va^{KFKF} (Figure 4B). Similar results were found with prothrombinase assembled with factor $Va^{KF/4A}$ and factor Va^{6A}. However, substituting all amino acids with alanine had a less detrimental effect on factor Va cofactor activity than when charge reversal mutations were introduced. The data demonstrate a differential effect of the mutations depending on both their nature and their location. Mutations in region 334–335 alone are detrimental to the

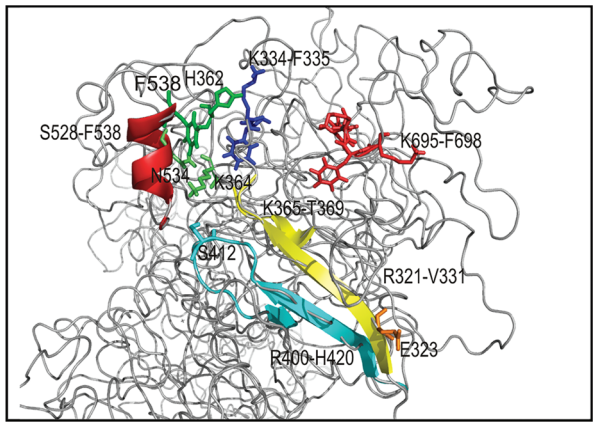


FIGURE 5: Structural analysis of factor Va heavy chain. The figure illustrates the final snapshot (3 ns) of factor Va^{3K} with the mutated amino acid residues K^{334} and F^{335} depicted as blue sticks and amino acid residues K^{695}, F^{696}, K^{697}, and F^{698} as red sticks. The region encompassing amino acid residues $S^{528}-F^{538}$ (α-helix) is represented as a red cartoon. The antiparalel β-sheet structures of amino acid residues $R^{321}-V^{331}$ and $K^{365}-T^{369}$ are colored yellow. Amino acid region $R^{400}-H^{420}$ is shown as cyan ribbons. Amino acid residues H^{362} and F^{538} are shown as green sticks. S^{412} is shown as cyan sticks. N^{534} and K^{364} are shown as lime sticks. E^{323} is shown as orange sticks.

function of prothrombinase regardless of their nature (*23*), while mutations in the COOH-terminal portion of the heavy chain of the molecule alone are perceived as beneficial since they result in increased k_{cat} values because of delayed cleavage at Arg^{271} and persistence of meizothrombin (*21*). We must thus conclude that the overall lower rate of α-thrombin formation by prothrombinase assembled with factor Va^{3K} as compared to prothrombinase assembled with factor Va^{Wt} is the direct result of decreased productive collisions between enzyme and substrate. Thus, these six amino acid residues from the heavy chain of factor Va stabilize the interaction of factor Xa with the cofactor and appear to be directly involved in the activity of prothrombinase because they may be also responsible for the interaction with prothrombin.

Structural Consequences of the Mutations. Figure 5 was constructed using the final snapshot of the model taken at 3 ns. The figure shows amino acid residues D^{334} and Y^{335} as blue sticks and amino acid residues $K^{695}-F^{698}$ as red sticks. The α-helix structure of amino acid region $S^{528}-F^{538}$ of the factor Va^{3K} molecule was found to be preserved during the simulation (Figure 5, red cartoon). The center of mass of the α-helix from the factor Va^{3K} molecule is shifted by 6.8 Å as compared with the same structure in the unmodified factor Va molecule. The closest distance between the $S^{528}-F^{538}$ α-helix and the 695−698 region was found to be ~24 Å in the case of factor Va^{3K} as compared to ~33 Å in the case of unmodified factor Va. Such a small distance between these amino acid groups in the factor Va^{3K} molecule is probably due to the stronger electrostatic interaction deriving from the charge reversal mutations (i.e., D → K at positions 695 and 698).

DISCUSSION

Our data demonstrate that amino acid region 334–335 together with amino acid region 695−698 of factor Va heavy chain work in concert for efficient conversion of prothrombin to α-thrombin by factor Xa within prothrombinase. We have recently demonstrated that amino acid region D^{334}-Y^{335} is critical for optimum rearrangement of enzyme and substrate required for efficient catalysis of prothrombin by prothrombinase (*23*). Mutation of these residues results in a molecule that is impaired in its cofactor capabilities for prothrombin activation. We have also established that residues ^{695}DYDY^{698} from the COOH terminus of factor Va heavy chain are involved in cofactor function and regulate the rate of meizothrombin cleavage by prothrombinase at Arg^{271} (*20, 21*). The data presented here demonstrate that when all six residues are mutated simultaneously, there is a significant effect on cofactor activity. However, depending on the nature of amino acid substitutions and their location, the effect may be large (nearly 6-fold reduction in the catalytic efficiency of prothrombinase assembled with factor Va^{3K}) or modest (1.7-fold reduction in the catalytic efficiency of prothrombinase made with factor Va^{6A}). In contrast, substitution of the amino acids in the two regions separately results in an 8-fold activity decrease or a 1.2-fold activity increase for prothrombinase assembled with either factor Va^{KF} or factor Va^{KFKF}, respectively (ref *23* and data presented here). Interestingly, while prothrombinase assembled with factor Va^{AA} is still severely impaired in its catalytic efficiency, prothrombinase assembled with factor Va^{4A} has an ~40% higher catalytic efficiency than prothrombinase assembled with the wild-type molecule. As a consequence, a less detrimental effect for prothrombin activation was observed with prothrombinase assembled with factor Va^{6A} as demonstrated by our assay using a chromogenic substrate to assess α-thrombin generation. All these findings can explain the dramatic loss of clotting activity of the mutant cofactors because of sustained meizothrombin generation (*21*). Meizothrombin has poor clotting activity because it has not yet exposed ABE-II that is covered by fragment 2 which is released upon cleavage at Arg^{271} (*33, 34*). Overall, the results support our early hypothesis that amino acid regions 334–335 and 695−698 are on the surface of factor Va heavy chain and act in concert for optimal cofactor effect during prothrombin activation (*15*).

We have recently reported that the distances between the centers of masses of the C^{α} atoms between amino acids 334 and 335 and the extremities of the factor Xa binding site taken separately, one by one, did not significantly change (*23*). However, while distance analysis between the center of masses of amino acid regions $D^{695}-Q^{699}$ and D^{334}–Y^{335} showed an approximate distance of 16.1 Å in factor Va^{Wt}, a similar distance analysis between amino acid groups K^{334}-F^{335} and $D^{695}-Q^{699}$ yielded a distance of 13.6 Å (*23*). Our previous study also revealed that there was a significant difference in the α-helix motif encompassing the region $S^{528}-F^{538}$ in factor Va^{KF} and factor Va^{AA}. However, in the case of factor Va^{3K}, the $S^{528}-F^{538}$ α-helix and the $R^{321}-V^{331}$/$K^{365}-T^{369}$ β-sheet appear to be unaffected by the mutations. Although one would expect the immediate milieu of factor Va^{3K} to be similar to factor Va^{KF} in the region encompassing amino acids 334 and 335, it appears that because of the second set of mutations (in region 695−698) factor Va^{3K} adopts a different conformation. Thus, although the secondary structures described in factor Va^{3K} were not affected structurally, the general conformation of the molecule in that region changed significantly. These data suggest a rearrange-

ment of amino acids belonging to both sites following amino acid substitution. These rearrangements are detrimental to cofactor activity as demonstrated by our kinetic experiments and the thermodynamic data. This, in turn, can be attributed to the impaired interaction between factor Va and factor Xa (illustrated by the decreased affinity of the mutant molecules for factor Xa).

Earlier data have demonstrated that meizothrombin has increased catalytic efficiency toward chromogenic and fluorogenic substrates usually employed to assess α-thrombin formation (*3, 35*−*37*) and that factor Va interacts with the substrate, prothrombin, via the heavy chain of the molecule (*8, 10*). A synthetic peptide, representing amino acid residues 697−709 from the COOH terminus of the heavy chain of factor Va, was found to interact directly with α-thrombin-agarose, indicating that these amino acid residues of the cofactor provide a binding site for α-thrombin and/or prothrombin (*18, 19*). Keeping all these findings in mind, we have recently established that the acidic COOH-terminal portion of factor Va heavy chain controls the rate of meizothrombin cleavage at Arg^{271} by factor Xa within prothrombinase, providing a productive interactive site of the cofactor with prothrombin, and thus contributes significantly to the enhanced function of prothrombinase (*20, 21*). In contrast, Toso and Camire using recombinant proteins have reported that the acidic COOH-terminal region of factor Va heavy chain does not contribute to the increased catalytic activity of prothrombinase with respect to prothrombin activation (*38*). This conclusion was surprising and at odds with the data provided in their work since they show that prothrombinases assembled with recombinant cofactor molecules with deletions at the carboxy-terminal end of factor Va heavy chain, factor Va^{692} (missing amino acid residues 693−709) and factor Va^{678} (missing amino acid residues 679−709), display k_{cat} values that are 1.3- and 1.5-fold increased, respectively, compared to the catalytic efficiency of prothrombinase assembled with recombinant factor Va^{Wt} (*38*). Experiments provided here also demonstrate that prothrombinases assembled with recombinant factor Va^{KFKF} and factor Va^{4A} display 1.2- and 1.4-fold increased k_{cat} values, respectively, compared to the catalytic efficiency of prothrombinase assembled with recombinant factor Va^{Wt} because of sustained meizothrombin formation during prothrombin activation. These results are in complete agreement with earlier data (*18, 39*−*41*) and our recent findings (*21*), and the combined findings clearly demonstrate that the sequence ^{695}DYDY^{698} controls meizothrombin concentration by controlling the rate of cleavage at Arg^{271} during prothrombin activation by prothrombinase.

The bulk of the data published by our laboratory provide strong evidence pointing to the fact that the COOH-terminal acidic region of factor Va heavy chain and in particular amino acid sequence DYDY is crucial for efficient α-thrombin generation at the place of vascular injury. Bearing in mind the poor fibrinogen clotting properties of meizothrombin, we can hypothesize that following vascular injury, individuals with a factor Va molecule lacking the COOH-terminal region of the heavy chain will produce more meizothrombin, rather than α-thrombin, at the place of vascular injury, resulting in mild bleeding tendencies. This hypothetical situation is verified by clinical data obtained from patients that are homozygous for an amino acid substitution in the prothrombin gene at Arg^{271} (*42*−*46*). All these patients that have a prothrombin molecule that is unable to be cleaved at Arg^{271} circulating in their plasma were identified because of their hemorrhagic syndrome clinically resembling mild hemophilia. Thus, while meizothrombin can competently substitute for most of α-thrombin's procoagulant and anticoagulant functions locally at the place of vascular injury in the presence of an adequate membrane surface (*33, 47*−*52*), the molecule still lacks the ultimate procoagulant function (i.e., efficient fibrin formation). All these observations together with the fact that prothrombin can be activated through two distinct pathways that differ in terms of rate constants and intermediates may allow for the design of novel anticoagulant molecules with the potential to act as a control switch and able to modulate specific events during α-thrombin (and meizothrombin) formation according to a precise need. DYDYQ may be the prototype for such a class of anticoagulants, and we have recently reported that DYDYQ inhibits prothrombin activation on the surface of endothelial cells (*28*), attesting to its physiological potency. Thus, if the requirement for anticoagulant therapy is to slow generation of α-thrombin activity without eliminating its production, thus generating a mild hemophilia phenotype in patients with thrombotic tendencies, a low concentration of DYDYQ will be enough to alleviate α-thrombin formation through the meizothrombin pathway (*20*). In contrast, if complete arrest of α-thrombin formation is required, higher concentrations of DYDYQ will completely eliminate α-thrombin generation through the prethrombin 2 pathway as well. In conclusion, pentapeptide DYDYQ provides an ideal backbone for exosite-directed anticoagulant molecules that could attenuate or fully suppress α-thrombin formation in individuals with thrombotic tendencies.

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