Contribution of Amino Acid Region 334–335 from Factor Va Heavy Chain to the Catalytic Efficiency of Prothrombinase†

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ABSTRACT: We have demonstrated that amino acids E323, Y324, E330, and V331 from the factor Va heavy chain are required for the interaction of the cofactor with factor Xa and optimum rates of prothrombin cleavage. We have also shown that amino acid region 332–336 contains residues that are important for cofactor function. Using overlapping peptides, we identified amino acids D334 and Y335 as contributors to cofactor activity. We constructed recombinant factor V molecules with the mutations D334 → K and Y335 → F (factor VKF) and D334 → A and Y335 → A (factor VAA). Kinetic studies showed that while factor VaKF and factor VaAA had a K_D for factor Xa similar to the K_D observed for wild-type factor Va (factor VaWT), the clotting activities of the mutant molecules were impaired and the k_cat of prothrombinase assembled with factor VaKF and factor VaAA was reduced. The second-order rate constant of prothrombinase assembled with factor VaKF or factor VaAA for prothrombin activation was ~10-fold lower than the second-order rate constant for the same reaction catalyzed by prothrombinase assembled with factor VaWT. We also created quadruple mutants combining mutations in the amino acid region 334–335 with mutations at the previously identified amino acids that are important for factor Xa binding (i.e., E323Y324 and E330V331). Prothrombinase assembled with the quadruple mutant molecules displayed a second-order rate constant up to 400-fold lower than the values obtained with prothrombinase assembled with factor VaWT. The data demonstrate that amino acid region 334–335 is required for the rearrangement of enzyme and substrate necessary for efficient catalysis of prothrombin by prothrombinase.

The mainstay of the hemostasis process is the generation of thrombin, which in turn promotes the assembly of the fibrin plug following vascular injury. The prothrombinase complex is composed of the enzyme, factor Xa, and the protein cofactor, factor Va, associated in the presence of divalent metal ions on a membrane surface (I–3). This complex catalyzes the activation of prothrombin to its active form, thrombin. Factor Xa alone can activate prothrombin by two sequential proteolytic cleavages at Arg709 and Arg1018 resulting in the intermediates fragment 1·2 and prothrombin 2 (4–14). The overall rate of this reaction is not compatible with survival; however, incorporation of the cofactor, factor Va, into prothrombinase reverses the order of the proteolytic cleavages and increases the catalytic activity of factor Xa by 5 orders of magnitude (7, 8, 15). The first, factor Va-dependent cleavage at Arg320 produces the intermediate meizothrombin. Further cleavage at Arg271 generates thrombin and fragment 1·2 (4–14). The regulation of this key step in the blood coagulation cascade has been under intense investigation for many years.

Plasma factor V circulates as an inactive protein cofactor with an M_r of 330000 that does not participate in the prothrombinase complex (16). Factor V must first be activated to factor Va by thrombin through three sequential proteolytic cleavages at Arg709, Arg1018, and Arg1545 to produce a heavy chain, composed of amino acid residues 1–709 (M_r = 105000) and a light chain composed of amino acid residues 1546–2196 (M_r = 74000) (17) (Figure 1). The crystal structure of the bovine factor Va molecule lacking the entire A2 domain and the acidic COOH terminus of the heavy chain (factor Va_i) has been determined (18). Using this crystal structure as a template, we have provided a complete model of the human cofactor in solution (19). This model allows for the identification of the structural perturbations of point mutations on the overall tertiary cofactor structure.

Both chains of factor Va have been shown to be required for the interaction of the cofactor with factor Xa (20, 21). While the binding sites on the light chain remain to be identified, several binding sites for factor Xa on the factor
Materials, Reagents, and Proteins. Ditospropyl fluorophosphate (DFP), O-phenylenediamine (OPD) dihydrochloride, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (Hepes), Trizma (Tris base), and Coomassie Blue R-250 were purchased from Sigma (St. Louis, MO). 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 the chromogenic substrates Spectrozyme-TH and Spectrozyme-Xa were from American Diagnostica Inc. (Greenwich, CT). RecombiPlasTin was purchased from Instrumentation Laboratory (Lexington, MA). Human factor Xa was from Enzyme Research Laboratories (South Bend, IN). Factor V-deficient plasma was from Research Proteins Inc. (Essex Junction, VT). Dapsylarginine-N-(3-ethyl-1,5-pentanediyl) amide (DAPA), RVV-factor V activator, human thrombin, and human prothrombin were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Factor V cDNA (ATCC catalog no. 40515 pMT2-V) was from American Type Culture Collection (Manassas, VA). All restriction enzymes were from New England Biolabs (Beverly, MA). All molecular biology and tissue culture reagents and media were from Gibco, Invitrogen Corp. (Grand Island, NY), or as indicated. Monoclonal antibodies αHFVHcL17 and αFHVIE9 and monoclonal antibody αHFV1 coupled to Sepharose were provided by K. G. Mann (Department of Biochemistry, University of Vermont, Burlington, VT).

**Thrombin Generation Assay Using a Fluorescent Thrombin Inhibitor.** Thrombin generation assays were performed using the fluorescent thrombin inhibitor DAPA as described previously (23). The buffer used was composed of 20 mM Tris, 0.15 M NaCl (pH 7.4), and 5 mM CaCl2 (TBS, Ca2+) and was made fresh and filtered before each use. Fluorescence was measured on a Perkin-Elmer LS-50B luminescence spectrometer (Perkin-Elmer Corp., Norwalk, CT) with a λex of 280 nm, a λem of 550 nm, and a 500 nm long-pass filter in the emission beam (Schott KV-500). The data points were captured using FL WinLab (Perkin-Elmer Corp.) and subsequently analyzed and plotted using Prism (GraphPad, San Diego, CA). Peptides AP3 and AP4 were made in the analytical facility of A. Kurosky (University of Texas, Medical Branch, Galveston, TX). Peptides IWDYA, AP5, AP4, AP5m(DY-KP), AP6, and the control P15H were purchased from New England Peptide (NEP, Gardner, MA). Peptides were dissolved in water or buffer. Accurate peptide concentrations were

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1 Abbreviations: PS, L-α-phosphatidylserine; PC, L-α-phosphatidylcholine; PCPS, small unilamellar phospholipid vesicles composed of 75% PC and 25% PS (w/w); HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; [Og488]-EGR-hXa, factor Xa labeled in the active site with Oregon Green 488; factor VaWT, recombinant wild-type human factor Va; factor VaM, recombinant human factor Va with the mutation W333F; factor VaK, recombinant human factor Va with the mutations E330K and Y335F; factor VaMI, recombinant human factor Va with the mutations E323K, D334K, and Y335F; factor VaMI/AA, quadruple mutant of factor Va with the mutations D334K, V331I, and Y335F; factor VaFF, recombinant human factor Va with the mutations E323F, D334F, and Y335F; factor VaMI/KF, quadruple mutant of factor Va with the mutations D334K, V331I, and Y335F; factor VaMI/AA, quadruple mutant of factor Va with the mutations D334K, V331I, and Y335F; factor VaMI/AA, quadruple mutant of factor Va with the mutations D334K, V331I, and Y335F.
determined by amino acid composition in the analytical facility of A. Kurosky as detailed previously (30).

Fluorescence Anisotropy Measurements. The fluorescence anisotropy of [OGG]₆-EGR-hXa was measured using a Perkin-Elmer LS-50B luminescence spectrometer in L-format essentially as extensively described previously (23). Anisotropy measurements were performed in a quartz cuvette under constant stirring (low) with a λₑₑ of 490 nm, a λₑₘₚ of 520 nm, and a long-pass filter (Schott KV-520) in the emission beam. All experiments were performed in the dark. The data obtained were instantly analyzed using FL WinLab (Perkin-Elmer Corp.).

PCR Site-Directed Mutagenesis and Transient Expression of Factor V Molecules. Factor VKF was constructed using the mutagenic primers 5'-ATT TGG AAG TTT GCA CCT G-3' (forward) and 5'-C AGG TGC AA A CT T CCA AAT G-3' (reverse) (bold underlined letters identify the mutated bases) in a two-stage PCR method and characterized as previously described (29). The resulting ampiclon was subcloned into pGEM-T, and the mutations were confirmed by DNA sequencing (DNA Analysis Facility, Cleveland State University). The pGEM-T plasmid was digested with restriction enzymes Bsu36I and XcmI to remove the factor V insert, and the insert containing the mutation was ligated into pMT2-FV at the same restriction sites. The remaining recombinant plasmids were constructed using Stratagene’s QuickChange XL site-directed mutagenesis kit according to the manufacturer’s instructions. Factor VAA was constructed with the primers 5'-GAG GAA GTT ATT TGG GCC GC C CCT GCA GAT A-3' (forward) and 5'-TAT TAC AGG TGC GGC GGC CCA AAT GAC TTC CTC-3' (reverse). Factor VMKRF was constructed with the primers 5'-GAA TAC TTC ATT GCT GCA GAG GAA-3' (forward) and 5'-TAT TAC AGG TGC AAT GCC CCT GTA-3' (reverse). Factor VAA was constructed with the primers 5'-CAG ATT AAG TGG TGT TTC TTC TTC ATT GCT GCA GAG GAA GTT ATT TGG GCC GC C CCT GCA GAT A-3' (forward) and 5'-TAT TAC AGG TGC AAT GCC CCT GTA-3' (reverse). Factor VAA was constructed with the primers 5'-CAG ATT AAG TGG TGT TTC TTC TTC ATT GCT GCA GAG GAA GTT ATT TGG GCC GC C CCT GCA GAT A-3' (forward) and 5'-TAT TAC AGG TGC AAT GCC CCT GTA-3' (reverse). Factor VAA was constructed with the primers 5'-GAG GAA GTT ATT TGG GCC GC C CCT GCA GAT A-3' (forward) and 5'-TAT TAC AGG TGC CT CTC TGC AGC AAT GAA GT A-3' (reverse). Factor VAA was constructed with the primers 5'-GAG GAA GTT ATT TGG GCC GC C CCT GCA GAT A-3' (forward) and 5'-TAT TAC AGG TGC CT CTC TGC AGC AAT GAA GT A-3' (reverse). The recombinant molecules were partially purified and assayed as described in detail in ref 29. The recombinant molecules were fully characterized in ref 31.

Table 1: Functional Properties of Recombinant Factor Va Molecules

| Factor Va species                  | clotting activity (units/mg)a | Kᵥᵥp (nM)b | Kᵥᵥa (µM)c |
|-----------------------------------|-------------------------------|------------|------------|
| factor Va₁₈₅₂₄₃₆ Ma                 | 30                            | 0.35 ± 0.07| 0.16 ± 0.1 |
| factor Va₁₈₅₂₄₃₆ WT               | 240                           | 0.4 ± 0.1  | 0.13 ± 0.06|
| factor Va₂₁₅₀₅₄ Ma                | -                             | 0.24 ± 0.12| 0.16 ± 0.1 |
| factor Va₁₈₅₂₄₃₆ RF               | -                             | 0.58 ± 0.2 | 0.42 ± 0.06|
| factor Va₁₈₅₂₄₃₆ Rd               | -                             | 0.55 ± 0.13| 0.06 ± 0.01|
| factor Va₁₈₅₂₄₃₆ A               | 550                           | 0.6 ± 0.15 | 0.62 ± 0.07|
| factor Va₁₈₅₂₄₃₆ DF              | -                             | 0.7 ± 0.3  | 0.10 ± 0.01|
| factor Va₁₈₅₂₄₃₆ Nd              | -                             | 1.8 ± 0.7  | 0.14 ± 0.01|
| factor Va₁₈₅₂₄₃₆ FKd              | -                             | 1.3 ± 0.4  | 0.66 ± 0.02|
| factor Va₁₈₅₂₄₃₆ FFd             | -                             | 2.5 ± 0.8  | 0.6 ± 0.3  |
| factor Va₁₈₅₂₄₃₆ Dkd             | ~30                           | 5.5 ± 1.1  | 0.65 ± 0.35|
| factor Va₁₈₅₂₄₃₆ Mkd             | ~46                           | 4.6 ± 0.8  | 0.14 ± 0.25|
| factor Va₁₈₅₂₄₃₆ IM             | ~60                           | 3.33 ± 0.06| 0.39 ± 0.07|
| factor Va₁₈₅₂₄₃₆ AM             | 2103 ± 360                    | 0.37 ± 0.08| 0.35 ± 0.05|

- Two-stage clotting assays of recombinant factor V molecules were performed as described in Experimental Procedures. Apparent dissociation constants of recombinant factor Va for plasma-derived factor Xa (Kᵥᵥp) were determined as described in Experimental Procedures at limiting factor Xa concentrations (15 pM). Apparent Kᵥᵥp and Kᵥᵥa (shown in Figure 3B) values were determined as described in Experimental Procedures at limiting factor Xa concentrations (5 pM) and saturating concentrations of factor Va. The recombinant molecules were partially purified and assayed as described in detail in ref 29. The recombinant molecules were fully characterized in ref 31.

Purification of Recombinant Proteins. The medium containing the recombinant proteins was centrifuged to remove any cellular debris. Recombinant factor VKF, factor VAA, factor V₁₈₅₂₄₃₆ A, factor V₁₈₅₂₄₃₆ D, and factor V₁₈₅₂₄₃₆ WT were purified and quantified as recently described in detail (32). In some instances, the quadruple recombinant factor V mutants, factor VKF, as well as factor V₁₈₅₂₄₃₆ WT were partially purified and quantified as extensively detailed previously (29, 31). The activities of the partially purified recombinant factor Va molecules (29, 31) and the activities of the recombinant cofactor molecules purified to homogeneity (32) were similar and are reported in Table 1. The proteins were stored at −80 °C in small aliquots to prevent repeated freeze–thaw cycles. The activity and 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.

Prothrombin Activation Analysis by Gel Electrophoresis. Prothrombin (1.4 µM) was incubated in a reaction mixture containing 20 µM PCPS, 50 µM DAPA, and various recombinant factor Va molecules at 10 nM (activated with thrombin at a 1/50 enzyme/substrate ratio) in TBS, Ca²⁺. 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 added to 2 volumes of 0.2 M glacial acetic acid and treated as described previously (31). The dried samples were reconstituted in 0.1 M Tris base (pH 6.8), 1%
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SDS, and 1% β-mercaptoethanol and heated for 75 s at 95 °C. A total of 5 µg of total protein was loaded per lane and analyzed via 9.5% SDS–PAGE.

**Gel Electrophoresis and Western Blotting.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analyses were carried out using 9.5% or 4 to 12% gradient gels following reduction with 2% β-mercaptoethanol as described previously (33). In some cases, the protein was transferred to polyvinylidene difluoride (PVDF) membranes as described previously (34). Factor Va heavy and light chains were probed with the appropriate monoclonal antibodies and visualized with chemiluminescence, or the protein was visualized by staining with Coomassie Brilliant Blue R-250, followed by destaining in a solution of methanol, acetic acid, and water. In some experiments, gels were analyzed by scanning densitometry using UN-SCAN-IT gel (Silk Scientific, Orem, UT) as described previously (30, 31).

**Assay Measuring Thrombin Formation.** The ability of the recombinant factor V molecules to assemble in the prothrombinase complex was assessed in a discontinuous assay described in detail elsewhere (31). In short, all recombinant factor V molecules were activated with thrombin (1/50 described in detail elsewhere (35). Reaction mixtures contained PCPS vesicles (20 µM), DAPA (3 µM), factor Xa (varying concentrations), and recombinant factor Va species in reaction buffer [HEPES, 0.15 M NaCl, 50 nM CaCl2, and 0.01% Tween 20 (pH 7.40)]. DAPA (at twice the concentration of prothrombin) was included in all mixtures to prevent the action of thrombin during the course of the assay on factor Va, on prothrombin, and on itself. The rate of thrombin generation was subsequently measured using an excess of chromogenic substrate, Spectrozyme-TH (0.4 mM). The initial rate of thrombin generation was analyzed with Prizm (GraphPad), and kinetic constants were extracted directly from the graphs.

For the 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 (from 25 pM to 5 nM). For the determination of the kinetic constants of prothrombinase assembly, $K_m$ and $k_{cat}$, experiments were executed with a limiting amount of factor Xa (5 pM) in the presence of a fixed amount of the various recombinant factor Va molecules (10 nM) and a varied amount of substrate (prothrombin). Experiments performed with the quadruple mutants used saturating concentrations of factor $V^{FF/KF}$, factor $V^{MK/KF}$, factor $V^{MF/AA}$, and factor $V^{MM/AA}$ and varying amounts of the substrate prothrombin (from 25 nM to 4 µM). The concentration of each mutant cofactor molecule necessary to saturate factor Xa was calculated with the quadratic equation (eq 1) described in the literature (36, 37) and given below.

$$X_a = \frac{1}{2} \left[ \frac{n[V_a]_T + [Xa]_T + K_D - \sqrt{(n[V_a]_T + [Xa]_T + K_D)^2 - 4n[V_a]_T[Xa]_T}}{2} \right]$$

where $[V_a]_T$ and $[Xa]_T$ are the total concentrations of factor Va and factor Xa, respectively. The apparent dissociation constant ($K_D$) for the bimolecular interaction between factor Va and factor Xa on a phospholipid surface was obtained from the functional titration described above and the total concentration of ligand ([Va]$_T$) modified as appropriate.

**Additive Effect of the Mutations on Prothrombinase Function.** The change in transition-state stabilization free energy, which measures the effect that mutations in the cofactor of the prothrombinase complex have on the catalytic site of the enzyme, was calculated for the quadruple mutants as extensively described previously (38-42), and recently (31). In brief, the perturbation to the function of prothrombinase assembled with wild-type factor Va (state A) caused by a mutation in factor Va (state B) affecting the transition state can be defined in general as follows:

$$\Delta G_B = \Delta G_B - \Delta G_A$$

(2)

Since prothrombinase activity (assembled with each of the recombinant factor Va proteins) is being measured against the same substrate (prothrombin), the transition-state stabilization free energy ($\Delta G^{\#}_{A-B}$) during catalysis induced by a mutation in factor Va can be determined from the following equation:

$$\Delta \Delta G^{\#}_{int} = \Delta G^{\#}_{A-B} - C - (\Delta G^{\#}_{A-B} + \Delta G^{\#}_{A-C})$$

(4)

A positive value of $\Delta \Delta G^{\#}_{int}$ indicates that the interaction of these amino acid side chains reduces the catalytic efficiency of prothrombinase, while a negative value demonstrates that the mutations are better for prothrombinase, resulting in an increase in the catalytic efficiency of the enzyme. A value of zero would indicate no effect.

**Molecular Dynamics Simulation Setup.** Factor Va$^{KF}$ and factor Va$^{AA}$ were modeled using the recent factor Va model (PDB entry 1y61) (19). Briefly, amino acid residues Asp$^{334}$ and Tyr$^{335}$ were substituted with both lysine and phenylalanine, respectively, or with alanine. The amino acid change was performed using the “mutate” tool from the SwissPDBViewer package (43). Wild-type factor Va was left unchanged. The molecular dynamics simulation of each factor Va model consisted of a three-step setup: (1) the solvation of the protein followed by energy minimization, (2) position restraint molecular dynamics simulation, and (3) production run molecular dynamics simulation. Simulations were run and analyzed using GROMACS version 3.2.1 (44, 45). The system was solvated using the SPC water model (46). The force used to describe the interaction parameters was set to GROMOS-87 implemented in GROMACS as “ffgmx” (47). Molecular topologies for both wild-type factor Va and factor Va$^{KF}$ were generated using the atom types described in the “ffgmx” force field. Bonds were constrained using the LINCS algorithm (48). Long-range electrostatic interactions were evaluated with the particle mesh Ewald summation method performed in all three dimensions and using a fourth-order Fourier interpolation on a 1.6 Å spaced grid (49, 50). Following the minimization procedure, the
systems were subjected to a 20 ps molecular dynamics simulation with the restriction that only water molecules were allowed to move freely and the protein was restrained. The simulation temperature was set to 300 K and was maintained using the Berendsen temperature coupling with the coupling parameter set to 0.1 ps \(51\). The constant reference pressure of 1 bar was maintained throughout the simulation using the Parrinello–Rahman isotropic pressure coupling scheme with the compressibility set to \(4.5 \times 10^{-5} \text{ bar}^{-1} \) \(52\). Simulations were run until the systems reached equilibrium. Equilibration of each system was confirmed when the root-mean-square displacement of the protein reached a plateau and the drift was less than 1 Å during a whole nanosecond simulation time. The last nanosecond of the equilibrated trajectories was analyzed using tools included in GROMACS \(44, 45\).

RESULTS

Inhibition of Prothrombinase Function by Synthetic Peptides from the Central Portion of the Factor Va Heavy Chain.

We have previously shown that a peptide representing amino acid residues 323–331 inhibits factor Va cofactor activity \(23\). We have extensively characterized the important amino acids from this region by site-directed mutagenesis \(29\). We have also shown that under our experimental conditions, peptides encompassing residues 327–336 (AP5) and 332–341 (AP6) of the factor Va heavy chain also inhibit factor Va cofactor activity, while the pentadecapeptide P15H (representing amino acids 337–351 of factor V) did not have any effect on factor Va activity and was a good negative control. Bold and underlined residues are the amino acid residues mutated in this study.

\[\text{AP3} \quad \text{RHMKWYFI} \]
\[\text{AP4'} \quad \text{EYFIAEV} \]
\[\text{AP5} \quad \text{AEYFIAE} \]
\[\text{AP6} \quad \text{EYFIAE} \]
\[\text{AP7} \quad \text{EYFIAE} \]
\[\text{N5A} \quad \text{EYFIAE} \]

\[\text{P15H} \quad \text{PVIPANMAK} \]

**Figure 2:** Factor Va heavy chain peptides. At the top, the sequence of N42R is illustrated (part of the hatched box in Figure 1). The underlined sequence (323–335, 13 amino acids) is the regulatory amino acid sequence of the factor Va heavy chain. Overlapping peptides from the central portion of the factor Va heavy chain [AP3–AP7 \(23\)] are also shown. The role of the underlined amino acid region included in the box (i.e., Arg934-Tyr935) and common to IWDYA, AP5 and AP6, is under investigation here. Bold and underlined residues are the amino acid residues mutated in this study.

\[\text{AP5} \quad \text{DY} \]
\[\text{AP6} \quad \text{DY} \]

**Figure 3:** Inhibitory potential of factor Va heavy chain peptides. (A) Inhibition of prothrombinase activity. Increasing concentrations of AP5, AP6, P15H, IWDYA, and AP5DY–KF were preincubated with factor Xa and assayed for prothrombinase activity as described in Experimental Procedures. P15H (■) represents the control peptide containing amino acids 337–351 of the human factor Va heavy chain (see Figure 2 for details). AP5 (○) represents amino acids 327–336 of factor Va, AP5DY–KF (△) represents amino acid residues 327–336 with residues 334 and 335 mutated from D and Y to K and F, respectively. IWDYA (□) represents amino acid residues 332–336 of the human factor Va heavy chain. Data for AP6 are shown with ●. The concentration of peptide given on the x axis represents its final concentration in the prothrombinase mixture. The data represent the average of the results found in three independent experiments. The apparent inhibition constant \(K_i\) was calculated as described in Experimental Procedures. (B) Inhibition of prothrombinase assembly. Prothrombinase was assembled with [OG488]-EGR-hXa (10 nM), PCPS vesicles (10 μM), and increasing concentrations of human factor Va (up to 25 nM) in the presence of 2 mM Ca\(^{2+}\). Once a plateau was obtained, the preformed complexes were titrated with increasing concentrations of P15H (■), AP3 (△), AP4′ (▲), AP5 (○), and AP6 (●). \(\Delta r\) was calculated as described in Experimental Procedures and plotted as extensively detailed previously \(23\). The data represent all the data points from two independent experiments performed in triplicate.

Concentrations as high as 500 μM (not shown). Complete inhibition of prothrombinase by AP5 occurred at 200 μM peptide (not shown). The \(K_i\) for the inhibition of prothrom-
binase by AP5 calculated from the IC₅₀ was found to be 5.9 μM. This constant represents the dissociation constant of AP5 for prothrombinase. We have also assessed the effect of AP5 and AP6 for their ability to inhibit the direct binding of factor Va to [OG₄₈₈]-EGR-hXa. Both peptides at concentrations as high as 100 μM were unable to inhibit the direct interaction of factor Va with [OG₄₈₈]-EGR-hXa [Figure 3B (● and ○, respectively)]. In contrast, the nonapeptide control [Figure 3B, AP4′ (▼)] was a good inhibitor of the interaction of factor Va with [OG₄₈₈]-EGR-hXa as previously described (23). In addition, AP3 that contains the NH₂-terminal portion of AP4′ also inhibited the direct interaction of factor Va with [OG₄₈₈]-EGR-hXa [Figure 3B (▲)], albeit less efficiently than AP4′. It is noteworthy that AP3 is a noncompetitive inhibitor of prothrombinase (Figure S1) with respect to substrate, like AP4′ (23). Kinetic analyses demonstrated that in the presence of increasing concentrations of inhibitor, the Kₘ remained constant (0.4 ± 0.06) while the Vₘₐₓ of the enzymatic reaction decreased (Figure S1A). Using the mathematical transformation associated with the Dixon plots, the apparent Kᵢ of prothrombinase with AP3 was found to be ~15.6 μM (Figure S1B). Finally, it is important to underline that control experiments demonstrated that AP5 did not have any effect on the catalytic activity of factor Xa, since high concentrations of peptide were unable to inhibit factor Xa activity toward both a chromogenic substrate and prothrombin (data not shown).

The mechanism of prothrombinase inhibition by AP5 was further investigated by examining the effect of the peptide on the kinetic parameters of prothrombinase assembly and function (Kₘ and Vₘₐₓ) in the presence of varying concentrations of the inhibitor (Figure 4). In the presence of increasing concentrations of peptide, both the apparent Kₘ and Vₘₐₓ changed; the inhibitor lowered the Vₘₐₓ and increased the Kₘ (Figure 4A). Analysis of the kinetic data using the Dixon plots demonstrated that AP5 inhibition of prothrombinase followed a model of linear mixed-type inhibition (Figure 4B). The apparent Kᵢ of prothrombinase extrapolated from the graph was 10.5 μM (Figure 4B) which is in good agreement with the Kᵢ value obtained from the IC₅₀ value determined above (5.9 μM). The inhibition model of a mixed-type inhibitor is defined as unequal binding of the inhibitor to E and ES and suggests that AP5 that contains a portion of the factor Xa binding site may also interact with prothrombin in association with prothrombinase (53). However, it is also possible that binding of prothrombin to prothrombinase alters the affinity of AP5 for the enzyme.

In view of these results, we have synthesized an additional peptide (IWDA) and assayed its ability to inhibit prothrombinase activity. Peptide IWDA inhibited cofactor activity, although not completely [Figure 3A (□)]. Even at 500 μM peptide there was still approximately 25% cofactor activity remaining (not shown). The data demonstrated that IWDA has an IC₅₀ of 130 μM which corresponds to a Kᵢ of 69 μM. Interestingly, AP6 [Figure 3A (●)] has an IC₅₀ of ~120 μM corresponding to a Kᵢ of 64 μM. The similar Kᵢ values between AP6 and IWDA suggest that the inhibitory effect of the peptides is a consequence of the shared amino acid sequence IWDA [Figure 2 (boxed sequence)]. However, it is important to note that the data provided in Figure 3A also demonstrate that while AP6 yields a Kᵢ value similar to that of pentapeptide IWDA for prothrombinase inhibition (~60 μM), AP5 which also shares this sequence shows a 10-fold lower Kᵢ value (~6 μM). These data suggest that amino acid residues NH₂-terminal to pentapeptide IWDA are functionally important. This conclusion is strengthened by our earlier findings demonstrating the important contribution of amino acid stretch AAEV³³¹ to factor Va–factor Xa interaction (29). More recently, we have provided strong evidence supporting the notion that amino acids Glu³³⁰ and Val³³¹ are significant contributors to prothrombinase complex function (31).

To identify the important amino acid residues of this region for cofactor activity, we have synthesized another peptide with the sequence DY replaced by KF (AP5DY–KF, AP5m) and assayed the peptide for prothrombinase inhibition. We have arbitrarily chosen to mutate these two amino acids because they are the only charged amino acid residues of

![Figure 4: Kinetic analyses of prothrombin activation by prothrombinase in the presence of AP5. (A) Michaelis–Menten plots. Prothrombin generation experiments were performed in the absence (■) and presence of increasing concentrations of AP5 [10 (●), 15 (▲), and 20 μM (□)], as described in Experimental Procedures using prothrombin concentrations varying from 40 nM to 1 μM. Initial rates of thrombin formation are plotted as a function of substrate concentration. (B) Analysis of the data using the Dixon plots. The data were analyzed and plotted as 1/Vₒ, as a function of inhibitor concentration (AP5, Dixon plots). For mixed-type inhibition, a Dixon plot of 1/Vₒ as a function of increasing concentration of inhibitor is linear at a fixed enzyme and substrate concentration (53). The apparent inhibition constant (Kᵢ) reported in the text is the value derived from the intercept of each of the four graphs (~10.5 μM). The lines drawn represent the best fit through the points with an R² varying from 0.9812 (worst) to 0.9981 (best). The following concentrations of prothrombin were used in the experiments: 80 (●), 100 (●), 150 (□), and 200 nM (△).]
this pentapeptide. However, the function of the other two amino acids (I<sup>332</sup>W<sup>333</sup>) was also established using recombinant technology and shown below. AP5m had an only weak effect on prothrombinase function with ~60% cofactor activity remaining at concentrations of peptide as high as 500 μM [Figure 3A (△)], with a K<sub>i</sub> value of ~530 μM (assuming an IC<sub>50</sub> of ~1 mM). These data indicate that amino acid region 334–335 is important for the expression of the inhibitory potential of AP5. Taken together, the results indicate that (1) peptide AP5 from the central portion of the factor Va heavy chain is a potent inhibitor of factor Va cofactor activity and (2) amino acid region 323–335 (13 amino acids) from the factor Va heavy chain plays an important role in the optimal expression of prothrombinase activity (Figure 2).

Expression and Activation of Recombinant Human Factor V Molecules. We next employed a recombinant protein scheme to further assess the importance of amino acid region 334–335 of the factor Va heavy chain for cofactor activity. We used one charge reversal mutation (D334K) and one conservative mutation (Y335F) to obtain recombinant factor VKF. We have also replaced both amino acids with alanine to produce factor VAA. In addition, we have combined these mutations with mutations at the extremities of the factor Xa binding site on factor Va. We have thus obtained the quadruple mutants, factor VMI/KF, factor VFF/KF, factor VMI/AA, and factor VFF/AA (see Figure 1 for more details) (31).

To assess the importance of the other two amino acids of peptide IWDYA, i.e., I<sup>332</sup> and W<sup>333</sup>, for cofactor function, we have created two additional mutant molecules with these amino acids changed to alanine (factor V<sup>332A</sup> and factor V<sup>W333A</sup>). All eight recombinant factor V molecules along with wild-type factor V (factor V<sup>WT</sup>) were expressed, purified, and assayed in a two-stage clotting assay, and the results are reported in Table 1. Factor Va<sup>WT</sup> displayed normal clotting activity, compared to plasma-derived factor Va. Conversely, while factor Va<sup>KF</sup> and factor Va<sup>AA</sup> had 5-fold reduced clotting activity under similar experimental conditions, all the quadruple mutants were practically devoid of clotting activity (~30 units/mg). We have previously demonstrated that factor Va<sup>F</sup> and factor Va<sup>M</sup> are impaired in their interaction with factor Xa (31). Results with these mutants are reported in Table 1 for comparison. The data demonstrate that while mutation of amino acid region 334–335 impair clotting activity, these mutations alone are not enough to completely abolish factor Va clotting activity. However, combining these mutations with mutations at the extremities of the previously described factor Xa binding site results in cofactor molecules that are devoid of clotting activity. The clotting data obtained with the recombinant proteins also demonstrate that amino acids I<sup>332</sup> and W<sup>333</sup> are not essential for expression of factor Va clotting activity as the mutant molecules have clotting capabilities comparable to that of the wild-type molecule.

Effect of Recombinant Factor Va Molecules on Prothrombin Activation by Prothrombinase. The capability of the recombinant factor Va molecules to be incorporated into prothrombinase and activate prothrombin was investigated by gel electrophoresis. Prothrombinase assembled with factor Va<sup>WT</sup> displayed normal activation pattern compared to the plasma-derived cofactor with initial cleavage at Arg<sup>320</sup> producing fragment 1-2-A (Figure S2), indicative of meizothrombin generation. Activation of prothrombin by prothrombinase assembled with recombinant purified factor Va<sup>KF</sup> or factor Va<sup>AA</sup> showed delayed thrombin formation as evidenced by delayed formation of both fragment 1-2-A and B chain of thrombin (Figure S2). Under similar experimental conditions, prothrombinase assembled with factor Va<sup>FF/KF</sup>, factor Va<sup>MIF/KF</sup>, factor Va<sup>FF/AA</sup>, and factor Va<sup>MI/AA</sup> did not activate prothrombin to any significant extent, following a 3 h incubation period (data not shown). These results indicate that substitution of amino acids 334 and 335 of the factor Xa binding site on factor Va...
Va heavy chain results in delayed prothrombin activation by factor Xa within prothrombinase assembled with factor VaKF or factor VaAA.

Kinetic Analyses of Prothrombinase Assembled with Recombinant Factor Va. The ability of the recombinant factor Va molecules to be incorporated into prothrombinase was investigated using an assay employing purified reagents. Under similar experimental conditions, factor VaD332A, factor VaW333A, factor VaKF, and factor VaAA displayed K\textsubscript{D} values comparable to the value obtained with the wild-type molecule (Table 1). Conversely, the affinities of factor VaFF/KF, factor VaFF/AA, factor VaMI/KF, and factor VaMI/AA were 6-, 10-, 23-, and 20-fold, respectively, lower than the affinity of factor VaWT for factor Xa (Table 1). These data demonstrate that amino acids 332–335 do not participate in the interaction of the cofactor with factor Xa.

Figure 5 and Table 1 show the original raw data (Figure 5A), the apparent k\textsubscript{cat} (Figure 5B), the apparent K\textsubscript{m} (Table 1), and the second-order rate constants (Figure 5C) of prothrombinase assembled with the recombinant factor Va molecules. Knowing the K\textsubscript{D} of each recombinant molecule for factor Xa (Table 1), we calculated the appropriate amount of factor Va to saturate factor Xa using the quadratic equation (eq 1) described in the literature (36, 37) and provided in Experimental Procedures. Thus, the titrations were performed under conditions where all factor Xa was saturated with factor Va, and as a consequence, any observed deficiency in the catalytic activity of prothrombinase was attributed to a defective factor Va molecule rather than to an impaired factor Va–factor Xa interaction. The data demonstrate that prothrombinase assembled with purified factor VaKF and factor VaAA was characterized by an ∼10-fold decrease in second-order rate constants (Figure 5B,C) compared to prothrombinase assembled with factor VaWT. Prothrombinase assembled with the quadruple mutant molecules, factor VaMI/KF, factor VaMI/AA, and factor VaFF/AA, demonstrated a significant decrease in the k\textsubscript{cat} and the second-order rate constant of the enzyme [up to 400-fold decrease (Figure 5C)]. The kinetic data obtained with the recombinant proteins also demonstrate that amino acids I\textsuperscript{332} and W\textsuperscript{333} are not required for expression of factor Va cofactor activity since the kinetic constants obtained with prothrombinase assembled with these two mutant molecules are similar to the data obtained with prothrombinase assembled with wild-type factor Va. Overall, the data show that substitution of amino acids 334 and 335 in factor Va has an effect on the catalytic efficiency of prothrombinase.

Effect of the Mutations on Prothrombin Catalysis. Prothrombinase is composed of factor Va and factor Xa assembled on a membrane surface in the presence of divalent metal ions. We can thus hypothesize that prothrombinase is an enzyme composed of two subunits: a catalytic subunit (factor Xa) and a regulatory subunit (factor Va). Any perturbation in the interaction between the two subunits or any perturbations in the interaction of prothrombinase with the substrate caused by a mutation in the regulatory subunit may influence (modify) the stability of the catalytic site of the catalytic subunit. Thus, the consequences of mutations in factor Va affecting factor Xa catalytic efficiency can be measured relative to the change in the transition-state stabilization free energy of the enzyme. The kinetic data showed that the DY → KF/AA mutation in factor Va results in a decrease in the catalytic efficiency of factor Va which in turn is translated by positive values of ∆∆G\textsuperscript{WT→KF} and ∆∆G\textsuperscript{WT→AA} of 1.24 and 1.5 kcal/mol, respectively (Scheme 1). To quantify the interaction between these mutations and the mutations in the previously described factor Xa binding site (31) and their synergistic effects on prothrombinase function, we have calculated the difference in free energy of the transition-state analogue (∆∆G\textsubscript{int}) for each of the quadruple mutants according to eqs 3 and 4. We have also constructed a thermodynamic cycle of prothrombinase (Scheme 1). The positive value of ∆∆G\textsubscript{int} for the combination of mutations at amino acids 323 and 324 and amino acids 330 and 331 with the DY → KF mutation indicates that these substitutions in the heavy chain of factor Va are detrimental to the catalytic activity of prothrombinase.

Structural Consequences of the Mutations. We next used the model of the factor Va molecule to understand the structural consequences of the mutations at positions D\textsuperscript{334} and Y\textsuperscript{335}. It is important to underline that our MD simulations are tentative, especially with reference to interactions within the A2 domain, as this region is not present in any crystal structures and is derived from modeling (19). The MD simulation of factor VaWT reached equilibrium after 1 ns and was extended for an additional 1 ns for analysis. The simulation of factor VaKF reached equilibrium after 2.5 ns, and the simulation was extended to 3.5 ns for analysis of the last equilibrated nanosecond. The simulation of factor VaAA reached equilibrium at 2 ns, and the last 500 ps was used for analysis. Figure 6A shows the final snapshot of factor VaWT with amino acid residues D\textsuperscript{334} and Y\textsuperscript{335} shown as blue sticks. The corresponding amino acids in factor VaKF [K\textsuperscript{334} and E\textsuperscript{335}, respectively (Figure 6B)] and in factor VaAA [A\textsuperscript{334} and A\textsuperscript{335}, respectively (Figure 6C)] are also represented as blue sticks. The MD data show that the antiparallel β-sheet contained in the region from R\textsuperscript{321} to V\textsuperscript{331} (first strand) and K\textsuperscript{366} to T\textsuperscript{369} (second strand) is preserved in the case of factor VaWT during the simulation. In contrast, in the case of factor VaAA and factor VaKF, the β-sheets are
FIGURE 6: Structural analyses of the factor V heavy chain. A portion of the prothrombin interactive site containing amino acids D334–Q339 (30, 54, 55) is shown as magenta spheres. The α-helix contained in S328–E338 is colored red. This region contains a secondary binding site for factor Xa (24). The β-sheet contained in the R321–V331/K365–T369 sequence that is important for expression of factor Va cofactor activity (22, 23, 29) is colored yellow. The loop contained within the sequence of amino acid residues 400–420 is shown with cyan ribbons. Amino acid residues F538 and H536 are shown as green sticks. S335 is shown as cyan sticks. N334 and K364 are shown as lime sticks. Amino acid residues Y530 and E330 are shown as olive sticks, and E323 is shown as orange sticks. The final snapshot at 2 ns of factor VaWT is shown in panel A with amino acid residues D334 and Y335 represented as blue sticks. Panel B shows the final snapshot at 3.5 ns of factor VaKF with the mutated amino acid residues K334 and Phe335 as blue sticks and R315 and S312 as cyan sticks. Panel C is a snapshot at 2 ns of factor VaAA with the mutated amino acid residues A334 and A335 colored blue.

extended at the V331 and K365 ends by three amino acids. This β-sheet contains the critical amino acid sequence 323–331 previously shown to regulate factor Va cofactor activity (22, 23, 29). Elongation of the R321–V331/K365–T369 β-sheet to R321–A334/H362–T369 in the factor VaAA molecule positioned H362 in a conformation that ensured hydrogen bonding with F538, thus directly affecting the stability of the S328–F538 α-helix. The S328–F538 helix is a continuation of the amino acid stretch that contains a secondary binding site of the cofactor for factor Xa that is located between amino acids 493 and 506 (24).

DISCUSSION

Collectively, through a systematic approach, using both kinetic studies with synthetic peptides and recombinant proteins, our data demonstrate that amino acid region 334–335 plays an important role in the expression of factor Va cofactor activity. Our data also demonstrate the cofactor requirement for the efficient rearrangement of enzyme (factor Xa) and substrate (prothrombin) within prothrombinase necessary for efficient catalysis of prothrombin at two spatially distinct sites. However, it is important to note that our study does not provide a definitive mechanism detailing the involvement of amino acid region 334–335 of factor Va in cofactor activity.

The mechanism of inhibition of prothrombinase function by AP5, AP5DY–KF, AP6, and IWDYA was investigated by assessing factor Va cofactor activity in the presence of increasing concentrations of synthetic peptide. These data show that the IWDYA motif shared by AP5 and AP6 contains the amino acids that account for the inhibitory effect of the peptides. This is evident in the similar Kᵩ values of peptides AP6 and IWDYA. AP5 has an ∼10-fold lower Kᵩ value than AP6 and IWDYA, because it also contains amino acids Glu330 and Val331, which have been previously shown to be involved in factor Xa binding (29). Analysis of the mode of inhibition of prothrombinase by AP5 suggests that the peptide is a mixed-type inhibitor and interacts with both prothrombinase and prothrombinase bound to the substrate (prothrombin). Thus, while it is possible that AP5 interacts with prothrombinase in the presence and absence of prothrombin, the possibility that AP5 also binds prothrombin when the enzyme is in complex with the substrate must be kept in mind. Finally, within the peptide sequence IWDYA, amino acids D and Y are contributing to its function, since substitution of these two amino acids with K and F, respectively (AP5DY–KF), results in the almost complete loss of the inhibitory potential of AP5.

Site-directed mutagenesis was used to assess the importance of amino acid region 334–335 from the factor Va heavy chain during prothrombin catalysis. The data show that mutating this amino acid region results in a factor Va molecule that when incorporated into prothrombinase produces an enzyme with an overall 10-fold decrease in its second-order rate constant. Since the Kᵩ values of factor VaKF and factor VaAA for factor Xa are similar to the Kᵩ of factor VaWT for the enzyme, the data strongly suggest that the inability of prothrombinase assembled with factor VaKF and factor VaAA to function optimally can be explained by the inability of factor Xa to efficiently convert prothrombin to thrombin because of a diminished number of productive collisions.
The results obtained from the MD simulations of wild-type factor Va, factor VaKF, and factor VaAA were able to offer an explanation both for the similar \( K_D \) values found for the interaction of wild-type factor Va and factor VaKF (or factor VaAA) with factor Xa and for the impaired clotting and cofactor activity of both mutant molecules. The modeling data demonstrate that the distances between the C\(^\text{a}\) atoms of amino acids 334 and 323, 334 and 324, 335 and 323, and 335 and 324 analyzed separately do not change significantly when comparing wild-type factor Va with the mutant molecules. Thus, there are no changes in the overall conformation of the segment of amino acids 323–331 because of the mutations. The combined data rather suggest that the difference in the distance of the centers of mass between the two amino acid segments observed is due to the presence of different amino acid side chains that interact with each other. Coincidentally, the thermodynamic and kinetic data obtained herein also suggest that combination of the mutations at the extremities of the factor Xa binding site in factor Va, \(^{334}\text{DYE}^{335}\) (factor VaKF) and \(^{330}\text{EV}^{331}\) (factor VaMD), with mutations in the \(^{334}\text{DY}^{335}\) region (factor VaKF or factor VaAA) have an additive detrimental effect on prothrombinase with respect to prothrombin activation. It is also important to note that replacement of an aspartic acid with an alanine appeared to be more detrimental to cofactor activity than replacement with lysine. These data would suggest that the negative charge per se at residue 334 is not critical for cofactor activity. Finally, we must point out that tentative explanations using our theoretical model are suggestive rather than conclusive and await verification that will be obtained from the crystal structure of the recombinant mutated cofactor molecule.

At this point, it is important to underline the complementarities of all the data provided in this paper. Our findings using the MD simulations demonstrate that in the presence of the mutations there is a relocation of the COOH-terminal region of factor VaKF and factor VaAA as compared with the wild-type cofactor molecule. However, these calculations are unable to distinguish whether the changes will enhance or decrease the catalytic efficiency of the prothrombinase complex assembled with the mutated cofactor molecule. On the other hand, the kinetic, thermodynamic, and molecular biology data demonstrate a detrimental effect of the mutations on cofactor activity without providing a structural explanation. Together, the data suggest that the internal rearrangement at the COOH terminus of factor VaKF and factor VaAA is detrimental to the overall catalytic efficiency of factor Xa as part of prothrombinase. Therefore, the MD simulations using the only complete structure of factor Va in solution available in the literature are useful in providing a theoretical explanation for the decrease in the second-order rate constant of prothrombinase assembled with the mutant cofactor molecules.

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SUPPORTING INFORMATION AVAILABLE

Figure S1 shows the kinetic analyses of prothrombin activation by prothrombinase in the presence of AP3. Panel A shows the Michaelis–Menten plots, while panel B shows the mathematical transformation of the data using Dixon plots. Figure S2 shows prothrombin activation by prothrombinase assembled with recombinant factor Va molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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