Role of the \(\alpha\)-Helix 163–170 in Factor Xa Catalytic Activity*

Received for publication, June 12, 2007, and in revised form, August 24, 2007. Published, JBC Papers in Press, August 28, 2007. DOI 10.1074/jbc.M704837200

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Factor Xa (FXa) is a key protease of the coagulation pathway whose activity is known to be in part modulated by binding to factor Va (FVα) and sodium ions. Previous investigations have established that solvent-exposed, charged residues of the FXa \(\alpha\)-helix 163–170 (h163–170), Arg\(^{165}\) and Lys\(^{169}\), participate in its binding to FVα. In the present study we aimed to investigate the role of the other residues of h163–170 in the catalytic functions of the enzyme. FX derivatives were constructed in which point mutations were made or parts of h163–170 were substituted with wild-type FXa. Purified FXa derivatives were compared with wild-type FXa. Kinetic studies in the absence of FVα revealed that, compared with wild-type FXa, the catalytic activity toward prothrombin and tripeptidyl substrates or prothrombin indicated that assembly of these FXa derivatives was improved their low catalytic efficiency. These data indicate that residues in the NH\(_2\)-terminal portion of the FVα-binding pocket of the enzyme and to the Na\(^+\)-binding site of the protease and that residues Val\(^{163}\) and Ser\(^{167}\) play a key role in this interaction.

Factor X (FX)\(^4\) is a vitamin K-dependent, two-chain glycoprotein that plays a central role in blood coagulation. During this process, FX is activated to FXa and forms a high affinity macromolecular complex with other components of the prothrombinase complex, factor Va (FVα), negatively charged phospholipid surfaces, and calcium to activate prothrombin to thrombin (1–6). These macromolecular interactions lead to an increase of 5 orders of magnitude increase in the catalytic efficiency of FXa toward prothrombin (2, 7). Enhancement of the \(k_{cat}\) of the reaction is mainly due to the cofactor function of FVα. Two basic residues of h163–170\(^5\) of the protease domain of FXa, namely Arg\(^{165}\) and Lys\(^{169}\), directly interact with FVα (8, 9). All known sequences from different species in this surface-exposed helix of FXa are similar. Interestingly despite being stimulated by different cofactors, the catalytic domains of other blood coagulation proteins, such as factor IXa (FIXa) and factor VIIa (FVIIa), share the same cofactor-dependent activity binding site based on the structural equivalences with chymotrypsin (10–12).

Like other serine proteases of blood coagulation, small ligands such as calcium and sodium can allosterically modulate the activity and the specificity of FXa (13–20) by binding to several exposed surface loops near or remote from the catalytic pocket of the enzyme (21). According to the three-dimensional structure of FXa (22), the FXa Na\(^+\)-binding site is close to the catalytic pocket of the enzyme and to the FVα-binding h163–170 (Fig. 1). Furthermore there is some evidence that both the FVα- and Na\(^+\)-binding sites of FXa are energetically linked (20, 23). Altogether these observations suggest that there is an allosteric linkage between the Na\(^+\)-binding site and FVα-binding h163–170.

In the current study, the relationship between h163–170, which is a crucial FVα-binding site, and the Na\(^+\)-binding site were investigated. To this end, FXa derivatives were designed in which h163–170 was either substituted by the corresponding known sequences from different species in this surface-exposed (Arg\(^{165}\)) or to be proximate to the Na\(^+\)-binding site (Val\(^{163}\) and Ser\(^{167}\)). The FXa derivatives were expressed in mammalian cells, and the purified activated forms were functionally characterized.

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\(^*\) This study was supported in part by INSERM, by the Ministère de l’Éducation Nationale, de la Recherche, et de la Technologie (to S. L. and F. T.), and by Groupe d’Etude sur l’Hémostase et la Thrombose (GEHT) (to S. L.). 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.

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\(^4\) The abbreviations used are: FX, factor X; FV, factor V; FVII, factor VII; FVIIa, factor VIII; FIX, factor IX; h163–170, \(\alpha\)-helix 163–170; RVV-X, purified small derivatives of FVIIIa; wt-FX, wild-type factor X; PS, phosphatidylserine; PC, phosphatidylcholine; BSA, bovine serum albumin; S-2238, CH\(_3\)CO\(_2\)-D-CHGly-Gly-Arg-pNA; D-FFR-CK, methanesulfonyl-D-phenyl-phenyl-arginyl-carbonyl-cyclohexylglycyl-glycyl-arginine-pNA; Spectrozyme FXa; PAB, paraaminobenzamidine; P-FFR-CK, methanesulfonyl-o-phenyl-phenyl-arginyl-chloromethyl ketone; ATIII, antithrombin III.

\(^5\) Amino acid sequence numbering of FXα and of all the proteases mentioned in this study is based on the three-dimensional topological equivalences with chymotrypsin.
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The catalytic triad residues, located in the center of this representation, are (from left to right) Asp189, His57, and Ser195 (chymotrypsin numbering) and are shown as black ball-and-stick; the S1 site (Asp189) is also shown. The h163–170, which is an FVa-binding site (8, 9), and the two loop segments 183–189 and 221–225, which constitute a Na+ binding site (35), are shaded black.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were obtained from Invitrogen and Genosys Biotechnologies (Saint-Quentin-Yvelines, France). Restriction enzymes, DNA-modifying enzymes, Dulbecco’s modified Eagle’s medium/F-12, and insulin-transferrin-selenium-X were purchased from Invitrogen, and TripleMaster™ polymerase was from Eppendorf (Le Pecq, France). H-D-Phenylalanyl-L-pipecolyl-L-arginine-para-nitroanilinedihydrochloride (H-D-Phe-Pip-Arg-pNA-2HCl), product name S-2238, and benzyloxy carbonyl-D-arginyl-L-glycyl-L-arginine-para-nitroanilinedihydrochloride (Z-D-Arg-Gly-Arg-pNA), product name S-2765, were from Chromogenix (MaxiSorp™ surface) and culture flasks were from Nunc (Roskilde, Denmark), and polystyrene microtiter plates were from Greiner Merck Eurolab (Strasbourg, France). Immobilon™ polyvinylidene difluoride membranes for Western blotting applications and Amicon Ultra 30,000 molecular weight cutoff filters were obtained from Millipore (Saint-Quentin-Yvelines, France). Madin-Darby canine kidney cells were purchased from ATCC (Manassas, VA). Benzamidine was from Acros Organics (Noisy-le-Grand, France). Methanesulfonat-D-phenyl-phenyl-arginyl-chloromethyl ketone (D-FFR-CK) was purchased from Calbiochem. CNBr-activated Sepharose 4 Fast Flow, HiTrap™ Q- and heparin-Sepharose columns, benzamidine-Sepharose, and Q-Sepharose Fast Flow were obtained from Amersham Biosciences.

Proteins—Polyclonal antibodies against FX conjugated or not with horseradish peroxidase were obtained from Dako (Dako-patts, Glostrup, Denmark). Purified human plasma-derived FX (pd-FX), antithrombin III (ATIII), human prothrombin, FX-activating enzyme from Russell’s viper venom (RVV-X), bovine trypsin, and human thrombin were obtained from Kordia (Leiden, The Netherlands). Human FVa was purchased from Hematologic Technologies Inc. (Essex Junction, VT).

Enzyme-linked immunosorbent assay using anti-FX polyclonal antibodies was used to assay pd-FX and FX derivative proteins. FX was expressed in units where 1 unit is the amount present in 1 ml of normal human plasma. Proteins were quantified by the method of Bradford (24) using BSA as a standard. Molar concentrations of FXa (recombinant or plasmatic) and α-thrombin were determined by active site titration (see below).

Recombinant FX Derivatives—The mammalian expression plasmid pKG5 containing human FX cDNA and encoding wild-type (wt) FX (25) was used as a template for standard PCR mutagenesis to generate cDNAs encoding FX mutants carrying specific residues of FIX or FVII named FX/FVII163–170 FX/FVII163–167, FX/FVII168–170 FX/FIX163–170 and FXR165A (Fig. 2). The mutated full-length cDNAs cloned into the expression vector pKG5 were checked by DNA sequence analysis using the ABI PRISM Dye Terminator Cycle Sequencing Reaction kit version 3.1 (Applied Biosystems Applera, Courtaboeuf, France) on an ABI PRISM 310 DNA sequencer according to the manufacturer’s specifications. All constructs were transfected into Madin-Darby canine kidney cells using calcium phosphate precipitation, and cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Stable cell lines producing recombinant FX derivatives and wt-FX were prepared as already described (25) and maintained in 300-cm² flasks for protein production in serum-free Dulbecco’s modified Eagle’s medium/F-12 supplemented with 800 μg/ml Gentamicin G-418, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml vitamin K₃, and 1% insulin-transferrin-selenium-X. FX-containing medium was harvested every 48 h. Benzamidine and phenylmethanesulfonyl fluoride were added to a final concentration of 10 and 2 mM, respectively, and the medium was centrifuged (6000 g), passed over cellulose acetate membranes (0.45 μm) to eliminate cell debris, and stored at −80 °C. Conditioned medium was thawed at 37 °C. EDTA was added to a final concentration of 5 mM. The medium was diluted to bring the final NaCl concentration to 60 mM. The mixture was then
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stirred at room temperature for 30 min with QAE-Sephadex A-50 beads to achieve a final concentration of 0.25% (w/v). Beads were washed before elution with 50 mM Tris (pH 7.4), 500 mM NaCl, and 10 mM benzamidine. Recombinant FX contained in the eluted fractions (enzyme-linked immunosorbent assay) was immediately dialyzed against 50 mM Tris (pH 7.4), 15 mM NaCl containing 10 mM benzamidine, and 4 mM EDTA. Proteins were incubated at 4 °C overnight with Q-Sepharose Fast Flow resin (1 ml of resin/50 ml of concentrated proteins). The resin was pre-equilibrated in 15 mM Tris (pH 7.4), 15 mM NaCl containing 10 mM benzamidine, and 4 mM EDTA. The resin was loaded on a column, washed with 200 ml of equilibration buffer containing 2 mM EDTA, and then washed with 100 ml of equilibration buffer without EDTA. Recombinant FX was eluted from the column with a 0–60 mM CaCl2 gradient at a flow rate of 1 ml/min. Recombinant FX fractions were pooled, concentrated on Amicon Ultra 30,000 molecular weight cutoff filters, dialyzed extensively against 50 mM Tris (pH 7.4) and 50 mM NaCl, and stored at −80 °C. Before analysis, a final pass over a benzamidine-Sepharose column equilibrated with 50 mM Tris (pH 7.4) and 50 mM NaCl was used to eliminate trace contaminants of FXa that may have been generated during production or purification of the recombinant protein. Furthermore 1 h prior to use as a zymogen, FX derivatives were incubated with 1 mM phenylmethylsulfonyl fluoride to neutralize any trace of FXa. Control experiments indicated that after 30 min in Tris-HCl buffer phenylmethylsulfonyl fluoride was fully hydrolyzed and would not interfere with other reactions. Protein purity was assessed using 15% SDS-polyacrylamide gel electrophoresis analysis of the FX derivatives under reducing (50 mM dithiothreitol, final concentration) and non-reducing conditions followed by staining with Coomassie Brilliant Blue R-250. NH2-terminal sequence analysis was carried out after conditions followed by staining with Coomassie Brilliant Blue R-250. NH2-terminal sequence analysis was carried out after light chains were excised and sequenced using an Applied Biosystems Procise model 494 sequencer in the sequencing facility of the Institut de Biologie et Chimie des Protéines (Lyon, France). This analysis indicated that for all FX derivatives the signal sequence and the propeptide were accurately and efficiently removed before secretion. In addition, sequence analysis disclosed that the average yield for the two glutamic acid residues at the NH2 terminus was less than 5% of the average yield of the two subsequent residues. Because γ-carboxylation reduces the yield of Glu residues, these data indicate that the glutamic residues were properly modified. Activation of FX by RVV-X—Recombinant FX derivatives and wt-FX were activated by RVV-X (26); 1 mg of it was coupled to 1 ml of CNBr-activated Sepharose 4B according to the manufacturer’s instructions. Recombinant FX derivatives (1 μM) were incubated with coupled RVV-X (30 nm) in 50 mM Tris (pH 7.4) and 100 mM NaCl containing 10 mM CaCl2. Time course analysis of the activation reactions by SDS-PAGE indicated that all FXzymogens were fully converted to their active forms within 1 h under these experimental conditions. After 2 h, the reaction was stopped by addition of 15 mM EDTA. Activated recombinant FX derivatives were loaded onto a 1-ml HiTrap heparin-Sepharose column and eluted with 50 mM Tris-HCl (pH 7.4) containing 0.5 mM NaCl and 5 mM CaCl2. The eluate was immediately dialyzed against 50 mM Tris (pH 7.4) and then against 50 mM Tris (pH 7.4) and 75 mM NaCl and precipitated by addition of solid ammonium sulfate to 80% saturation. The precipitated proteins were dissolved in 50 mM Tris (pH 7.4) and 10 mM NaCl, and the protein solution was immediately dialyzed against the same buffer containing 50% glycerol (v/v) and stored at −20 °C until use. Recombinant FXa appeared pure by SDS-PAGE and was indistinguishable from commercially available pd-FXa (Kordia). Furthermore wt-FXa was identical to pd-FXa with respect to its Kcat and km values for the hydrolysis of the chromogenic substrates S-2765 and SpeFXa as well as the rate of prothrombin activation within prothrombinase. Recombinant wt-FXa and commercial pd-FXa were nevertheless systematically compared in all experiments reported in this study and found to be virtually identical.

Interaction of FXa with D-FFR-CK—The rate constant km for the interaction between D-FFR-CK and pd-FXa, wt-FXa, or recombinant FXa derivatives was evaluated as follows. First titrated α-thrombin, with p-nitrophenyl-p’-guanidinobenzoate hydrochloride, was used to determine the precise concentration of D-FFR-CK aliquots in 1 mM HCl. Briefly 250 nM α-thrombin in kinetic buffer (50 mM Tris-HCl (pH 7.5) containing 75 mM NaCl, 5 mM CaCl2, 0.1% (w/v) BSA, and 0.1% polyethylene glycol 8000) was incubated for 3 h at room temperature with various amount of D-FFR-CK (0.03–6 mM). The reaction mixture was diluted 1:10 in kinetic buffer containing 100 mM S-2238, and the remaining enzyme concentration was estimated from the rate of A405 increase. The release of para-nitroanilide (pNA) was recorded at 405 nm as a function of time (i.e. the initial rate of S-2238 hydrolysis) in a kinetics microplate reader (Bio-Tek Instruments, Winooski, VT). The initial concentrations of D-FFR-CK aliquots were deduced from the intercept to the x axis of a linear plot of the remaining activity versus the amount of inhibitor added.

Subsequently a sufficient amount of activated enzyme (30–500 nM, estimated from the published extinction coefficient of FXa of 1.25 ml mg−1 cm−1 at 280 nm (27)) to obtain a readily detectable amidolytic activity (10% hydrolysis of S-2765 in 30 min) was incubated for a given period of time (10 s to 5 h) in the presence of a fixed concentration of D-FFR-CK in kinetic buffer at 25 °C. Typically three concentrations of D-FFR-CK were used that correspond to 10, 20, and 40 times the target concentration to ensure that the reaction occurs under pseudo-first order conditions. At the end of each incubation, 100 or 500 μM S-2765 was added, and the residual amidolytic activity was monitored at 405 nm in a kinetics microplate reader. By plotting the rate of S-2765 hydrolysis as a function of the incubation time of D-FFR-CK and its target enzyme, a curve was obtained. By non-linear regression, using Equation 1, the rate constant for inactivation of the target enzyme can be estimated.

\[ A_t = A_{\text{min}} + A_0 e^{-k/n} \]  

(Eq 1)

The parameters A_t, A_0, and A_{\text{min}} represent the residual activity at time t, the initial activity at time 0, and the activity at infinite time, respectively. Under pseudo-first order condition, the
value obtained for $k$ is equal to the concentration of inhibitor multiplied by its $k_{on}$ for the enzyme.

**Titration of FXa**—The active site concentrations of FXa (plasmatic and activated recombinant FX derivatives) were determined by titration with known concentrations of $d$-FFR-Ck (see above). For FXa (plasmatic and recombinant derivatives) titration, 1 mM enzyme (determined from the published extinction coefficient of FXa at 280 nm (27)) was incubated with 50 nM to 10 mM $d$-FFR-Ck in kinetic buffer at room temperature. The incubation was sustained until the reaction was complete, i.e. a minimum of 10 half-lives were covered. The half-life of the reaction is equal to the natural logarithm of 2 divided by the product of the $k_{on}$ value of $d$-FFR-Ck for each FXa derivatives (see above) and the concentration of the inhibitor. At the end of this incubation, remaining free enzyme was measured with 100 $\mu$M S-2765 as substrate. The residual amidolytic activity was monitored at 405 nm in a kinetics microplate reader. By plotting the rate of S-2765 hydrolysis as a function of $d$-FFR-Ck concentration, a straight line was obtained for which the abscissa at the origin corresponds to the initial concentration of the active enzyme.

**Activation of Prothrombin by FXa**—The initial rate of prothrombin activation by FXa (plasmatic and activated recombinant FX derivatives) was measured in both the absence and presence of FVa as described previously (28). Briefly in the absence of the cofactor, the concentration dependence of prothrombin activation was studied by incubating each FXa derivative (5–10 nM) with increasing concentrations of the zymogen (20–3000 nM) in the presence of a saturating concentration (30 $\mu$M) of phospholipid vesicle preparation (PC:PS, 3:1) in 50 mM Tris (pH 7.5) and 100 mM NaCl with 5 mM CaCl$_2$ and 0.2% (w/v) BSA at 25 °C. Phospholipid vesicles (PC:PS, 3:1) of nominal 100-nm diameter were synthesized by the method of membrane extrusion (29). Phospholipid concentration was determined by phosphate analysis. Aliquots of the reaction mixture were taken at specified times, and the reaction was stopped in EDTA (10 mM final concentration) at the specified times. In experiments using various concentrations of prothrombin (0–4000 nM) and a saturating concentration of FVa (25 nM), the same protocol as described above was followed. During all assays, it was ensured that less than 10% of prothrombin was converted to thrombin, and thrombin formation was linear. Thrombin concentration was calculated as described above.

Initial velocity measurements of prothrombin hydrolysis by FXa derivatives alone or in the prothrombinase complex were analyzed by fitting the data to the Henri-Michaelis-Menten equation to yield fitted values for $k_m$ and $k_{cat}$. Non-linear regression was used to derive apparent dissociation constants $K_d(app)$ for the interaction between FXa derivatives and phospholipid vesicle-bound FVa using the initial rate of thrombin formation in the presence of different concentrations of FVa and a single ligand-binding site equation.

**Chromogenic Substrate Cleavage by FXa**—The steady-state kinetics of hydrolysis of S-2765 and SpeFXa by FXa derivatives were assayed at 37 °C in kinetic buffer with various salt combinations. Kinetics parameters of substrate hydrolysis were determined using enzyme concentrations of 0.5, 2.0, or 6.0 nM and various substrate concentrations ranging from 20 to 2000 $\mu$M. The release of pNA was monitored at 405 nm at 37 °C in a kinetics microplate reader.

Apparent dissociation constants $K_d(app)$ for the interaction between FXa derivatives and Na$^+$ were obtained from the dependence of the initial rate of pNA formation on the concentration of Na$^+$ (10–800 mM) in the presence of various concentrations of S-2765 (25–2500 $\mu$M). It has been demonstrated previously that the amidolytic activity of FXa is not dependent on the ionic strength of the reaction buffer in the range of NaCl used (19, 23, 30). Thus, no compensating chloride salt was added to the reactions. The data were fitted to a single ligand-binding site with a defined background amidolytic activity in the presence of 10 mM Na$^+$ using Equation 2,

$$y = k_{cat(min)} + k_{cat}x/(K_{d(app)} + x) \quad (Eq. 2)$$

where $K_{d(app)}$ is the apparent dissociation constant of Na$^+$ for an FXa derivative in the kinetic buffer used, $y$ is the $k_{cat}$ of the substrate hydrolysis at a given Na$^+$ concentration depicted by $x$, $k_{cat(min)}$ is the corrected background $k_{cat}$ in the absence (in fact 10 mM) of Na$^+$, and $k_{cat}$ is the corrected $k_{cat}$ at saturating concentration of Na$^+$.

In the presence of a saturating concentration of FVa (25 nM), the concentration dependence of SpeFXa cleavage was studied by incubating each FXa derivative (0.5 and 2 nM) with increasing concentrations of the substrate (10–2000 $\mu$M) on phospholipid vesicles prepared at a saturating concentration (50 $\mu$M). Initial velocity measurements of pNA generated by FXa derivatives alone or in the prothrombinase complex were analyzed by fitting the data to the Henri-Michaelis-Menten equation to yield fitted values for $K_m$ and $k_{cat}$.

**Inhibition of FXa Derivatives Alone or in the Prothrombinase Complex by PAB**—The inhibitory constant ($K_i$) of PAB for FXa derivatives alone or in the prothrombinase complex was assessed assuming classical competitive inhibition by initial
velocity measurements of S-2765 hydrolysis by each enzyme using increasing concentrations of substrate (0–1000 μM) at different fixed concentrations of PAB (0–500 μM) as described previously (31). Initial velocities were measured at 37°C in kinetic buffer using enzyme concentrations of 0.5, 2.0, or 6.0 nM with or without 25 nM FVa and 50 μM phospholipid vesicles (PS/PC). The concentrations were chosen to saturate the prothrombinase complexes with the FXa derivatives based on the equilibrium constants determined above. The linear dependence of rate on the concentration of FXa at saturating concentrations of FVa and PS/PC was established in separate experiments. Initial velocity measurements of substrate hydrolysis by FXa derivatives alone or in the prothrombinase complex were analyzed according to the rate expression for linear competitive inhibitor to yield the fitted values for $K_m$, $V_{max}$, and $K_I$.

Inhibition of FXa Derivatives Alone or in the Prothrombinase Complex by Antithrombin III—The rate of inactivation of FXa derivatives alone or in the prothrombinase complex by ATIII was measured under pseudo-first order rate conditions by a discontinuous assay. For the inhibition of FXa derivatives, ATIII (0–0.7 μM) was incubated with FXa (5 or 10 nM) in kinetic buffer up to 120 min (12 time points). At the end of the time course, S-2765 was added (100 μM final concentration) to monitor residual enzyme activity. For the inhibition of FXa derivatives in the prothrombinase complex, ATIII (0–7 μM) was incubated with FXa (5 nM), FVa (30 nM), and phospholipid vesicles (50 μM) in kinetic buffer for up to 60 min (12 time points). Residual enzyme activity was monitored as described above. By plotting the rate of S-2765 hydrolysis as a function of the incubation time of ATIII and its target enzyme, a curve was obtained. By non-linear regression, using Equation 1 (see above), the rate constant for inactivation of the target enzyme was estimated. In both assays, each measurement was made in three separate experiments.

Molecular Modeling—To explore the possible structural consequences of the FXaV163A and FXaS167A mutations near the interface with Tyr225, energy minimization and molecular dynamics simulations were performed with GROMACS 3.3.1 (32) based on the 2.2-Å structure of wt-FXa (Protein Data Bank accession code 1HCG (22)). The wild-type structure was used as a control and treated in an identical fashion to the mutants. A strategy using positional restraints was chosen to broadly constrain the conformation of the protein to that observed in the crystal structures of FXa while permitting deviation particularly in the vicinity of Tyr225, Val163, and Ser167. Side-chain Cγ1 and Cγ2, and Oγ atoms were deleted to generate the FXaV163A and FXaS167A mutants. The two mutants and wild-type protein were modeled in an 81-Å cubic box with boundaries at least 9.5 Å from the protein, solvated using the Simple Point Charge model for solvent with 18,800 water molecules, and subjected to 1000 steps of steepest descent energy minimization using a grid search and default parameters. Subsequent molecular dynamics simulations used periodic boundary conditions, 2-6 steps, weak coupling (0.1 s⁻¹) to a 300 K temperature bath separately for protein and solvent, coupling (0.5 s⁻¹) to a pressure sink (1 bar), Linear Constraint Solver restraints on bond lengths, the Particle Mesh Ewald model for electrostatic interactions with a 10-Å cutoff, and the ffG43a1 (GROMOS96) force field (33). Initially a 100-ps molecular dynamics simulation applying medium side-chain/high main-chain (1000 kJ mol⁻¹ nm⁻²/10,000 kJ mol⁻¹ nm⁻²) positional restraints was undertaken. Following this, these restraints were relaxed to 10/100 for residues 145–151 and 224–227 and 100/1000 for residues within 10 Å with a smooth transition to 100/1000 at a distance of 18 Å or more. A 1-ns molecular dynamics simulation was then performed. For each variant, the average structure was determined from the simula-
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TABLE 1
Kinetic constants for the cleavage of prothrombin by FXa derivatives in the absence and presence of FVa and the apparent dissociation constants for their interaction with FVa

The kinetic parameter $k_{cat}/K_{m(app)}$ values were determined from the concentration dependence of prothrombin activation by FXa derivatives on phospholipid vesicles in the absence and presence of saturating concentration of FVa (25 nM) as described under "Experimental Procedures." The apparent dissociation constant values for the interaction with FVa were determined from FVa concentration dependence of thrombin generation by each FXa derivative in the presence of a saturating concentration of prothrombin (2 μM) as described under "Experimental Procedures." Mean values ± S.D. for at least three experiments are presented.

| FXa/FVII163–170 | Prothrombin, PC/PS, Ca\(^{2+}\) | 2.5 \(\times\) 10\(^{-9}\) | 0.18 ± 0.03 |
| Wild-type FXa | Prothrombin, PC/PS, Ca\(^{2+}\) | 2.5 \(\times\) 10\(^{-9}\) | 0.18 ± 0.03 |
| FXa/FIX163–170 | Prothrombin, PC/PS, Ca\(^{2+}\) | 2.5 \(\times\) 10\(^{-9}\) | 0.18 ± 0.03 |
| FXa/FIX163–167 | Prothrombin, PC/PS, Ca\(^{2+}\) | 2.5 \(\times\) 10\(^{-9}\) | 0.18 ± 0.03 |

RESULTS

Recombinant Proteins—All recombinant FX proteins were subjected to digestion by RVV-X under conditions similar to those of pd-FX. All FX derivatives could be converted into their active form, and the final activated preparations were more than 90% active as determined by active site titration. Mutations introduced within h163–170 of the protease domain are shown in Fig. 2.

Prothrombin Activation with Increasing FVa Concentrations—Previous studies have indicated that substitution of two basic residues exposed at the surface of h163–170 interferes with the formation of the FXa-FVa complex (8, 9, 34). We decided to explore the effect of substituting other residues within h163–170 on prothrombin activation by the FXa-FVa complex. For this purpose, the rate of thrombin generation was studied as a function of increasing FVa concentration in the presence of an excess of phospholipid (50 μM) and prothrombin (2 μM). Under these conditions, prothrombin represents a saturating concentration of substrate for all constructs (see results below) and twice the physiological concentration found in plasma. For all the recombinant FXa constructs tested, the rate of thrombin formation was saturable and dependent on the concentration of FVa (Fig. 3).

The apparent affinity ($K_d$) of wt-FXa for FVa was determined to be 0.11 nM, and a similar value (0.09 nM) was obtained for the FX/FIX163–170 chimera (Table 1). In contrast, substitution of h163–170 by the corresponding residues of FVII had a signifi-
TABLE 2
Kinetic constants for the cleavage of peptidyl substrates by FXa derivatives alone or in the prothrombinase complex

Hydrolysis of S-2765 and SpeFXa by FXa derivatives alone or in the prothrombinase complex was measured in the presence of 150 mM NaCl. $K_m$ and $k_{cat}$ values were determined using enzyme concentrations of 0.5, 2.0, or 6.0 nM and various substrate concentrations (20–2000 μM). For the experiments in which the prothrombinase complex was used, 2.0 nM FXa derivatives were incubated with 25 nM FVa, 50 μM phospholipid vesicles (PS/PC), and increasing concentrations of SpeFXa substrate (10–2000 μM). ND, experiment not done. Details of experimental designs can be found under "Experimental Procedures." Mean values ± S.D. for at least three experiments are presented.

|          | Peptidyl substrates |          |          |          |
|----------|---------------------|----------|----------|----------|
|          | S-2765              | SpeFXa   |          |          |
|          | $K_m$ (μM)          | $k_{cat}$ (μM s$^{-1}$) | $k_{cat}/K_m$ (μM$^{-1}$ s$^{-1}$) |          |
| pd-FXa   | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 160 ± 16 | 185 ± 24 | 1.15     | 97 ± 15  | 210 ± 21 | 2.16     |
| wt-FXa   | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 153 ± 12 | 186 ± 26 | 1.21     | 109 ± 16 | 215 ± 25 | 1.98     |
| FXa/FIX163–170 | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 132 ± 11 | 194 ± 23 | 1.46     | 90 ± 10  | 222 ± 15 | 2.47     |
| FXa/FVII163–170 | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 177 ± 21 | 161 ± 17 | 0.90     | 217 ± 40 | 185 ± 20 | 0.85     |
| FXa/FVII163–167 | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 318 ± 46 | 110 ± 11 | 0.34     | 431 ± 97 | 126 ± 15 | 0.29     |
|          | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 350 ± 43 | 77 ± 6   | 0.22     | 560 ± 152| 89 ± 12  | 0.16     |
|          | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 165 ± 13 | 186 ± 22 | 1.12     | 101 ± 12 | 213 ± 19 | 2.10     |
|          | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 314 ± 31 | 146 ± 15 | 0.46     | 388 ± 52 | 171 ± 18 | 0.44     |
|          | Ca$^{2+}$, PC/PS, FVa, Ca$^{2+}$ | 307 ± 30 | 152 ± 17 | 0.50     | 353 ± 48 | 178 ± 19 | 0.50     |

In contrast, the absence of FVa yielded a different profile of prothrombinase activity among the FXa variants. The rate of thrombin formation was assessed in the presence of saturating concentrations of phospholipids (50 μM) and various concentrations of prothrombin but in the absence of FVa. Under these conditions, the FXa/FIX163–170 chimera displayed the same catalytic efficiency as wt-FXa toward prothrombin (Fig. 4A) as observed in the presence of an excess of FVa (Fig. 4B). Similarly and as previously reported (9), FXaR165A had the same catalytic efficiency as wt-FXa (Table 1 and Fig. 4A). In contrast, substitution of h163–170 by the corresponding residues of FVII and the V163A and S167A mutations had a significant effect on the catalytic efficiency of FXa toward prothrombin in the absence of FVa (Table 1 and Fig. 4A). These results suggest that h163–170, more specifically the residues at the amino-terminal part of FVa, plays a critical role in the catalytic activity of FXa toward prothrombin. The assembly of the defective FXa derivatives in the prothrombinase complex, containing 25 nM FVa, was found to correct their catalytic efficiency toward prothrombin.

**Prothrombin Activation with Increasing Prothrombin Concentration**—Next the rate of thrombin generation was studied as a function of increasing concentrations of prothrombin in the presence of an excess of phospholipids (50 μM) and a concentration of FVa representing the physiological concentration of FV found in plasma (25 nM). Under these conditions, the assembly of the FXa derivatives in the prothrombinase complex partially restored, when it was defective, the catalytic efficiency of the enzyme toward prothrombin to that observed with wt- and pd-FXa (Table 1).

In contrast, the absence of FVa yielded a different profile of prothrombinase activity among the FXa variants. The rate of thrombin formation was assessed in the presence of saturating concentrations of phospholipids (50 μM) and various concentrations of prothrombin but in the absence of FVa. Under these conditions, the FXa/FIX163–170 chimera displayed the same catalytic efficiency as wt-FXa toward prothrombin (Fig. 4A) as observed in the presence of an excess of FVa (Fig. 4B). Similarly and as previously reported (9), FXaR165A had the same catalytic efficiency as wt-FXa (Table 1 and Fig. 4A). In contrast, substitution of h163–170 by the corresponding residues of FVII and the V163A and S167A mutations had a significant effect on the catalytic efficiency of FXa toward prothrombin in the absence of FVa (Table 1 and Fig. 4A). These results suggest that h163–170, more specifically the residues at the amino-terminal part of h163–170 but not Arg165, had a deleterious effect on the catalytic groove of these two constructs is identical to that of pd- and wt-FXa. In contrast, an increase in $K_m$ and a decrease in $k_{cat}$ were observed for FXa/FVII163–170, FXa/FVII163–170, FXaV163A, FXaS167A, and to a lesser extent FXa/FVII163–170 compared with those of wt- and pd-FXa (Table 2). These data indicate that these mutations altered the amidolytic activity of FXa, and hence suggest that the substrate binding cleft (S1–S3 site) is changed in some fashion.

**Amidolytic Activity of Complex-bound Protease**—In contrast, the assembly of the FXa derivatives in the prothrombinase complex restored or partially restored their catalytic efficiency
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**TABLE 3**

Inhibition kinetics of synthetic substrate cleavage by FXa derivatives alone or in the prothrombinase complex

Inhibition kinetics of FXa derivatives alone or in the prothrombinase complex were determined from initial velocity studies conducted with the peptide substrate S-2765. The \( k_{cat} \) values of D-FFR-CK for activated forms of the FXa derivatives were estimated under pseudo-first order conditions. The \( k_{cat} \) values were determined from the residual activities of the enzymes toward S-2765 after their incubation with the inhibitor. The constant values are indicated along with standard errors (expressed as a percentage of the value obtained). ND, experiment not done. As well, the second-order rate constants, \( k_{cat} \), for ATIII inhibition of FXa derivatives were determined from the residual activities of the enzymes toward S-2765 after their incubation with the inhibitor. The \( K_i \) values for PAB were determined by measuring the decrease in the initial rate of hydrolysis at varying concentrations of S-2765. Global fitting of data to a classical competitive binding equation yielded the \( K_i \) values for PAB. Details of experimental designs and concentrations of reagents can be found under "Experimental Procedures." Mean values ± S.D. for at least three similar experiments are presented.

| FXa derivatives | D-FFR-CK \( k_{cat} \) ± S.D. (%) | ATIII \( k_i \) ± S.D. × 10^-3 | PAB \( K_i \) ± S.D. |
|-----------------|----------------------------------|-------------------------------|------------------|
| **Plasma-derived FXa** |                                  |                               |                  |
| Ca²⁺, PC/PS, FVa, Ca²⁺ | 2423 ± 24                        | 3.13 ± 0.27                  | 55.1 ± 3.8       |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.43 ± 0.12                  | 62.5 ± 3.3       |
| **Wild-type FXa** |                                  |                               |                  |
| Ca²⁺, PC/PS, FVa, Ca²⁺ | 2351 ± 16                        | 3.33 ± 0.24                  | 60.6 ± 6.1       |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.45 ± 0.11                  | 62.1 ± 5.0       |
| **FXa/FIX163–170** |                                  |                               |                  |
| Ca²⁺ | 2250 ± 15                        | 3.02 ± 0.24                  | 63.3 ± 5.5       |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.40 ± 0.11                  | 67.3 ± 6.1       |
| **FXa/FVII163–167** |                                  |                               |                  |
| Ca²⁺ | 1077 ± 19                        | 1.55 ± 0.75                  | 126.5 ± 9.3      |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.40 ± 0.12                  | 73.3 ± 7.2       |
| **FXa/FVII163–167** |                                  |                               |                  |
| Ca²⁺ | 575 ± 13                         | 0.83 ± 0.22                  | 225.1 ± 23.2     |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.23 ± 0.09                  | 122.7 ± 15.1     |
| **FXa/FVII163–170** |                                  |                               |                  |
| Ca²⁺ | 313 ± 8                          | 0.45 ± 0.15                  | 688.5 ± 65.6     |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.11 ± 0.08                  | 310.3 ± 21.9     |
| **FXaR165A** |                                  |                               |                  |
| Ca²⁺ | 2268 ± 25                        | 3.21 ± 0.25                  | 64.3 ± 5.5       |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.41 ± 0.12                  | 72.6 ± 8.1       |
| **FXaS167A** |                                  |                               |                  |
| Ca²⁺ | 688 ± 14                         | 0.94 ± 0.20                  | 208.3 ± 20.1     |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.33 ± 0.09                  | 80.8 ± 10.6      |
| **FXaR165A** |                                  |                               |                  |
| Ca²⁺ | 635 ± 15                         | 0.93 ± 0.22                  | 227.5 ± 22.6     |
| PC/PS, FVa, Ca²⁺ | ND                              | 0.31 ± 0.12                  | 91.0 ± 10.1      |

**FIGURE 5.** Na⁺ dependence of the amidolytic activity toward S-2765 of FXa derivatives. The amidolytic activity toward S-2765 (10–2500 µM) of each FXa derivative was monitored in the presence of different concentrations of Na⁺, 10 mM in panel A and 150 mM in panel B, as described under "Experimental Procedures." The concentrations of enzymes were 0.5 or 2 nM for wt-FXa (open circles), FXa/FX163–170 (closed circles), or FXaR165A (closed squares) and 2 or 6 nM for FXa/FVII163–170 (open triangles), FXa/FVII163–167 (closed triangles), FXa/ FVII168–170 (open squares), FXaR165A (open diamonds), or FXaS167A (closed diamonds). Data represent mean values of at least three experiments.

against the peptidyl substrate tested to that observed with wt- and pd-FXa with the exception of FXa/FVII163–167 and FXa/FVII163–170. This was mediated through changes in \( K_m \) values, whereas the \( k_{cat} \) values were not significantly changed (Table 2). The minimal restoration of the catalytic efficiency observed for FXa/FVII163–170 and FXa/FVII163–170 will be discussed later. In conclusion, the data obtained are consistent with the hypothesis that prothrombinase complex assembly corrects defective binding of peptidyl substrates introduced by mutations in h163–170 of FXa.

**Interaction of a Probe with the S1 Site**—The binding of a well defined S1 probe, PAB, to FXa was also investigated. FXa/FIX163–170 and FXaR165A displayed affinity for PAB similar to that of wt-FXa or pd-FXa, whereas FXa/FVII163–167, FXa/ FVII168–170, FXa/FVII163–170, FXaV163A, and FXaS167A displayed lower rates of affinity, indicating an alteration of their S1 specificity pocket (Table 3). Similar results were also observed for the interaction with both D-FFR-CK and ATIII (Table 3). In contrast, assembly of the FXa derivatives in the prothrombinase complex, containing an excess of FVa, restored ATIII and PAB binding to that observed with wt- and pd-FXa with the exception of FXa/FVII163–167 and FXa/FVII163–170 (Table 3). In conclusion, the data obtained show that prothrombinase complex assembly corrects defective binding of peptidyl substrates into the S1 pocket by molecules containing mutations in h163–170.

**The Effect of Sodium Ions on Amidolytic Activity**—Previous results (20, 23) have indicated that there is an allosteric linkage between the FVa-binding site(s) and the Na⁺-binding site of FXa. To test the hypothesis that h163–170, which is an FVa-binding site, is linked to the Na⁺-binding site, Na⁺ dependence of the amidolytic activity toward S-2765 of pd-FXa, wt-FXa, and recombinant FXa derivatives was assayed. Na⁺ improved the amidolytic activity of wt-FXa and all FXa derivatives (Fig. 5). At low (10 mM) Na⁺ concentration, the \( k_{cat} \) values for FXa/FVII163–170 and FXa/FVII163–167 were reduced compared with wt-FXa, whereas all the other FXa derivatives had \( k_{cat} \) values similar to that of wt-FXa (Fig. 5A). At 150 mM Na⁺, which is the physiological concentration tightly regulated in blood, only FXa/FIX163–170 and FXaR165A displayed catalytic efficiency similar to that of wt-FXa, whereas the other FXa derivatives displayed reduced catalytic efficiencies (Fig. 5B). Comparison of the catalytic parameters as a function of Na⁺ concentration showed that Na⁺ diminished the \( K_m \) values and to a lesser extent increased the \( k_{cat} \) values (Fig. 6). Notably it was observed that Na⁺ reduced the differences between the catalytic parameters of the variants toward the peptidyl substrate tested (S-2765) compared with wt- and pd-FXa. This was affected predominantly through changes in \( K_m \) values. When \( k_{cat} \) and \( K_m \) values are


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TABLE 4

| Factor Xa Derivative | Apparent Dissociation Constant (K_{d_{app}}) | Mean ± S.D. |
|----------------------|---------------------------------------------|-------------|
| Wild-type FXa        | 60 ± 13                                     |             |
| FXa/FIX^{163–170}    | 46 ± 9                                      |             |
| FXa/FIX^{166–170}    | 67 ± 14                                     |             |
| FXa/FIX^{166–167}    | 371 ± 114                                   |             |
| FXa/FIX^{166–167}    | 400                                         |             |
| FXa/FIX^{V163A}      | 74 ± 17                                     |             |
| FXa/FIX^{V163A}      | 324 ± 72                                    |             |
| FXa/FIX^{S167A}      | 352 ± 105                                   |             |

DISCUSSION

In the present investigation, we extended the finding of previous studies that have implicated the basic residues of h163–170 in the regulation of FXa by cofactors (8, 9). Indeed our data show (Fig. 3) that FXa derivatives with a mutation at position Arg^{165} exhibited a severe defect in thrombin formation in the presence of small amounts of FV. The same defect was observed, albeit to a lesser extent, in FXa/FIX^{163–170} bearing the substitutions K169L and L170Q. Surprisingly replacement of h163–170 with the FIXa h163–170 was without effect on FVa binding (Fig. 2). This is consistent with the structure of FXa and the data presented in this study, however. Both Lys^{169} and Leu^{170} are solvent-exposed and protrude away from the enzyme; hence neither mutation would be expected to alter the local conformation of h163–170. It has also been reported that basic residues are important for the interaction of FXa with its cofactor (8), and FIXa possesses an Arg at position 170; essentially the hydrophobic and charged side chains have been transposed. Furthermore consideration of the three-dimensional structures of FXa and FIXa (21, 22) suggests that the other residues of h163–170 are equivalent in the two enzymes. In particular, residue Ser^{167} is replaced in FIXa by threonine, which is able to adopt a similar rotamer and form the hydrogen bond with Asp^{164} identified in this work as important to the function of h163–170. Thus, the absence of effect on prothrombinase activity observed in FXa/FIX^{163–170} can be explained by the presence of a surface-exposed basic residue at position 169 or 170 (as in FXa or FXa/FIX^{163–170}).

plotted as a function of Na^{+} concentration (Figs. 6, A and B, respectively) the midpoint of the curves yields the apparent K_{d} for Na^{+} interaction with wt-FXa and FXa derivatives in the amidolytic assay using S-2765. Calculated K_{d} values show a significant decrease of the affinity of FXa^{V163A}, FXa^{S167A}, FXa/FIX^{166–170}, and FXa/FIX^{166–167} for Na^{+} compared with wt-FXa (Table 4). Altogether these results support the notion that in the presence of 150 mM Na^{+} (close to the physiological concentration found in plasma) the impairment of the amidolytic activity of FXa derivatives, when observed, is caused by their impaired interaction with Na^{+}.

FIGURE 6. K_{m} and K_{cat} as a function of Na^{+} of the amidolytic activity toward S-2765 of FXa derivatives. The catalytic parameters of the amidolytic activity toward S-2765 of each FXa derivative (wt-FXa (open circles), FXa/FIX^{163–170} (closed circles), or FXa^{V163A} (open squares)) were determined in the presence of 10 mM Na^{+} as a function of Na^{+} concentration. The open squares, closed circles, and open triangles show the amidolytic activity of FXa derivatives toward S-2765 of each FXa derivative (wt-FXa (open circles), FXa/FIX^{163–170} (closed circles), or FXa^{V163A} (open squares)).

TABLE 4

Apparent dissociation constants (K_{d_{app}}) for Na^{+} interaction with FXa derivatives

| Factor Xa Derivative | K_{d_{app}} (mM) |
|----------------------|------------------|
| Wild-type FXa        | 60 ± 13          |
| FXa/FIX^{163–170}    | 46 ± 9           |
| FXa/FIX^{166–170}    | 67 ± 14          |
| FXa/FIX^{166–167}    | 371 ± 114        |
| FXa/FIX^{166–167}    | 400              |
| FXa/FIX^{V163A}      | 74 ± 17          |
| FXa/FIX^{V163A}      | 324 ± 72         |
| FXa/FIX^{S167A}      | 352 ± 105        |

The present study demonstrates that FXa positions other than the surface-exposed arginine and lysine residues of h163–170 are important for the interaction with FVas. When Val^{163} or Ser^{167} were changed to alanine, FXa displayed a defect in thrombin formation in the presence of small amounts of FVas (Fig. 3 and Table 1). The effect of the mutations could occur because Val^{163} or Ser^{167} is directly important for FVa binding. This notion is supported by the reduced affinity of FXa^{V163A} and FXa^{S167A} for FVas (Table 1 and Fig. 3). Furthermore the same observation has been reported previously at these two positions in FIXa-FVIIIa interaction (12). Residues Val^{163} and Ser^{167} are on the same side of h163–170, making contacts with
FIGURE 7. Schematic representation of the crystal structure of the catalytic domain of FXa, indicating the position of the helix 163–170 and the point mutants investigated. Panel A shows a representation (Protein Data Bank code 1HCG (22)) oriented to highlight the position of h163–170 (yellow) and loops that coordinate the sodium ion (dark blue tubes). Other helices are pale blue, and strands are pink. The positions of the calcium ion (green sphere) and sodium ion (red sphere), inferred from crystal structures (Protein Data Bank codes 2J4I and 2BOK) are also shown along with their coordinating bonds. The S1 subsite (gray transparent surface) and the catalytic residues (orange stick), on the opposite face of the enzyme, are partially visible. Tyr225, Val163, Asp164, and Ser167 are shown in black-and-red stick, and Arg165 is shown in blue stick. In panel B, a close-up view of the FVa-binding region of wt-FXa is shown, illustrating the close contacts between Val163 and Tyr225 (gray sticks inside transparent van der Waals spheres). The sodium ion and S1 subsite are shown for reference. In panel C, the equivalent region in the FXaV163A mutant shows both a difference in packing and a change in the orientation of Tyr225. In panel D, a graphical representation of the relative mobility of the Tyr225 side chain during the molecular dynamics simulation for wt-FXa (blue) and the V163A mutant (red) is shown. The abscissa reflects the relative dihedral angle around the Ca–Cβ bond with respect to that of the initial, energy-minimized structure of wt-FXa; the ordinate reflects the relative angle around the Cβ–Cγ bond. It can be seen that the FXaV163A mutant is able to explore a much greater angular range than the wild-type protein. In panel E, the FVa-binding region of the FXaS167A mutant highlights the loss of the hydrogen bond between Ser167 and Asp164 visible for wt-FXa (A) with the consequence that the side chain of the aspartic acid residue is no longer held in place. Structural figures were prepared using PyMOL (36).

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residues inside the enzyme (22). There is a hydrogen bond between Ser167 and Asp164 that appears to orient the acidic side chain toward h163–170 (Fig. 7B). Val163 makes symmetric van der Waals contacts with the aromatic ring of Tyr225, restricting the conformational mobility of the side chain (Fig. 7B).

A molecular dynamics simulation, undertaken to explore a potential change in conformational freedom of Tyr225, revealed that in FXaV163A the tyrosine side chain would gain additional mobility (Fig. 7D, red circles) with a corresponding predicted shift in position (Fig. 7C) with respect to the wild-type enzyme (Fig. 7D, blue circles). In the case of FXaS167A under the same conditions, this increase in conformational freedom is not evident for Tyr225 (Fig. 7E). However, loss of the hydrogen bond with Asp164 and the concomitant increase in mobility of this side chain may still elicit structural consequences. For example FVa binding may cause a slight shift in the position of h163–170; loss of the Ser167–Asp164 hydrogen bond would reduce the degree to which this shift is transmitted to the adjacent Val163 residue. The observation that a saturating concentration of FVa restored or partially restored the catalytic defect of FXa derivatives toward prothrombin (Table 1 and Fig. 4), chromogenic substrate (Table 2), and interaction with PAB, D-FFR-CK, and ATIII (Table 3) supports this notion.

The effect of these mutations is likely mediated indirectly through allosteric changes in the conformation of the S1 site of the protease. These defects were prominently observed for FXa derivatives with substitutions or mutations of residues at the amino-terminal end of h163–170 except Arg165. Therefore, the data presented demonstrate that FVa, through interaction with h163–170, optimizes the conformation of the S1 site for FXa interaction with its substrates in the activation complex.

Thermodynamic linkage analyses have shown an allosteric coupling between FVa and Na⁺ sites in FVa (20, 23). This study shows that the alteration of the interaction of FXaV163A and FXaS167A with their substrates or inhibitors paralleled their impaired Na⁺ binding (Fig. 6). The same phenomenon was observed for FXa/FVII163–167 and FXa/FVII163–170. Although it cannot be excluded that the more extensive changes in
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FVa-binding site, Na⁺, and S1 sites that is modulated by residues Val⁽¹⁶³⁾ and Ser⁽¹⁶⁷⁾.

Acknowledgments—We thank the following members of INSERM U770: Cécile Denis for critical reading of the manuscript and Jean-Marie Freyssinet and Dominique Meyer for support and useful suggestions.

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