Factor Va Alters the Conformation of the Na^+-Binding Loop of Factor Xa in the Prothrombinase Complex†

Likui Yang,‡ Chandrashekhara Manithody,‡ Shabir H. Qureshi, and Alireza R. Rezaie*

Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, Saint Louis, Missouri 63104

Received February 25, 2008; Revised Manuscript Received April 17, 2008

ABSTRACT: Structural and mutagenesis data have indicated that the 220-loop of thrombin is stabilized by a salt-bridge between Glu-217 and Lys-224, thereby facilitating the octahedral coordination of Na^+ with contributions from two carbonyl O atoms of Arg-221a and Lys-224. All three residues are also conserved in fXa and the X-ray crystal structure of fXa indicates that both Glu-217 and Lys-224 are within hydrogen-bonding distance from one another. To investigate the role of these three residues in the catalytic function of fXa and their contribution to interaction with Na^+, we substituted them with Ala and characterized their properties in both amidolytic and proteolytic activity assays. The results indicate that the affinity of all three mutants for interaction with Na^+ has been impaired. The mutant with the greatest loss of affinity for Na^+ (E217A or E217Q) also exhibited a dramatic impairment (~3−4 orders of magnitude) in its activity toward both synthetic and natural substrates. Interestingly, factor Va (fVa) restored most of the catalytic defect with prothrombin, but not with the synthetic substrate. Both Glu-217 mutants exhibited a near normal affinity for fVa in the prothrombinase assay, but a markedly lower affinity for the cofactor in a direct-binding assay. These results suggest that, similar to thrombin, an ionic interaction between Glu-217 and Lys-224 stabilizes the 220-loop of fXa for binding Na^+. They further support the hypothesis that the Na^+ and fVa-binding sites of fXa are energetically linked and that a cofactor function for fVa in the prothrombinase complex involves inducing a conformational change in the 220-loop of fXa that appears to stabilize this loop in the Na^+-bound active conformation.

Factor Xa (fXa[1]) is a multidomain vitamin K-dependent trypsin-like serine protease in plasma that, upon complex formation with factor Va (fVa) on the membrane surfaces expressing negatively charged phospholipids (prothrombinase complex), converts prothrombin to thrombin at the final stage of the clotting cascade (*1–5*). The assembly of fXa into the prothrombinase complex enhances the catalytic efficiency of fXa by greater than 5 orders of magnitude by improving both the K_m and k_{cat} of the substrate activation reaction (*6, 7*). The improvement in K_m (~2 orders of magnitude) has been demonstrated to arise from the γ-carboxyglutamic acid (Gla) domain-dependent binding of both fXa and prothrombin on the same membrane surfaces (*1, 6, 7*). The remaining ~3 orders of magnitude improvement in the rate of prothrombin activation by fXa has been attributed to the cofactor effect of fVa which increases the k_{cat} of the reaction (*2, 7*). The mechanism by which fVa promotes the k_{cat} of prothrombin activation by fXa is not well understood. Results from several studies indicate that fVa binds to both fXa (*8, 9*) and prothrombin (*10, 11*) to stabilize the enzyme−substrate complex in an extended conformation above the membrane surface (*12, 13*), thereby facilitating the optimal docking of the two scissile bonds of the substrate into the active-site pocket of the protease (*14*). Numerous studies attempting to detect a conformational change in the active-site pocket of fXa upon its interaction with fVa have not been successful. Nevertheless, it is relatively well established that the activity of fXa is allosterically modulated by fVa as well as the metal ions Na^+ and Ca^{2+} (*1, 15–18*). Na^+ plays a critical role in the structure and function of fXa and other coagulation proteases in plasma (*18–20*). Recent mutagenesis and kinetic data have indicated that the Na^+-binding site of fXa (as well as the Na^+-binding sites of thrombin (*21*) and activated protein C (*22*)) is energetically linked to the primary S1 specificity pocket (nomenclature of Schechter and Berger (*23*)) of the protease (*16, 17*). In addition to the S1 subsite, it is also known that a thermodynamic linkage between the Na^+-binding site and the Ca^{2+}-binding site of fXa, which is located on the 70−80 loop ~30 Å away, modulates the structure and catalytic function of fXa (*19, 24*). Interestingly, recent results from several laboratories suggest that the binding of fVa to a conserved helical structure on fXa (160-helix) (*8, 9, 24*) is also associated with allosteric

† The research discussed herein was supported by grants awarded by the National Heart, Lung, and Blood Institute of the National Institutes of Health (HL 62565 and HL 68571 to A.R.R.).

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, 1100 S. Grand Blvd., St. Louis, MO 63104. Phone: (314) 977-9240. Fax: (314) 977-9205. E-mail: rezaiear@slu.edu.

‡ These authors contributed equally to this study.

[1] Abbreviations: fXa, activated factor X; fVa, activated factor V; Gla, γ-carboxyglutamic acid; E217A, R221aA, K222A, and K224A, fXa mutants in which the indicated residues have been replaced with Ala in the chymotrypsinogen numbering system (*25*); AT, antithrombin; H_5, pentasaccharide; RVV-X, factor X-activating enzyme from Russell's viper venom; PAB, *p*-aminobenzamidine; OG_{488}, Oregon Green 488; PC, dioleoylphosphatidylcholine; PS, dioleoylphosphatidylserine, PC/PS, phospholipid vesicles containing 80% PC and 20% PS.

10.1021/bi800319r CCC: $40.75
Published on Web 05/06/2008

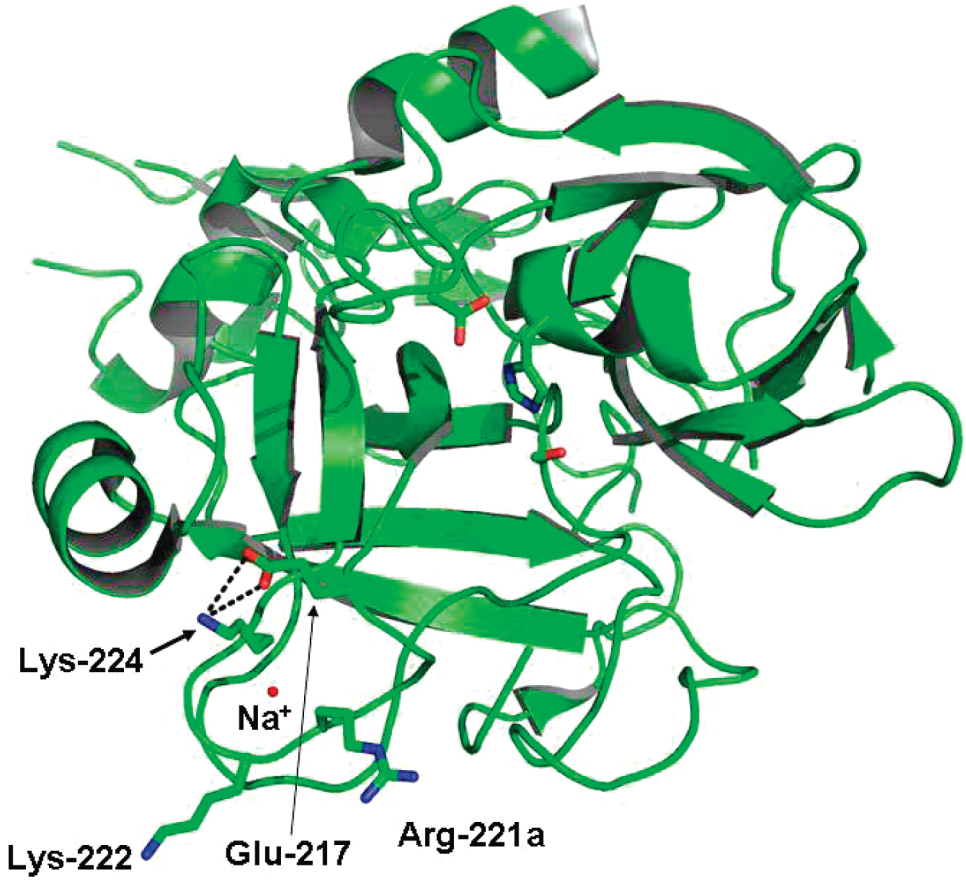

FIGURE 1: Crystal structure of the catalytic domain of fXa. The side chains of three basic residues (Arg-221a, Lys-222 and Lys-224) and one acidic residue (Glu-217) of the Na^+-binding loop of fXa are shown by arrows. The side chains of the catalytic triad residues in the center of the molecule from top to bottom Asp-102, His-57 and Ser-195 are marked. The coordinates (Protein Data Bank ID code 2BOK) were used to prepare the figure (*39*).

changes in the structure of the Na^+-binding 220-loop (chymotrypsin numbering (*25*)) of the protease (*15, 16*). The existence of such a network of allosterically linked regions in fXa, which are all subject to modulation by the cofactor functions of fVa, monovalent and divalent cations, is a testimony to the complex nature of the question under study, possibly providing an explanation for some of the difficulties we have in understanding the exact mechanism by which fVa improves the catalytic efficiency of fXa in the prothrombinase complex.

The Na^+-binding 220-loops of both fXa and thrombin have considerable sequence homologies, with both proteases having two conserved basic residues at positions 221 and 224, and fXa having an additional basic residue at position 222 (*24, 25*). In the case of thrombin, structural data have indicated that the 220-loop is stabilized by a salt-bridge between Glu-217 and Lys-224 (*26, 27*). The formation of this salt-bridge is essential for Na^+ binding to thrombin since, in addition to stabilizing the loop, it also positions the carbonyl O atom of Lys-224 in a configuration so that it can contribute to the octahedral coordination of Na^+ in the 220-loop (*26*). Thus, the substitution of Glu-217 with Lys or Ala has been demonstrated to disrupt this interaction, thereby abolishing the procoagulant activity of the mutant thrombin and stabilizing the protease in the Na^+-free slow conformation that is capable of activating protein C with near normal activity in the presence of thrombomodulin (*28, 29*). Glu-217 has also been conserved in fXa, and the examination of the X-ray structure of the catalytic domain of the protease suggests that it is also located within hydrogen-bonding distance from Lys-224 (Figure 1). To investigate the role of these residues in fXa and to determine whether a similar salt-bridge between Glu-217 and Lys-224 stabilizes the 220-loop to facilitate its interaction with Na^+, we substituted these residues with Ala in individual constructs and characterized their properties in kinetic assays in the absence and presence of fVa. The Lys-224 mutant was not expressed to a sufficient yield for full characterization. However, the results with the Glu-217 mutant suggest that, similar to thrombin, an ionic interaction between Glu-217 and Lys-224 may be required for Na^+ binding to fXa, as the mutation of this residue dramatically impaired the interaction of the metal ion with the protease. While the activation of prothrombin by both E217A and E217Q mutants was dramatically impaired (>2000-fold), nevertheless, fVa restored nearly all of the catalytic defects, suggesting that fVa induces conformational changes in the 220-loop to stabilize fXa in the Na^+-bound fast conformation.

MATERIALS AND METHODS

Mutagenesis and Expression of Recombinant Proteins. The expression and purification of wild-type factor X (fX) in human embryonic kidney (HEK-293) cells has been described previously (*30*). The fX mutants in the chymotrypsinogen numbering (*25*) [Glu-217 → Ala or Gln (E217A or Q), Arg-221a → Ala (R221aA), Lys-222 → Ala (K222A) and Lys-224 → Ala (K224A)] corresponding to residues Glu-

441, Arg-445, Lys-446, and Lys-448 in the fX cDNA numbering (*31*) were generated in the same expression/purification vector system by standard PCR mutagenesis methods as described (*30*). After confirmation of the accuracy of the mutagenesis by DNA sequencing, the constructs were introduced into HEK-293 cells and the mutant proteins were isolated from 20-L cell culture supernatants by a combination of immunoaffinity and ion exchange chromatography using the HPC4 monoclonal antibody and a Mono Q ion exchange column as described (*30*). The fully γ-carboxylated proteins were eluted from the ion exchange column at ~0.4 M NaCl as described previously (*32*). fX derivatives were converted to active forms by the factor X-activating enzyme from Russell's viper venom (RVV-X) and further purified by a heparin-Sepharose column chromatography as described (*30, 32*). Active-site concentrations of fXa derivatives were determined by an amidolytic activity assay and titrations with known concentrations of antithrombin (AT) assuming a 1:1 stoichiometry as described (*30*).

Human plasma proteins including fVa, fXa, prothrombin, AT, RVV-X and Oregon Green 488 (OG_{488})-labeled glutamylglycinylarginyl chloromethyl ketone (EGR) active-site-blocked human fXa (OG_{488}-EGR-fXa) were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Tissue factor pathway inhibitor (TFPI) was from Monsanto Chemical Co. (St. Louis, MO). Recombinant tick anticoagulant peptide (rTAP) was a generous gift from Dr. G. Vlasuk (Wyeth Research, Collegeville, PA). The active AT-binding pentasaccharide (H_5) fragment of heparin (fondaparinux sodium) was purchased from Quintiles Clinical Supplies (Mt. Laurel, NJ). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (*33*). Heparin-Sepharose was purchased from Amersham Pharmacia (Piscataway, NJ). The chromogenic substrates S2765 and S2238 were purchased from Kabi Pharmacia/Chromogenix (Franklin, OH). The active-site directed serine protease inhibitor *p*-aminobenzamidine (PAB) was purchased from Sigma (St. Louis, MO).

Cleavage of Chromogenic Substrates. The steady-state kinetics of hydrolysis of S2765 (8−4000 μM) by fXa derivatives (1−20 nM) was determined in 0.1 M NaCl, 0.02 M Tris-HCl (pH 7.5) containing 0.1% polyethylene glycol (PEG) 8000 and 5 mM $CaCl_2$ (TBS/Ca^{2+}). The rate of hydrolysis was measured at 405 nm at room temperature by a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA) as described (*30*). The amidolytic activities were monitored at two NaCl concentrations of 0.1 and 0.5 M for all fXa mutants. Furthermore, the activity of mutants toward the chromogenic substrate was evaluated in the presence of a saturating concentration of fVa (25 nM) on PC/PS vesicles (25 μM). The K_m and k_{cat} values for substrate hydrolysis were calculated from the Michaelis−Menten equation. The specificity constant for each mutant was expressed as the ratio of k_{cat}/K_m.

Apparent Dissociation Constant ($K_{d(app)}$) for Na^+. The $K_{d(app)}$ values for Na^+ interaction with each protease was determined from the effect of varying concentrations of Na^+ on the activity of the proteases toward S2765 (50−200 μM) at room temperature in 20 mM Tris-HCl buffer containing 5 mM Ca^{2+} and increasing concentrations of Na^+ (0−1000 mM) as described (*19*). The concentration of the chromogenic substrate used in each reaction was near or below the K_m values. The protease concentrations ranged from 1−20 nM. The $K_{d(app)}$ values were calculated from the hyperbolic increase in the rate of substrate hydrolysis as a function of increasing concentrations of Na^+ as described (*19*). It has been previously demonstrated that the amidolytic activity of fXa is not dependent on the ionic strength of the reaction buffer (*19*). Thus, no compensating chloride salt was added to the reactions. The data were fit to the Langmuir binding isotherm to obtain the $K_{d(app)}$ values for Na^+. A similar procedure was used to evaluate the Na^+-dependence of fXa derivatives in the presence of a saturating concentration of fVa (25 nM) on PC/PS vesicles (25 μM).

Inactivation by Antithrombin. The rate of inactivation of fXa derivatives by AT in both the absence and presence of H_5 was measured under pseudo-first-order rate conditions by a discontinuous assay as described (*30*). In the absence of H_5, each protease (1−5 nM) was incubated with AT (0.5−4.0 μM) in TBS/Ca^{2+} for 10−120 min in 50 mL volumes in 96-well polystyrene assay plates at room temperature. In the presence of H_5, the reaction conditions were the same except that the proteases were incubated with AT (25−500 nM) in the presence of a saturating concentration of H_5 (2 μM) for 0.5−30 min in the same TBS buffer system. All reactions were stopped by addition of 50 mL S2765 (final concentration of 0.2 mM) in TBS containing 1 mg/mL Polybrene. The remaining activities of enzymes were measured by a V_{max} kinetic microplate reader at 405 nm as described above. The observed pseudo-first-order and second-order rate constants (k_2) were calculated as described (*30*).

Interaction with TFPI and rTAP. The ability of fXa derivatives to bind either TFPI or rTAP was evaluated by incubating each fXa (1−20 nM) with different concentrations of TFPI or rTAP (0.15−1000 nM) in TBS/Ca^{2+} in 80 mL volumes in 96-well polystyrene plates. The reactions were carried out both in the absence and in the presence of fVa (25 nM) on PC/PS vesicles (20 μM). Following 30 min incubation at room temperature, 20 mL S2765 was added to a final concentration of 100−1000 μM, and the K_i values were estimated by nonlinear regression analysis of data using the quadratic equation describing the tight binding interactions as described (*15*).

Inhibition by p-Aminobenzamidine (PAB). The affinity of fXa derivatives for interaction with the active-site directed serine protease inhibitor, PAB, was determined by incubating each protease (1−10 nM) with increasing concentrations of PAB (0−2500 μM) in the presence of different fixed concentrations of S2765 (12.5−800 μM) in TBS/Ca^{2+} at room temperature. The enzyme activities were measured from the cleavage rate of the chromogenic substrate as described above, and the K_i values were determined by global fitting of data to a competitive binding equation as described (*15*).

Activation of Prothrombin. The initial rate of prothrombin activation by wild-type and mutant fXa derivatives was measured in both the absence and presence of fVa as described (*15*). In the absence of the cofactor, the concentration dependence of prothrombin activation was studied by incubating each fXa derivative (5−400 nM) with increasing concentrations of the zymogen (15−2000 nM) on PC/PS vesicles (25 μM) in TBS/Ca^{2+} at room temperature. Following 30−60 min activation, small aliquots of the activation reactions were transferred into wells of a 96-well assay plate

Table 1: Kinetic Constants for the Cleavage of S2765, Apparent Dissociation Constants ($K_{d(app)}$) for Interaction with Na^+, and the Second-Order Association Rate Constants for the Interaction of fXa Derivatives with AT in TBS/Ca^{2+}[a]

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	$K_{d(app)}$ (mM Na^+)	k_2 (AT) (M^{-1} s^{-1})
fXa	51.7 ± 6.0	125.3 ± 10.9	2.4 ± 0.5	40 ± 5	(2.6 ± 0.8) × 10^3
E217A	3900 ± 500	27.1 ± 6.9	0.007 ± 0.002	>1000	(1.1 ± 0.1) × 10^1
(0.5 M NaCl)	2000 ± 80	57.2 ± 2.8	0.029 ± 0.002		
E217Q	2050 ± 180	94.3 ± 13.8	0.046 ± 0.01	>1000	(3.5 ± 0.3) × 10^1
(0.5 M NaCl)	950 ± 150	151.7 ± 9.8	0.16 ± 0.03		
R221aA	191.7 ± 5.5	154.4 ± 4.2	0.8 ± 0.04	77 ± 8	(0.5 ± 0.1) × 10^3
K222A	50.7 ± 13.4	99.2 ± 6.9	2.0 ± 0.7	30 ± 11	(1.9 ± 0.3) × 10^3

[a] The kinetic constants were determined from the steady-state kinetics of hydrolysis of S2765 (7.8−4000 μM) by each fXa derivative (1−20 nM) in TBS/Ca^{2+}. The $K_{d(app)}$ values for the interaction of Na^+ with fXa derivatives were calculated from the metal ion dependent enhancement of amidolytic activities toward S2765. The rate of reaction remained linear for both E217A and E217Q mutants for up to 1000 mM Na^+. The kinetic parameters for hydrolysis of S2765 by both E217A and E217Q in TBS/Ca^{2+} containing 0.5 M NaCl are also presented. The second-order rate constants for the AT inhibition of fXa derivatives were calculated as described in Materials and Methods. All values are the average of at least 3 measurements ± standard deviations.

containing S2238 (200 μM in TBS containing 20 mM EDTA, and in 100 mL final volume) and the initial rate of thrombin generation was monitored at 405 nm by a V_{max} kinetic microplate reader. The concentration of thrombin generated in each activation reaction was determined from a standard curve prepared from the cleavage rate of S2238 by known concentrations of thrombin under exactly the same conditions.

In the presence of fVa, first the apparent affinity of fXa derivatives for interaction with the cofactor was evaluated in a prothrombinase assay as described (*15*). Briefly, fXa (0.1 nM) was mixed with varying concentrations of human fVa (0−10 nM) on PC/PS vesicles (25 μM) in TBS/Ca^{2+} at room temperature. The activation reaction was initiated with 1 μM human prothrombin for 1−4 min, following which it was terminated by addition of EDTA to a final concentration of 20 mM. The concentrations of thrombin generated in the activation reactions were determined from a standard curve as described above. The concentration dependence of prothrombin activation in the presence of fVa on PC/PS vesicles was also studied by a similar prothrombinase assay. In this case, fXa (50 pM) in complex with a saturating concentration of fVa (25 μM in all reactions) on PC/PS vesicles (25 μM) was incubated with varying concentrations of human prothrombin (7.8−1000 nM) in TBS/Ca^{2+}. Following 0.5−1 min of incubation at room temperature, EDTA was added to a final concentration of 20 mM and the concentrations of thrombin generated were determined by an amidolytic activity assay as described above.

Fluorescence Anisotropy Measurements. The affinities of fXa derivatives for interaction with fVa were evaluated by their ability to displace OG_{488}-EGR-fXa from the bound fVa at equilibrium as monitored by a decrease in the fluorescence anisotropy of the labeled fXa−fVa complex using an Aminco-Bowman series 2 spectrophotometer (Spectronic Unicam, Rochester, NY) as described (*15*). The competitive assay was carried out by the addition of small aliquots of increasing concentrations of the wild-type or mutant fXa derivatives to a fixed concentration of OG_{488}-EGR-fXa (10 nM) and fVa (25 nM) on PC/PS vesicles (10 μM) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5 containing 0.1% PEG-8000 and 5 mM Ca^{2+} with monitoring the decrease in the fluorescence intensity of the reaction upon the displacement of the labeled fXa from the cofactor. The excitation and emission wavelengths were set at 490 and 520 nm, respectively. The bandwidths were set at 8 nm for both excitation and emission. The titration was performed by addition of 1−2 μL of stock solution of fVa (1−4 μM) to the labeled fXa in a quartz cuvette in 1 mL volumes at 25 °C. The titration volume did not exceed 2% of the total volume of the reaction. The decrease in the anisotropy of the OG_{488}-EGR-fXa−fVa complex upon interaction with the unlabeled fXa derivatives was recorded for up to 55 nM fXa and the K_D values were estimated using a competitive binding equation as described (*10*).

RESULTS

Amidolytic Activity of fXa Derivatives. The kinetic parameters for the hydrolysis of S2765 by fXa derivatives in TBS/Ca^{2+} are presented in Table 1. The catalytic efficiency of both E217A and E217Q mutants of fXa toward the chromogenic substrate was dramatically impaired. A similar extent of impairment in the amidolytic activity of K224A was observed; however, a detailed kinetic analysis with this mutant was not feasible due to its poor yield in our expression system. Nevertheless, the amidolytic activity of the mutant at a single concentration of the substrate was similar to the activity of the E217A mutant. The catalytic efficiency of the isosterically substituted E217Q mutant of fXa was 6−7-fold higher than that of the E217A mutant (Table 1). In contrast to a dramatic impairment in the activities of E217A, E217Q and K224 mutants, the activity of K222A toward S2765 was normal and it was impaired only ~3-fold for the R221aA mutant (Table 1).

To determine whether the defect in the amidolytic activity of fXa derivatives is due to an altered interaction with the Na^+ ion, their apparent dissociation constants for interaction with the metal ion were calculated by an amidolytic activity assay as described (*15*). With the exception of the K222A mutant, which exhibited a normal affinity for Na^+, all other mutants bound Na^+ with lower affinities which ranged from an ~2-fold lower affinity for R221aA and greater than 25-fold lower affinity for the E217Q and 217A mutants (Table 1). The impairment in the affinity of the K224A mutant for Na^+ was also dramatic, but a lower expression yield for this mutant precluded a detailed analysis, thus no further data for this mutant will be presented below. These results suggest that the dramatic impairments in the amidolytic activity of the mutants may be due to their inability to effectively interact with Na^+. In support of this proposal, increasing the concentration of NaCl in TBS/Ca^{2+} from 0.1 to 0.5 M had minimal effect in the activity of wild-type, but improved the catalytic efficiencies of both E217A and E217Q ~4-fold (Table 1). To determine whether the mutagenesis of the

residues under study influences the S1 site specificity of the mutants, their ability to bind the S1 site specific probe of the trypsin-like serine proteases *p*-aminobenzamidine (PAB) was examined. In contrast to a K_i of ~80 μM for both wild-type and K222A fXa, a K_i value of ~250 μM was observed for the PAB inhibition of R221aA, thus the mutant exhibited ~3-fold impairment in interaction with the inhibitor that is identical to its extent of impairment toward the chromogenic substrate. Similar to the dramatic effect of mutagenesis on the chromogenic activity of E217A and E217Q mutants, the PAB inhibition of these mutants was also dramatically impaired (K_i > 1 mM), suggesting that the mutagenesis has altered the conformation of the S1 site of the mutant proteases. These results support the previous conclusion that the Na^+-binding and S1 sites of fXa are energetically linked (*16, 17*).

To determine whether the cofactor function of fVa can restore the catalytic defect of fXa derivatives toward hydrolysis of S2765, the chromogenic activity assays were also monitored in the presence of a saturating concentration of fVa on PC/PS vesicles. Factor Va did not influence the activities of mutants toward S2765, thus exhibiting similar degrees of impairment in the hydrolysis of the chromogenic substrate in both the absence and presence of the cofactor (data not shown). Interestingly, however, fVa eliminated the Na^+ requirement for the amidolytic activity of all fXa derivatives (data not shown). These results support the hypothesis that conformations of the Na^+-binding and fVa-binding sites of fXa are energetically linked. The Na^+-binding properties of fXa derivatives were not influenced by the PC/PS vesicles alone.

Inhibition by AT, TFPI and rTAP. Similar to their amidolytic activities and interaction with Na^+, both E217A and E217Q mutants exhibited dramatic impairments of ~225-fold and ~45-fold, respectively, in their reactivity with AT (Table 1). In the case of R221aA, the impairment in reactivity with AT was modest (~5-fold) and the K222A exhibited near normal reactivity with the serpin (Table 1). The reactivity of the mutant with AT was also evaluated in the presence of the high-affinity AT binding pentasaccharide fragment of heparin. A similar 200−300-fold improvement in the reactivity of wild-type and mutants in the presence of pentasaccharide suggested that the Na^+-binding loop does not provide a recognition site for specific interaction with the activated conformation of the serpin (data not presented). Kinetic studies monitoring the inhibition of fXa derivatives by tissue factor pathway inhibitor (TFPI) suggested a wild-type like inhibitor affinity for K222A (K_i = 350 pM and 258 pM for wild-type and K222A, respectively), ~5-fold lower affinity for R221aA (K_i = 1.8 nM) (Figure 2A) and a dramatically impaired affinity for the E217A and E217Q mutants (K_i = 72 nM and 15 nM, respectively) (Figure 2B). Thus, the inhibition profiles of mutants with TFPI closely correlate with their activity toward the chromogenic substrate S2765 (Table 1). The same inhibition studies in the presence of fVa on PC/PS vesicles indicated that fVa improves the affinity of E217Q mutant for interaction with TFPI 3-fold (K_i = 5 nM), but the cofactor did not influence the affinity of E217A for interaction with the inhibitor (Figure 2B). Factor Va also did not improve the affinity of wild-type or the other two mutants with TFPI, but rather slightly weakened the interactions (data not shown).

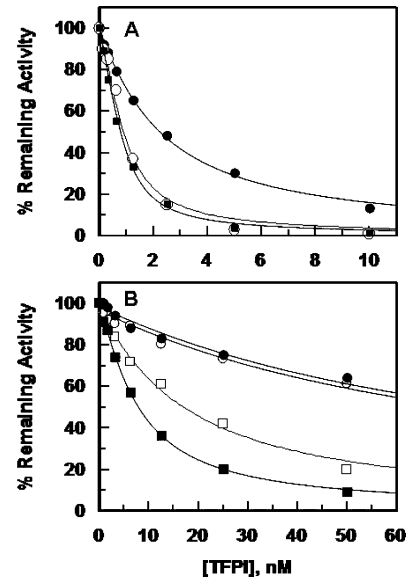

FIGURE 2: Inhibition of fXa derivatives by TFPI. A, the inhibition of wild-type fXa (○), R221aA (●) and K222A (■) (1 nM each) by increasing concentrations of TFPI was monitored in TBS/Ca^{2+}. The K_i values at equilibrium were determined from the remaining activities after 30 min incubation at room temperature by an amidolytic activity assay using S2765 as described in Materials and Methods. B, the same as A except that the remaining amidolytic activities of 10 nM E217A (○, ●) and 2 nM E217Q (□, ■) were monitored in the absence (open symbols) and presence of fVa (25 nM) on PC/PS vesicles (closed symbols). Solid lines in both panels are the best fit of kinetic data to a quadratic tight binding equation.

The X-ray crystal structure of recombinant tick anticoagulant peptide (rTAP) in complex with bovine fXa has indicated that an acidic region on the C-terminal helix of rTAP makes extended interactions with the basic residues of the 220-loop (*34*). To validate the structural data and to identify the residue(s) critical for the interaction, the rTAP concentration dependence of inhibition of fXa derivatives was studied. The results presented in Figure 3 suggested that Lys-222 makes no contribution to the interaction of the protease with the inhibitor since it exhibited an affinity for the inhibitor (K_i = 0.12 nM) that was slightly higher than that of wild-type fXa (K_i = 0.21 nM). On the other hand, the affinity of all other mutants for interaction with the inhibitor was dramatically impaired (Figure 3B). The impairment in affinities ranged from ~75-fold for R221aA (K_i = 15.9 nM) to ~415-fold for E217Q (K_i = 87.3 nM) and ~590-fold for E217A (K_i = 124.1 nM). Interestingly, fVa completely restored the impairment in the affinity of R221aA for rTAP on PC/PS vesicles so that the mutant interacted with the inhibitor with a K_i that was similar to that observed for the wild-type protease (Figure 3A, open triangles). On the other hand, while the affinity of both E217A and E217Q for interaction with rTAP was also improved ~10-fold in the presence of fVa + PC/PS vesicles, nevertheless, the effect was not mediated by fVa since similar results were obtained in the presence of PC/PS vesicles alone (data not shown). These results appear to suggest that the side chain of Arg-221a provides a specific recognition site for interaction with rTAP in the absence of fVa, however, a normal affinity of this mutant for the inhibitor in the presence of fVa suggests that a disordered conformation of the 220-loop and/or the S1 binding pocket of fXa may also be responsible for this

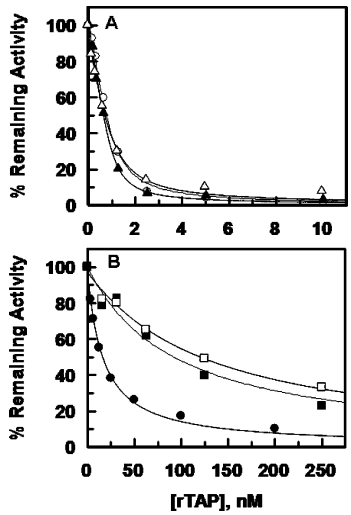

FIGURE 3: Inhibition of fXa derivatives by rTAP. A, wild-type fXa (○) or K222A (▲) (1 nM each) was incubated with increasing concentrations of rTAP in TBS/Ca^{2+} and the K_i values at equilibrium were determined from the remaining activities of enzymes using S2765 (100 μM) as described in Materials and Methods. The inhibition of one nM R221aA (△) was monitored in the presence of 25 nM fVa on 20 μM PC/PS vesicles. B, the same as A except that the inhibitions of R221aA (1 nM) (●), E217A (20 nM) (□) and E217Q (5 nM) (■) by rTAP were monitored. Solid lines in both panels are the best fit of kinetic data (average of three measurements) to a quadratic tight binding equation. All data with the exception of R221aA in panel A are derived from the kinetic studies in the absence of fVa.

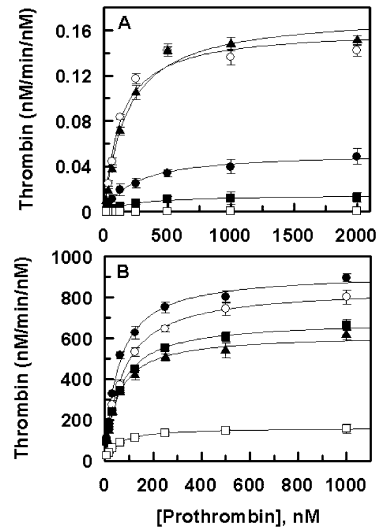

FIGURE 4: Prothrombin activation by fXa derivatives in the absence and presence of fVa. A, in the absence of fVa, different concentrations of human prothrombin (15−2000 nM) were incubated with each fXa derivative (5−400 nM): wild-type (○), R221aA (●), K222A (▲), E217A (□) and E217Q (■) on PC/PS vesicles (25 mM) in TBS/Ca^{2+}. Following 30−60 min activation at room temperature, EDTA was added to a final concentration of 20 mM and the rate of thrombin generation was measured from the cleavage rate of S2238 as described in Materials and Methods. B, the same as A except that the activation reactions by each fXa derivative (50 pM for all) were carried out in the presence of human fVa (25 nM) for 30−60 s. Solid lines in both panels are nonlinear regression fits of kinetic data (average of three measurements) to the Michaelis−Menten equation. The kinetic values are presented in Table 2.

observation. These results further support the existence of a linkage between fVa and Na^+ binding sites of fXa. It is not known whether Glu-217 and Lys-224 directly interact with the inhibitor or if their loss of affinity for the inhibitor is due to the disruption of the conformation of the 220-loop by the elimination of an ionic interaction between these two residues. Another possibility that cannot be ruled out is that the disruption of the ionic interaction between Glu-217 and Lys-224 may lead to a general closure of the active-site cleft of the mutants. In this context, a PC/PS-mediated conformational change in the active-site cleft of either mutant could account for the marked improvement in the affinity of both mutants for interaction with rTAP.

Activation of Prothrombin. The activities of fXa derivatives toward the natural substrate prothrombin were evaluated on PC/PS vesicles in both the absence and presence of fVa. The results in the absence of fVa suggested that K222 activates prothrombin with a catalytic efficiency similar to that of wild-type fXa, suggesting that Lys-222 makes no interaction with the substrate (Figure 4A). However, the activity of the other three mutants toward prothrombin was markedly impaired. The extent of impairment ranged from ~5-fold for R221aA, ~18-fold for E217Q and a dramatic >2300-fold for E217A (Table 2). Further studies in the presence of fVa indicated that the apparent dissociation constant $K_{d(app)}$ for the interaction of the fXa mutants with fVa has been minimally altered in the prothrombinase assay (Table 2). Interestingly, the concentration dependence of prothrombin activation by fXa derivatives in complex with saturating concentrations of fVa revealed that fVa restores most of the catalytic defects observed with mutant proteases (Figure 4B). Thus, in contrast to >2300-fold lower catalytic efficiency for E217A toward prothrombin, the activity of the mutant in the presence of fVa was only 3−4-fold lower than that of wild-type fXa (Table 2). The catalytic defects caused by either Arg-221a or Glu-217 mutagenesis were also completely restored (Figure 4B, Table 2).

The observation that fVa exhibited a normal affinity for interaction with the fXa mutants was surprising and not consistent with the suggestion that fVa and Na^+ binding sites are linked. Thus, the following two approaches were taken to determine whether the dramatically higher catalytic efficiency of fXa derivatives in the activity assays obscures a potential defect in the interaction of these mutants with fVa. In the first approach, prethrombin-2 (prothrombin derivative lacking Gla, kringle-1 and kringle-2 domains) was used as a substrate in the prothrombinase assay. Interestingly, in contrast to a $K_{d(app)}$ of 1.1 nM for wild-type fXa, the corresponding values were increased to 1.3 nM for both R221aA and K222A, 1.8 nM for E217A and 2.4 nM for E217Q, suggesting an ~1.7−2-fold impairment in the apparent affinity of the latter two mutants for interaction with the cofactor. These results indicate that the interaction of the Gla domain of prothrombin with fVa in the prothrombinase complex may influence the $K_{d(app)}$ measurements for fXa derivatives. It is known that prethrombin-2 also interacts with fVa (*10, 11*), thus we decided to evaluate the affinity of E217A and E217Q for binding to fVa by a competitive binding assay at equilibrium. It is known that fluorescence anisotropy of OG_{488}-EGR-fXa is associated with a saturable increase in the fluorescence of OG_{488} upon interaction with fVa with a K_D of ~1.6 nM (*15*). The ability of the fXa derivatives to compete with OG_{488}-EGR-fXa for binding to

Table 2: Kinetic Constants for the Activation of Prothrombin by fXa Derivatives in the Absence and Presence of fVa and the Apparent Dissociation Constants ($K_{d(app)}$) for Their Interaction with the Cofactor[a]

	$K_{m(app)}$ (nM)	k_{cat} (nM/min/nM)	$k_{cat}/K_{m(app)}$ (nM/min)	$K_{d(app)}$ (nM)
fXa				
prothrombin, PC/PS, Ca^{2+}	141.1 ± 17.2	0.2 ± 0.06	1.4×10^{-3}	
prothrombin, PC/PS, fVa, Ca^{2+}	71.9 ± 4.6	847.5 ± 15.2	11.8	0.55 ± 0.15
E217A				
prothrombin, PC/PS, Ca^{2+}	985.7 ± 178.0	$(0.58 \pm 0.04) \times 10^{-3}$	5.9×10^{-7}	
prothrombin, PC/PS, fVa, Ca^{2+}	49.1 ± 2.2	162.3 ± 1.9	3.3	0.65 ± 0.22
E217Q				
prothrombin, PC/PS, Ca^{2+}	207.0 ± 28.2	0.016 ± 0.004	7.7×10^{-5}	
prothrombin, PC/PS, fVa, Ca^{2+}	59.2 ± 4.2	686.3 ± 13.2	11.6	0.58 ± 0.24
R221aA				
prothrombin, PC/PS, Ca^{2+}	165.0 ± 47.7	0.046 ± 0.004	2.8×10^{-4}	
prothrombin, PC/PS, fVa, Ca^{2+}	55.2 ± 3.8	919.6 ± 16.8	16.6	0.64 ± 0.11
K222A				
prothrombin, PC/PS, Ca^{2+}	183.0 ± 21.2	0.17 ± 0.003	9.3×10^{-4}	
prothrombin, PC/PS, fVa, Ca^{2+}	52.2 ± 4.7	617.4 ± 14.7	11.8	0.34 ± 0.12

[a] The kinetic parameters $K_{m(app)}$ and k_{cat} were determined from the concentration dependence of prothrombin activation by fXa derivatives on PC/PS vesicles in TBS/Ca^{2+} in the absence and presence of a saturating concentration of fVa as described in Materials and Methods. The $K_{d(app)}$ values for the interactions with fVa were determined from the saturable cofactor concentration dependent thrombin generation by each fXa derivative at room temperature as described in Materials and Methods. All values are the average of at least 3 measurements ± standard deviations.

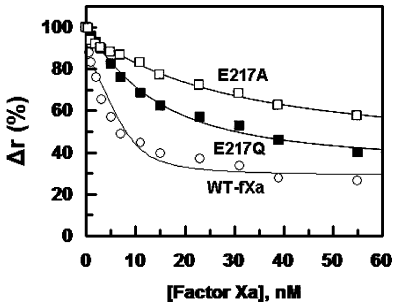

FIGURE 5: Fluorescence measurements of the competitive effect of the fXa derivatives on the interaction of OG_{488}-EGR-fXa with fVa. The competitive effect of increasing concentrations of fXa derivatives: wild-type (○), E217A (□) and E217Q (■) on the interaction of OG_{488}-EGR-fXa (10 nM) and fVa (25 nM) on PC/PS vesicles (10 μM) in TBS buffer containing 0.1% PEG 8000 and 5 mM Ca^{2+} was monitored from the decrease in the anisotropy (Δr) of the labeled fXa caused by its displacement from the cofactor. The fluorescent data from two independent experiments were analyzed according to a competitive binding equation yielding K_D values of 0.9 ± 0.8 nM for wild-type fXa, 23.0 ± 7.5 nM for E217A, and 8.3 ± 1.6 nM for E217Q.

fVa was evaluated from a decrease in the fluorescence of the labeled fXa-fVa complex as a function of increasing concentrations of wild-type fXa, E217A and E217Q. The unlabeled fXa displaced ~70% of the fVa-bound OG_{488}-EGR-fXa on PC/PS vesicles (Figure 5). The basis for an incomplete displacement of the labeled fXa has not been investigated, but it is consistent with previous results (*15*), possibly suggesting that the binding of the unlabeled fXa to fVa-bound PC/PS is influencing the anisotropy of free OG_{488}-EGR-fXa on phospholipid vesicles. Analysis of the binding data suggested that wild-type fXa competes with OG_{488}-EGR-fXa for interaction with fVa with a K_D of ~0.9 nM. Interestingly, both E217A and E217Q mutants were ineffective in displacing the labeled fXa, exhibiting markedly weaker K_D values of ~23 nM and ~8 nM, respectively (Figure 5). These results indicate that prothrombin masks the lower affinity of the mutants for interaction with fVa in the prothrombinase assay.

DISCUSSION

The catalytic activity of coagulation proteases with a conserved Tyr at position 225 (*20*) is modulated by a single Na^+ ion binding to the 220-loop located near the S1 specificity pocket and formed by four antiparallel beta-strands between Cys-182−Cys-191 and Val-213−Tyr-228 (*26, 27*). In fXa, two main chain carbonyl oxygen atoms from each loop (residues 185 and 185a from the former loop and residues 221a and 224 from the latter loop) along with two water molecules participate in the octahedral coordination of Na^+ (Figure 6A). By contrast, neither one of the residues of the first loop (186-loop) contributes to a direct interaction with Na^+ in thrombin, but rather two carbonyl oxygen atoms of Arg-221a and Lys-224 together with four water molecules are responsible for the coordination of the cation (Figure 6B). The structural data of thrombin has indicated that a salt-bridge between Glu-217 and Lys-224 stabilizes the 220-loop in order to orient the carbonyl oxygen atoms of Arg-221a and Lys-224 to appropriate positions so that they can participate in the coordination of Na^+ in this loop (*26*). The importance of this salt-bridge in Na^+ binding and in the catalytic activity of thrombin has been demonstrated by a near complete loss of the procoagulant activity of thrombin in mutants in which Glu-217 of the protease has been substituted with either an Ala or a Lys (*28, 29*). Interestingly, however, while the thrombin mutants have lost their procoagulant activity, they have retained a near normal anticoagulant activity as evidenced by their ability to activate protein C in the presence of thrombomodulin with only ~2-fold lower activity, suggesting that the disruption of this salt-bridge destabilizes the fast conformation of thrombin (*29*). In the X-ray crystal structure of the catalytic domain of fXa (*24*), Glu-217 and Lys-224 are located within hydrogen-bonding distance (3.45 Å and 3.49 Å for the two contacts) from one another (Figures 1 and 6). To determine whether a similar ionic interaction between these residues supports the binding of Na^+ to the 220-loop, we substituted them with Ala (also Gln in the case of Glu-217) and expressed the mutants in mammalian cells. We also replaced the conserved Arg-221a and Lys-222, which has not been conserved in thrombin, with Ala. All of these mutants with the exception

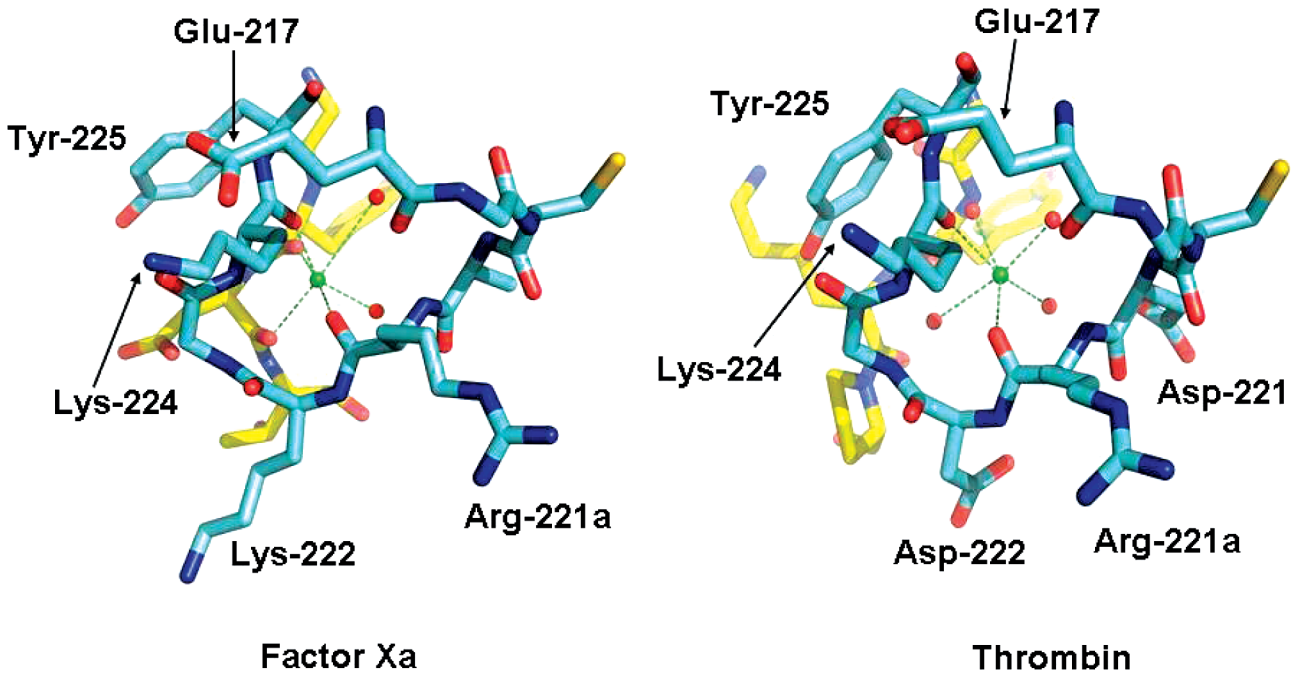

FIGURE 6: Crystal structures of the Na^+-binding loops of fXa and thrombin. Shown are the 217−225 (cyan) and 184−186 (yellow) segments of fXa (Protein Data Bank ID code 2BOK) (*39*) and thrombin (Protein Data Bank ID code 2THF) (*20*) with water molecules (red spheres) bound to Na^+ (green sphere).

of K224A were expressed to a sufficient yield for full characterization in the amidolytic and proteolytic activity assays. K224A was not expressed to a sufficient yield for full characterization, and thus only qualitative results could be obtained with this mutant. The basis for the deficient expression of K224A was not investigated; it is likely that the mutagenesis of this residue has the greatest destabilizing effect on the 220-loop of fXa.

The amidolytic activities of E217A and E217Q toward hydrolysis of S2765 were dramatically impaired, and the extent of loss of activities correlated well with the extent of loss of their affinity for Na^+ suggesting that, similar to thrombin, an ionic interaction between Glu-217 and Lys-224 may also be essential for the integrity of this loop to support its interaction with the monovalent cation. The R221aA mutant also exhibited ~3-fold lower activity toward the chromogenic substrate, which also correlated well with its extent of loss of affinity for Na^+, supporting the involvement of this residue in the coordination of Na^+, similar to that observed in thrombin. The observation that all mutants exhibited a similar extent of impairment in their affinity for the S1 subsite specific inhibitor PAB suggested that the defects in the amidolytic activity of the mutants may partially be caused by an alteration of the conformation of the primary specificity residue Asp-189, located near the 220-loop in the active-site groove of fXa. In support of this hypothesis, increasing the concentration of Na^+ partially restored the catalytic defect, however, the dramatic impairments in the affinities of these mutants for interaction with Na^+ (Table 1) and the insolubility of the chromogenic substrate at such high concentrations of Na^+ precluded detailed kinetic analysis with these mutants. These results are in agreement with the hypothesis that the Na^+-binding and the S1 specificity pocket of fXa are linked (*16, 17*). Furthermore, the observation that fVa eliminated the Na^+ requirement for the amidolytic activities of fXa derivatives in the prothrombinase complex supports the hypothesis that fVa and Na^+ binding sites are also energetically linked.

Similar to their loss of activity toward the chromogenic substrate, the same fXa mutants exhibited defects in their interaction with the fXa-specific inhibitors AT, TFPI and rTAP. While the extent of defects with both AT and TFPI correlated well with the extent of loss in the amidolytic activities for all mutants, nevertheless, R221aA exhibited a greater loss in its interaction with rTAP in the absence, but a normal affinity for the inhibitor in the presence of fVa on PC/PS vesicles. These results are consistent with the hypothesis that the loss of affinity for interaction with both AT and TFPI is primarily due to the alteration of the S1 specificity pocket of the fXa mutants; however, Arg-221a appears to make a specific interaction with rTAP to stabilize the inhibitor in the active-site groove of the protease. Nevertheless, based on the normal affinity of R221aA in the prothrombinase complex for interaction with rTAP, the possibility that a disordered conformation of the 220-loop is responsible for the binding defect with this mutant cannot be ruled out. In agreement with these mutagenesis data, the structural data do not predict any specific interaction for this loop with either AT (*35*) or TFPI (*36*), however, the structure of rTAP in complex with bovine fXa indicates that an acidic region on the C-terminal helix of the inhibitor can interact with the basic residues of the 220-loop, all of which have also been conserved in the structure of bovine fXa (*34*). Examination of the relative orientations of the side chains of the three basic residues of the 220-loop in the structure of the protease−inhibitor complex suggests that only Arg-221a and Lys-224 can interact with the inhibitor since the ammonium group of Lys-222 is pointing away from the inhibitor (*34*). The mutagenesis study in this manuscript validates these structural findings as evidenced by a normal affinity of rTAP for interaction with K222A and a dramatically lower affinity of other mutants for interaction with the

inhibitor. However, it is not known whether the dramatic loss of the affinity of the E217A and E217Q mutants for interaction with rTAP is due to a loss of specific side-chain interaction of Glu-217 with the inhibitor, or due to the loss of its ionic interaction with Lys-224 which results in the disruption of the structure of the 220-loop in the mutant protease. This uncertainty also applies to the underlying basis for the dramatic loss of the affinity of the Glu-217 mutants for interaction with TFPI. Nevertheless, the observation that fVa overcomes the inhibitor binding defects with some of the mutants suggests that fVa binding induces conformational changes in the Na^+-binding loop of the protease.

Another interesting observation of this study is that the E217A, E217Q and R221aA mutants exhibited a marked impairment in their activity toward the natural substrate prothrombin, however, the catalytic defect was essentially completely restored by fVa in the latter two mutants. The cofactor also decreased the >2300-fold catalytic defect of prothrombin activation by E217A to only ~3−4-fold. These results suggest that fVa induces a conformational change in the 220-loop of the mutants, which appears to stabilize the loop in the Na^+-bound active conformation, thereby rendering the activity of fXa derivatives independent of Na^+. The lack of Na^+ requirement for fXa in the presence of fVa suggests that Na^+ may not play a significant regulatory role in the procoagulant function of fXa in the prothrombinase complex. This is in contrast to the important role that Na^+ has in the regulation of the activity of thrombin in the clotting cascade, thus the Na^+-free form of the protease specifically functioning in the anticoagulant pathway (*18*). These results further support the hypothesis that the fVa-binding and Na^+-binding sites of fXa are allosterically linked (*15, 16*). Results of several mutagenesis studies have indicated that fVa interacts with basic residues of the 162-helix (*8, 9, 37*). Several residues of this helix appear to have intramolecular electrostatic (Ser-167) and hydrophobic (Val-163) interactions with the residues of the Na^+-binding loop that are important for the integrity of the S1 specificity pocket (*37*). Thus, based on the observation that fVa partially restores the catalytic defects of the 162-helix mutants of fXa, it has been hypothesized that fVa modulates the S1 specificity pocket of fXa (*37*). Nevertheless, the cofactor minimally influenced the activities of the mutants of this study toward the chromogenic substrate, yet it restored the dramatic catalytic defects in the activation of prothrombin, suggesting that the optimization of the S1 subsite may not significantly contribute to the cofactor function of fVa in the prothrombinase complex. Thus, the exact mechanism by which fVa overcomes the proteolytic defects of mutants through the modulation of the Na^+-binding loop is not understood. What is clear is that the side chains of the residues under study make no apparent contribution to the specificity of prothrombin recognition by fXa in the prothrombinase complex. It is possible that the Na^+-binding loop of fXa is yet energetically linked to another uncharacterized region(s) in the extended binding pocket of fXa which can specifically interact with prothrombin and that fVa restores the catalytic activity of mutants through the modulation of this site(s). Some support for this hypothesis may come from the spectral studies with both fXa and factor IXa containing a fluorescein dye covalently attached to His-57 of their active-sites via the tripeptide inhibitor, D-Phe-Pro-Arg-ck (*13, 38*). The structural data have indicated that such an active-site labeling would position the fluorescein dye (attached to the P3 Phe) ~15 Å away from Ser-195 in the active-site groove of these proteases (*38*). It has been demonstrated that the binding of either fVa to fXa or factor VIIIa to factor IXa alters the spectral properties of the fluorescein dye in the active-site grooves of these proteases, leading to the conclusion that the cofactor binding sites of both proteases are conformationally coupled to a region of the active-site pocket that is located some 15 Å from Ser-195 (*38*). Further studies will be required to test the validity of these hypotheses.

ACKNOWLEDGMENT

We would like to thank Audrey Rezaie for proofreading of the manuscript.

REFERENCES

1. Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu. Rev. Biochem. 57*, 915–956.
2. Rosing, J., Tans, G., Govers-Riemslag, J. W. P., Zwaal, R. F. A., and Hemker, H. C. (1980) The role of phospholipids and factor Va in the prothrombinase complex. *J. Biol. Chem. 255*, 274–283.
3. Furie, B., and Furie, B. C. (1988) The molecular basis of blood coagulation. *Cell 53*, 505–518.
4. Davie, E. W., Fujikawa, K., and Kisiel, W. (1991) The coagulation cascade: Initiation, maintenance and regulation. *Biochemistry 30*, 10363–10370.
5. Jackson, C. M., and Nemerson, Y. (1980) Blood coagulation. *Annu. Rev. Biochem. 49*, 765–811.
6. Nesheim, M. E., Kettner, C., Shaw, E., and Mann, K. G. (1981) Cofactor dependence of factor Xa incorporation into the prothrombinase complex. *J. Biol. Chem. 256*, 6537–6540.
7. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood 76*, 1–16.
8. Rezaie, A. R. (2000) Identification of basic residues in the heparin-binding exosite of factor Xa critical for heparin and factor Va binding. *J. Biol. Chem. 275*, 3320–3327.
9. Rudolph, A. E., Porche-Sorbet, R., and Miletich, J. P. (2001) Definition of a factor Va binding site in factor Xa. *J. Biol. Chem. 276*, 5123–5128.
10. Anderson, P. J., Nesset, A., Dharmawardana, K. R., and Bock, P. E. (2000) Role of proexosite I in factor Va-dependent substrate interactions of prothrombin activation. *J. Biol. Chem. 275*, 16435–16442.
11. Chen, L., Yang, L., and Rezaie, A. R. (2003) Proexosite-1 on prothrombin is a factor Va-dependent recognition site for the prothrombinase complex. *J. Biol. Chem. 278*, 27564–27569.
12. Lim, T. K., Bloomfield, V. A., and Nelsestuen, G. L. (1977) Structure of the prothrombin- and blood clotting factor X-membrane complexes. *Biochemistry 16*, 4177–4181.
13. Husten, E. J., Esmon, C. T., and Johnson, A. E. (1987) The active site of blood coagulation factor Xa: its distance from the phospholipid surface and its conformational sensitivity to components of the prothrombinase complex. *J. Biol. Chem. 262*, 12953–12962.
14. Krishnaswamy, S. (2004) Exosite-driven substrate specificity and function in coagulation. *J. Thromb. Haemostasis 3*, 54–67.
15. Rezaie, A. R., and Kittur, F. S. (2004) The critical role of the 185−189-loop in the factor Xa interaction with Na^+ and factor Va in the prothrombinase complex. *J. Biol. Chem. 279*, 48262–48269.
16. Camire, R. M. (2002) Prothrombinase assembly and S1 site occupancy restore the catalytic activity of FXa impaired by mutation at the sodium-binding site. *J. Biol. Chem. 277*, 37863–37870.
17. Underwood, M. C., Zhong, D., Mathur, A., Heyduk, T., and Bajaj, S. P. (2000) Thermodynamic linkage between the S1 site, the Na^+ site, and the Ca^{2+} site in the protease domain of human coagulation factor Xa. *J. Biol. Chem. 275*, 36876–36884.
18. Dang, Q. D., and Di Cera, E. (1996) Residue 225 determines the Na^+-induced allosteric regulation of catalytic activity in serine proteases. *Proc. Natl. Acad. Sci. U.S.A. 93*, 10653–10656.

19. Rezaie, A. R., and He, X. (2000) Sodium binding site of factor Xa: Role of sodium in the prothrombinase complex. *Biochemistry 39*, 1817–1825.
20. Guinto, E. R., Caccia, S., Rose, T., Futterer, K., Waksman, G., and Di Cera, E. (1999) Unexpected crucial role of residue 225 in serine proteases. *Proc. Natl. Acad. Sci. U.S.A. 96*, 1852–1857.
21. Prasad, S., Cantwell, A. M., Bush, L. A., Shih, P., Xu, H., and Di Cera, E. (2004) Residue Asp-189 controls both substrate binding and the monovalent cation specificity of thrombin. *J. Biol. Chem. 279*, 10103–10108.
22. He, X., and Rezaie, A. R. (1999) Identification and characterization of the sodium-binding site of activated protein C. *J. Biol. Chem. 274*, 4970–4976.
23. Schechter, I., and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun. 27*, 157–162.
24. Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R., Blankenship, D. T., Cardin, A. D., and Kisiel, W. (1993) Structure of human des (1-45) factor Xa at 2•2 Å resolution. *J. Mol. Biol. 232*, 947–966.
25. Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) The refined 1.9 Å crystal structure of human α-thrombin: interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment. *EMBO J. 8*, 3467–3475.
26. Zhang, E., and Tulinsky, A. (1997) The molecular environment of the Na^+ binding site of thrombin. *Biophys. Chem. 63*, 185–200.
27. Di Cera, E., Guinto, E. R., Vindigni, A., Dang, Q. D., Ayala, Y. M., Wuyi, M., and Tulinsky, A. (1995) The Na^+ binding site of thrombin. *J. Biol. Chem. 270*, 22089–22092.
28. Gibbs, C. S., Coutré, S. E., Tsiang, M., Li, W. X., Jain, A. K., Dunn, K. E., Law, V. S., Mao, C. T., Matsumura, S. Y., Mejza, S. J., Paborsky, L. R., and Leung, L. L. K. (1995) Conversion of thrombin into an anticoagulant by protein engineering. *Nature 378*, 413–416.
29. Carter, W. J., Myles, T., Gibbs, C. S., Leung, L. L., and Huntington, J. A. (2004) Crystal Structure of anticoagulant thrombin variant E217K provides insights into thrombin allostery. *Biol. Chem. 279*, 26387–26394.
30. Manithody, C., Yang, L., and Rezaie, A. R. (2002) Role of basic residues of the autolysis loop in the catalytic function of factor Xa. *Biochemistry 41*, 6780–6788.
31. Leytus, S. P., Foster, D. C., Kurachi, K., and Davie, E. W. (1986) Gene for human factor X: A blood coagulation factor whose gene organization is essentially identical with that of factor IX and protein C. *Biochemistry 25*, 5098–5102.
32. Chen, L., Manithody, C., Yang, L., and Rezaie, A. R. (2004) Zymogenic and enzymatic properties of the 70−80 loop mutants of factor X/Xa. *Protein Sci. 13*, 431–442.
33. Smirnov, M. D., and Esmon, C. T. (1994) Phosphatidylethanolamine incorporation into vesicles selectively enhances factor Va inactivation by activated protein C. *J. Biol. Chem. 269*, 816–819.
34. Wei, A., Alexander, R. S., Duke, J., Ross, H., Rosenfeld, S. A., and Chang, C.-H. (1998) Unexpected binding mode of tick anticoagulant peptide complexed to bovine factor Xa. *J. Mol. Biol. 283*, 147–154.
35. Johnson, D. J. D., Li, W., Adams, T. E., and Huntington, J. A. (2006) Antithrombin-S195A factor Xa-heparin structure reveals the allosteric mechanism of antithrombin activation. *EMBO J. 25*, 2029–2037.
36. Burgering, M. J. M., Orbons, L. P. M., van der Doelen, A., Mulders, J., Theunissen, H. J. M., Grootenhuis, P. D. J., Bode, W., Huber, R., and Stubbs, M. T. (1997) The second Kunitz domain of human tissue factor pathway inhibitor: Cloning, structure determination and interaction with factor Xa. *J. Mol. Biol. 269*, 395–407.
37. Levigne, S., Thiec, F., Cherel, G., Irving, J. A., Fribourg, C., and Christophe, O. D. (2007) Role of the α helix 163−170 in factor Xa catalytic activity. *J. Biol. Chem. 282*, 31569–31579.
38. Mutucumarana, V. P., Duffy, E. J., Lollar, P., and Johnson, A. E. (1992) The active site of factor IXa is located far above the membrane surface and its conformation is altered upon association with factor VIIIa: A fluorescence study. *J. Biol. Chem. 267*, 17012–17021.
39. Scharer, K., Morgenthaler, M., Paulini, R., Obst-Sander, U., Banner, D. W., Schlatter, D., Benz, J., Stihle, M., and Diederich, F. (2005) Quantification of cation-pi interaction in protein-ligand complexes: crystal-structure analysis of factor Xa bound to a quaternary ammonium ion ligand. *Angew. Chem., Int. Ed. 44*, 4400–4404.

BI800319R