Functional Mapping of Charged Residues of the 82−116 Sequence in Factor Xa: Evidence That Lysine 96 Is a Factor Va Independent Recognition Site for Prothrombin in the Prothrombinase Complex†

Chandrashekhara Manithody and Alireza R. Rezaie*

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

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ABSTRACT: It has been hypothesized that two antiparallel structures comprised of residues 82−91 and 102−116 in factor Xa (fXa) may harbor a factor Va- (fVa-) dependent prothrombin recognition site in the prothrombinase complex. There are 11 charged residues in the 82−116 loop of human fXa (Glu-84, Glu-86, Lys-90, Arg-93, Lys-96, Glu-97, Asp-100, Asp-102, Arg-107, Lys-109, and Arg-115). With the exception of Glu-84, which did not express, and Asp-102, which is a catalytic residue, we expressed the Ala substitution mutants of all other residues and evaluated their proteolytic and amidolytic activities in both the absence and presence of fVa. K96A and K109A activated prothrombin with 5−10-fold impaired catalytic efficiency in the absence of fVa. All mutants, however, exhibited normal activity toward the substrate in the presence of fVa. K109A also exhibited impaired amidolytic activity and affinity for Na^+; however, both fVa and higher Na^+ restored the catalytic defect caused by the mutation. Analysis of the X-ray crystal structure of fXa indicated that Glu-84 may interact by a salt bridge with Lys-109, explaining the lack of expression of E84A and the lower activity of K109A in the absence of fVa. These results suggest that none of the residues under study is a fVa-dependent recognition site for prothrombin in the prothrombinase complex; however, Lys-96 is a recognition site for the substrate independent of the cofactor. Moreover, the 82−116 loop is energetically linked to fVa and Na^+ binding sites of the protease.

Factor X (fX)[1] is a vitamin K-dependent serine protease zymogen in plasma that upon activation to factor Xa (fXa) forms a high-affinity complex with factor Va (fVa) on negatively charged membrane surfaces in the presence of calcium (prothrombinase) to generate thrombin from prothrombin (*1*−*5*). The catalytic efficiency of fXa in the prothrombinase complex is 5 orders of magnitude higher than that of fXa alone (*1*, *2*). The dramatic improvement in the catalytic efficiency of fXa is believed to arise from an approximately 2 orders of magnitude decrease in the K_m of substrate recognition due to the Gla-dependent assembly of proteins on the negatively charged membrane surfaces (*1*, *6*). The other 3 orders of magnitude enhancement in the k_{cat} is attributed to factor Va (fVa) interaction with fXa in the prothrombinase complex (*1*, *2*, *6*). The mechanism by which fVa improves the catalytic activity of fXa in the prothrombinase complex is not very well understood. Two different nonexclusive mechanisms have been presented. First, it has been proposed that fVa may provide a direct binding site for the substrate prothrombin in the prothrombinase complex (*7*, *8*). In support of this hypothesis, kinetics and mutagenesis data have indicated that the basic residues of proexosite 1 on prothrombin interact with an acidic hirudin-like sequence on fVa in the prothrombinase complex (*9*, *10*). Moreover, a role for the C-terminal residues of prothrombin in interaction with fVa in the prothrombinase complex has been demonstrated (*11*, *12*). Second, a hypothesis postulates that the binding of fVa to fXa allosteriocally exposes extended surfaces on fXa that are specific recognition sites for interaction with the substrate prothrombin in the prothrombinase complex (*13*−*15*). The identity of this cryptic exosite on fXa, which fVa makes available for interaction with prothrombin, is not known. Nevertheless, recent inhibition studies with a monoclonal antibody specific for bovine fXa have raised the possibility that the putative allosteric site on fXa may reside between residues 82−116 in the protease domain of the molecule (*14*).

The 82−116 loop of human fXa contains 11 charged residues including Glu-84, Glu-86, Lys-90, Arg-93, Lys-96, Glu-97, Asp-100, Asp-102, Arg-107, Lys-109, and Arg-115 which are mostly exposed to solvents. All of these residues are also conserved in bovine fXa. Noting that electrostatic interactions make significant contributions to the macromolecular complex assembly in coagulation reactions, we decided to neutralize the charges of these residues by substituting them with Ala and then assess whether any one

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* Corresponding author. Phone: (314) 977-9240. Fax: (314) 977-9205. E-mail: rezaiear@slu.edu.

[1] Abbreviations: fX, factor X; fXa, activated factor X; fVa, activated factor V; AT, antithrombin; E86A, K90A, R93A, K96A, E97A, D100A, R107A, K109A, and R115A, fXa derivatives which contain an Ala substitution at the indicated positions in the chymotrypsinogen numbering system (*18*); RVV-X, Russell's viper venom; PC/PS, phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine; PEG, poly(ethylene glycol); BSA, bovine serum albumin.

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of these residues interacts with prothrombin specifically in the presence of fVa. The catalytic Asp-102 was not mutated since this residue is essential for the catalytic function of fXa and all other serine proteases. The remaining mutants, with the exception of Glu-84, which did not express, were expressed in mammalian cells and purified to homogeneity, and their catalytic properties were characterized in prothrombin activation assays in both the absence and presence of fVa. It was discovered that two Lys-96 and Lys-109 mutants activated prothrombin in the absence of fVa with 5−10-fold impaired catalytic efficiency in comparison to wild-type fXa. On the other hand, the catalytic activities of all mutants including both Lys-96 and Lys-109 mutants toward prothrombin in the presence of fVa in the prothrombinase complex were comparable to the activity of wild-type fXa. Further studies revealed that, unlike all other mutants which exhibited normal amidolytic activity and reactivity with antithrombin (AT), the Lys-109 mutant also had defective catalytic activity in both of these reactions. The basis for the global catalytic defect of K109A is likely to be due to the elimination of a potential salt bridge between Lys-109 and Glu-84 by their mutagenesis to Ala, as evidenced by examination of the X-ray crystal structure of fXa (*16*). It was also discovered that the affinity of the Lys-109 mutant for interaction with both sodium and fVa has been impaired ∼4−6-fold. Interestingly, either 0.6 M sodium or a saturating concentration of fVa overcame the impairment in the amidolytic activity of the Lys-109 mutant. These results suggest that none of the charged residues of the 82−116 loop in human fXa is a fVa-dependent recognition site for prothrombin; however, Lys-96 is critical for the protease interaction with prothrombin independent of the cofactor. Furthermore, fVa interaction with the Lys-109 mutant is associated with conformational changes in the Na^+ binding and/or the catalytic cleft of K109A, leading to restoration of both amidolytic and proteolytic activities of the mutant protease. Thus, these sites are allosterically linked.

MATERIALS AND METHODS

Mutagenesis and Expression of Recombinant Proteins. The expression and purification of wild-type factor X (fX) in human embryonic kidney 293 cells were described previously (*17*). The fX mutants in the chymotrypsinogen numbering (*18*), Glu-86 → Ala (E86A), Lys-90 → Ala (K90A), Arg-93 → Ala (R93A), Lys-96 → Ala (K96A), Glu-97 → Ala (E97A), Asp-100 → Ala (D100A), Arg-107 → Ala (R107A), Lys-109 → Ala (K109A), and Arg-115 → Ala (R115A), corresponding to residues Glu-306, Lys-310, Arg-313, Lys-316, Glu-317, Asp-320, Arg-327, Lys-329, and Arg-335 in the fX cDNA numbering (*19*), were generated in the same expression/purification vector system by standard PCR mutagenesis methods as described (*17*). After confirmation of the accuracy of the mutagenesis by DNA sequencing, the constructs were introduced into the same cell line, 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 (*17*). In this vector system, the HPC4 epitope replaces the first 12 residues of the fX activation peptide on the heavy chain of the molecule, and since the activation peptide of the zymogen is cleaved off during activation by Russell's viper venom, the N-terminal HPC4 epitope is removed along with the activation peptide from all fXa derivatives. Thus, with the exception of specific mutations, all fXa derivatives have identical amino acid sequences as the plasma-derived fXa (*17*). The fully γ-carboxylated proteins were eluted from the ion-exchange column at ∼0.4 M NaCl as described previously (*17*). The expression and purification of recombinant human prethrombin-2 (prothrombin lacking the γ-carboxyglutamic acid and kringle-1 and kringle-2 domains) in the pNUT-PL2 expression/purification vector system in baby hamster kidney cells have been described previously (*20*).

Human plasma proteins including fVa, fXa, prothrombin, and antithrombin (AT) and the factor X-activating enzyme from Russell's viper venom (RVV-X) were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (*21*). Heparin−Sepharose was purchased from Amersham Pharmacia (Piscataway, NJ). The chromogenic substrate, spectrozyme FXa (SpFXa, MeO-CO-D-cyclohexylglycyl-Gly-Arg-*p*-nitroanilide), was purchased from American Diagnostica (Greenwich, CT), and S2238 (H-D-Phe-pipecolyl-Arg-*p*-nitroanilide) was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH).

Activation of Factor X Derivatives by RVV-X. Factor X derivatives were converted to active forms by RVV-X as described (*22*). Briefly, each fX derivative (∼10−50 μg) was incubated with RVV-X (10 nM) at 37 °C for 0.5−1 h in 0.1 M NaCl and 0.02 M Tris-HCl, pH 7.5, containing 2.5 mM Ca^{2+} (TBS/Ca^{2+}). Time course analysis of the activation reactions indicated that all fX zymogens were converted to their active forms under these experimental conditions. Factor Xa derivatives were separated from the snake venom by heparin−Sepharose column chromatography as described (*22*). Active site concentrations of fXa derivatives were determined by an amidolytic activity assay and titrations with human AT assuming a 1:1 stoichiometry as described (*22*).

Cleavage of Chromogenic Substrates. The steady-state kinetics of hydrolysis of SpFXa (7.8−1000 μM) by fXa derivatives (0.5−1 nM) was determined in TBS/Ca^{2+} containing 0.1 mg/mL bovine serum albumin (BSA) and 0.1% poly(ethylene glycol)- (PEG-) 8000. 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 (*17*). The K_m and k_{cat} values for the substrate hydrolysis were calculated from the Michaelis−Menten equation. The steady-state hydrolysis of the chromogenic substrate in the presence of fVa was also examined with certain mutants. In this case, the experimental conditions were the same except that cleavage reactions were carried out with fXa (1 nM) in complex with a saturating concentration of fVa (25 nM) on PC/PS vesicles (10 μM).

Apparent Dissociation Constant ($K_{d(app)}$) for Na^+. The $K_{d(app)}$ values for Na^+ interaction with each protease were determined from the effect of varying concentrations of Na^+ on the activity of the protease toward SpFXa (100 μM) at room temperature in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000. The background amidolytic activities at zero Na^+ were deducted from the activities in the presence of increasing concentrations of Na^+ (0−800 mM), and data were fit to the Langmuir binding isotherm to estimate $K_{d(app)}$ values as described (*23*).

Inactivation by Antithrombin. The rate of inactivation of fXa derivatives by AT was measured under pseudo-first-order rate conditions by a discontinuous assay as described (*17*). Briefly, each protease (1−2 nM) was incubated with AT (0.5−1 μM) in TBS/Ca^{2+} for 10−30 min in 50 μL volumes in 96-well polystyrene assay plates at room temperature. All reactions were stopped by addition of 50 μL of SpFXa (final concentration of 0.2 mM), and 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 (*17*).

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 (*23*). In the absence of the cofactor, the concentration dependence of prothrombin activation was studied by incubating each fXa derivative (2.5−5 nM) with increasing concentrations of the zymogen (7.8−1000 nM) on PC/PS vesicles (10 μM) in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000 at room temperature. Following 15−30 min activation in 96-well assay plates, EDTA was added to a final concentration of 20 mM, and the rate of thrombin generation was determined by an amidolytic activity assay using S2238 (100 μM) as described (*23*). 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 the cofactor, first the apparent affinity of fXa derivatives for interaction with fVa was evaluated in the prothrombinase assay as described (*23*). Briefly, fXa (100 pM) was mixed with varying concentrations of human fVa (0−20 nM) on PC/PS vesicles (10 μM) in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000 at room temperature. The activation reaction was initiated with the addition of 1 μM human prothrombin (final concentration) for 1 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. Next, the concentration dependence of prothrombin activation in the presence of fVa on PC/PS vesicles was studied by a similar prothrombinase assay. In this case, fXa (50−100 pM) in complex with a saturating concentration of fVa (25 nM in all reactions) on PC/PS vesicles (10 μM) was incubated with varying concentrations of human prothrombin (7.8−1000 nM) in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000. Following 30 s of incubation at room temperature, EDTA was added to a final concentration of 20 mM, and the concentration of thrombin generated was determined by an amidolytic activity assay as described above. The initial rate of prethrombin-2 activation by all fXa derivatives was also measured by a similar assay with the exception that the activation of different concentrations of prethrombin-2 (1−62 μM) by fXa (100 pM) in complex with fVa (25 nM) on PC/PS vesicles (10 μM) was monitored at room temperature for 3−4 min. The activation reactions were stopped by the addition of EDTA (20 mM), and the rate of thrombin generation was calculated as described above. It was ensured that less than 15% substrate was activated in all reactions.

Table 1: Kinetic Constants for Cleavage of Spectrozyme FXa by fXa Derivatives[a]

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	k_2(AT) ($\times 10^3 M^{-1} s^{-1}$)
WT	102.4 ± 6.2	203.2 ± 3.7	2.0 ± 0.2	3.1 ± 0.1
E86A	98.4 ± 3.2	205.7 ± 2.0	2.1 ± 0.1	3.1 ± 0.1
K90A	88.4 ± 4.1	207.0 ± 2.8	2.3 ± 0.1	3.7 ± 0.2
R93A	91.6 ± 2.7	204.6 ± 1.8	2.2 ± 0.1	3.1 ± 0.1
K96A	63.4 ± 3.9	141.9 ± 2.5	2.2 ± 0.1	5.0 ± 0.3
E97A	99.6 ± 9.1	173.7 ± 4.7	1.7 ± 0.2	2.4 ± 0.2
D100A	87.9 ± 4.5	184.3 ± 2.8	2.1 ± 0.1	3.2 ± 0.3
R107A	84.8 ± 3.8	179.9 ± 2.3	2.1 ± 0.1	3.2 ± 0.2
K109A	681.2 ± 39.1	134.1 ± 3.9	0.2 ± 0.02	0.4 ± 0.05
R115A	97.9 ± 4.9	155.9 ± 4.9	1.6 ± 0.1	3.0 ± 0.3

[a] The kinetic constants were determined from the steady-state kinetics of hydrolysis of SpFXa (7.8−1000 μM) by each fXa derivative (0.5−1 nM) in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000. The second-order rate constants (k_2) of the antithrombin (AT) inhibition were calculated from the remaining activity of enzymes (1−2 nM) following their incubation with AT (0.5−1 μM) for 10−30 min at room temperature as described under Materials and Methods. Kinetic values are the average of two to three measurements ± standard errors.

RESULTS

Amidolytic Activity and Reactivity with Antithrombin. The kinetic parameters for the hydrolysis of the chromogenic substrate SpFXa by the fXa derivatives are presented in Table 1. With the exception of K109A, which exhibited approximately an order of magnitude impairment in its specificity constant (k_{cat}/K_m) toward SpFXa, all other mutants cleaved the chromogenic substrate with a normal catalytic efficiency. The primary defect in substrate hydrolysis by K109A was in the K_m parameter (~7-fold elevation), suggesting that the chromogenic substrate cannot effectively bind to the active site cleft of the mutant protease. Examination of the X-ray crystal structure of the protease domain of fXa (*16*) revealed that the side chain of Lys-109 is oriented toward Glu-84 and is situated three dimensionally at a close distance (2.886 Å) to interact by a salt bridge with this residue, and thus mutation of either residue may result in misfolding and/or alteration of the conformation of the active site pocket of the mutant molecules. Indeed, this may explain our failure to express the E84A mutant in mammalian cells.

The 82−116 loop of fXa extends out of the Ca^{2+} binding 70−80 loop and becomes organized into two antiparallel structures comprised of residues 82−91 and 102−116 (*14*, *16*). It has been previously demonstrated that the 70−80 loop of fXa is allosterically linked to the Na^+ binding site of the protease (*24*). Moreover, it is known that the S1 pocket and the Na^+ binding site of fXa are energetically linked (*25*, *26*). To determine whether the conformation of the Na^+ binding site of K109A has also been affected, the chromogenic substrate assay was used to evaluate the affinity of the monovalent cation for interaction with K109A and other fXa derivatives. Consistent with previous results (*23*), wild type and all mutants, with the exception of K109A, exhibited similar $K_{d(app)}$ values of 80−140 mM for interaction with Na^+ (data not shown). On the other hand, relative to interaction with wild-type fXa ($K_{d(app)}$ = 113 ± 9 mM), the K109A mutant interacted with Na^+ with a much weaker affinity of 606 ± 68 mM (Figure 1). All mutants also reacted normally with the target protease inhibitor AT; however, similar to the amidolytic activity, the reactivity of the K109A mutant with the inhibitor was also impaired by an order of

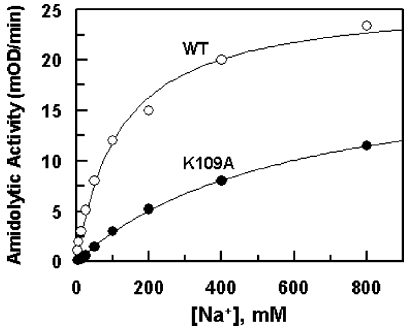

FIGURE 1: Na^+ dependence of the amidolytic activity of wild-type fXa and the K109A mutant of fXa. The amidolytic activity of wild-type (○) (1 nM) and mutant fXa (●) (1.4 nM) was monitored in the presence of increasing concentrations of Na^+ at room temperature using 100−500 μM SpFXa as described under Materials and Methods. The solid lines are nonlinear regression fits of kinetic data to the Langmuir isotherm equation.

magnitude (Table 1). Taken together, these results suggest that, with the exception of K109A, the mutagenesis did not adversely affect the conformation of either the active site cleft or the functionally critical Na^+ binding site of the fXa mutants.

Prothrombin Activation. The concentration dependence of prothrombin activation by the fXa derivatives on PC/PS vesicles in both the absence and presence of fVa is presented in panels A and B of Figure 2, respectively. In the absence of fVa, with the exception of two K96A and K109A mutants, all other mutants activated the substrate with a near normal catalytic efficiency. Analysis of data suggested that both the $K_{m(app)}$ and k_{cat} parameters with the K96A and K109A mutants have been impaired ~5−10-fold (Table 2). Similar to other reactions, the impairment in the proteolytic activity of the K109A mutant is most likely nonspecific and may be attributed to the mutagenesis-induced general conformational alterations in the structure of the mutant molecule. This is, however, not true for the K96A mutant since none of the other activities of this mutant were altered. Thus the lower proteolytic activity of this mutant is an indication that Lys-96 is a specific recognition site on fXa for interaction with prothrombin.

Before studying the proteolytic properties of fXa derivatives in the presence of fVa, we evaluated first the $K_{d(app)}$ for the interaction of mutants with the cofactor in the prothrombinase assay. The results presented in Table 2 indicate that most of the fXa derivatives interact with fVa with a $K_{d(app)}$ value of 1−2 nM in the prothrombinase complex. Noting that similar $K_{d(app)}$ values of 1−2 nM for the wild-type fXa interaction with fVa have been reported by this type of assay (*23*, *27*), the significance of slightly weaker $K_{d(app)}$ values for these mutants with fVa is not clear. However, the values with two mutants, K109A ($K_{d(app)}$ = 3.5 nM) and K93A ($K_{d(app)}$ = 2.7 nM), were impaired the most. In the case of the K109A mutant, similar to other activities, a global conformational change as the result of the disruption of a potential salt bridge between this residue and Glu-84 may account for the weaker affinity of the mutant for interaction with fVa. However, in the case of the R93A mutant, the result may indicate a specific interaction between Arg-93 and fVa in the prothrombinase complex. This is consistent with our previous report in which we showed that Arg-93 is critical for fXa interaction with both heparin and fVa in the prothrombinase complex (*22*) (further discussed below).

Next, the ability of fXa derivatives to activate prothrombin was evaluated in the presence of a saturating concentration of fVa (25 nM). The results presented in Figure 2B and Table 2 suggest that, with the exception of an ~2-fold impairment in the $K_{m(app)}$ of prothrombin activation by K109A, all other mutants activate the substrate with $K_{m(app)}$ and k_{cat} values similar to those observed for the wild-type fXa. These results suggest that the cofactor function of fVa can overcome the catalytic defects caused by the K96A and K109A mutations. Thus, in contrast to 5−10-fold impairment in k_{cat}/K_m of prothrombin activation by these mutants on the PC/PS vesicles, the k_{cat}/K_m value for K96A was comparable to that of wild-type fXa and was only lower by ~2-fold with the K109 mutant due to slightly elevated $K_{m(app)}$. Noting the dramatic effect of PC/PS vesicles on lowering the $K_{m(app)}$ of prothrombin activation by fXa, we decided to further assess the role of mutant residues using prethrombin-2, which is a good substrate for prothrombinase (*13*, *20*), yet it is not capable of interacting with the PC/PS vesicles. The results presented in Figure 3 suggest that, similar to prothrombin activation in the absence of fVa, the activity of the K96A mutant toward prethrombin-2 has been markedly impaired. Thus, relative to wild-type fXa, the catalytic efficiency (k_{cat}/K_m) of the K96A mutant toward prethrombin-2 was impaired ~10-fold (Table 2). There was also an ~3-fold impairment in the activity of K109A, which is consistent with the general lower activity of this mutant in all other reactions. The activities of all other mutants toward prethrombin-2 were either comparable or affected less than 2-fold. These results suggest that only Lys-96 is a recognition site on fXa for interaction with prothrombin. Nevertheless, the similar lower activity of this mutant toward the substrate in both the absence and presence of fVa suggests that its interaction with the substrate is independent of the cofactor.

Noting that fVa was capable of restoring the catalytic defect of the K109A mutant toward prothrombin in the prothrombinase complex, we decided to determine whether the cofactor can similarly influence the amidolytic activity of the mutant. The concentration dependence of the hydrolysis of SpFXa on PC/PS vesicles in both the absence and presence of a saturating concentration of fVa revealed that the cofactor elevates the $K_{m(app)}$ of the chromogenic substrate from ~100 to ~200 μM for wild-type fXa (Figure 4A). A similar fVa-mediated elevation in K_m for hydrolysis of the chromogenic substrates by fXa has been reported in the literature (*28*). Interestingly, unlike wild-type, fVa improved the $K_{m(app)}$ of SpFXa hydrolysis by the K109A mutant more than 3-fold (Figure 4B). Thus, in contrast to $K_{m(app)}$ of ~680 μM in the absence of fVa, this value was decreased to ~210 μM in the presence of the cofactor. To examine whether, similar to fVa, higher concentrations of Na^+ can also restore the amidolytic activity of the K109A mutant, the concentration dependence of SpFXa hydrolysis was also studied in 0.6 NaCl. Similarly, a 3-fold improvement in $K_{m(app)}$ of the chromogenic substrate hydrolysis by K109A was observed at the high salt concentration (Figure 4C). These results confirm the hypothesis that the neutralization of the basic charge of Lys-109 alters the conformation of both the catalytic cleft and the Na^+ binding loop (*29*) of the mutant protease, and thus these sites are energetically linked.

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

	$K_{m(app)}$ (nM)	k_{cat} (nM min^{-1} nM^{-1})	k_{cat}/K_m (nM^{-1} min^{-1})	$K_{d(app)}$ (nM)
fXa				
prothrombin, PC/PS, Ca^{2+}	115.6 ± 30.9	0.39 ± 0.03	0.003	
prothrombin, PC/PS, fVa, Ca^{2+}	57.6 ± 3.1	1506.8 ± 21.5	26.2	0.95 ± 0.1
prethrombin-2, PC/PS, fVa, Ca^{2+}	(10.6 ± 0.7) × 10^3	757.4 ± 16.7	0.071	
E86A				
prothrombin, PC/PS, Ca^{2+}	167.5 ± 30.7	0.43 ± 0.03	0.003	
prothrombin, PC/PS, fVa, Ca^{2+}	53.9 ± 4.8	1008.1 ± 42.6	18.7	0.7 ± 0.1
prethrombin-2, PC/PS, fVa, Ca^{2+}	(11.7 ± 1.2) × 10^3	657.2 ± 24.5	0.056	
K90A				
prothrombin, PC/PS, Ca^{2+}	139.9 ± 23.2	0.28 ± 0.02	0.002	
prothrombin, PC/PS, fVa, Ca^{2+}	59.8 ± 2.7	1285.3 ± 15.5	21.5	1.7 ± 0.1
prethrombin-2, PC/PS, fVa, Ca^{2+}	(16.2 ± 2.4) × 10^3	685.4 ± 40.8	0.042	
R93A				
prothrombin, PC/PS, Ca^{2+}	100.1 ± 21.7	0.32 ± 0.02	0.003	
prothrombin, PC/PS, fVa, Ca^{2+}	67.8 ± 1.9	1670.4 ± 67.8	24.6	2.7 ± 0.1
prethrombin-2, PC/PS, fVa, Ca^{2+}	(12.7 ± 1.1) × 10^3	743.1 ± 23.4	0.058	
K96A				
prothrombin, PC/PS, Ca^{2+}	229.9 ± 11.4	0.15 ± 0.03	0.0007	
prothrombin, PC/PS, fVa, Ca^{2+}	78.2 ± 12.5	1277.8 ± 58.9	16.3	1.0 ± 0.1
prethrombin-2, PC/PS, fVa, Ca^{2+}	(21.1 ± 1.8) × 10^3	152.5 ± 5.5	0.007	
E97A				
prothrombin, PC/PS, Ca^{2+}	137.8 ± 23.2	0.38 ± 0.02	0.003	
prothrombin, PC/PS, fVa, Ca^{2+}	73.7 ± 11.2	1091.1 ± 47.1	14.8	2.0 ± 0.4
prethrombin-2, PC/PS, fVa, Ca^{2+}	(11.8 ± 1.5) × 10^3	337.5 ± 15.0	0.029	
D100A				
prothrombin, PC/PS, Ca^{2+}	144.8 ± 25.2	0.39 ± 0.02	0.003	
prothrombin, PC/PS, fVa, Ca^{2+}	63.7 ± 3.6	1216.1 ± 18.7	19.1	1.7 ± 0.1
prethrombin-2, PC/PS, fVa, Ca^{2+}	(19.2 ± 3.0) × 10^3	723.12 ± 47.1	0.038	
R107A				
prothrombin, PC/PS, Ca^{2+}	130.2 ± 13.5	0.30 ± 0.01	0.002	
prothrombin, PC/PS, fVa, Ca^{2+}	60.0 ± 7.3	933.4 ± 3.1	15.6	1.7 ± 0.1
prethrombin-2, PC/PS, fVa, Ca^{2+}	(19.7 ± 2.0) × 10^3	712.7 ± 29.8	0.036	
K109A				
prothrombin, PC/PS, Ca^{2+}	442.2 ± 49.9	0.12 ± 0.01	0.0003	
prothrombin, PC/PS, fVa, Ca^{2+}	124.6 ± 28.0	1399.0 ± 101.8	11.2	3.5 ± 0.2
prethrombin-2, PC/PS, fVa, Ca^{2+}	(20.7 ± 4.6) × 10^3	433.7 ± 40.9	0.021	
R115A				
prothrombin, PC/PS, Ca^{2+}	105.0 ± 17.9	0.25 ± 0.01	0.002	
prothrombin, PC/PS, fVa, Ca^{2+}	59.8 ± 2.7	1098.9 ± 50.2	18.4	1.8 ± 0.1
prethrombin-2, PC/PS, fVa, Ca^{2+}	(9.5 ± 3.1) × 10^3	498.8 ± 55.1	0.053	

[a] The kinetic parameters $K_{m(app)}$ and k_{cat} were determined from the concentration dependence of prothrombin (or prethrombin-2) activation by fXa derivatives on PC/PS vesicles in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000 in the absence and presence of a saturating concentration of fVa as described under Materials and Methods. The $K_{d(app)}$ values for interaction with fVa were determined from the saturable cofactor concentration dependence of thrombin generation by each fXa derivative at room temperature as described under Materials and Methods. All values are the average of at least two to three measurements ± standard errors.

DISCUSSION

In this study, we have demonstrated that neither one of the charged residues of the 82−116 loop of fXa is a fVa-dependent recognition site for interaction with prothrombin in the prothrombinase complex. Nevertheless, the observation that the catalytic efficiency of the K96A mutant toward both prothrombin in the absence of fVa and prethrombin-2 in the presence of fVa was impaired 5−10-fold suggests that Lys-96 is a recognition site for interaction with prothrombin independent of the cofactor. The catalytic activities of both K96A and K109A mutants toward prothrombin were markedly impaired. However, fVa overcame the defects in both $K_{m(app)}$ and k_{cat} of prothrombin activation by the mutant proteases in the prothrombinase complex. Thus, our results do not provide additional support for the hypothesis that fVa interaction with fXa may expose extended surfaces on the 82−116 loop that could specifically interact with prothrombin in the prothrombinase complex (*14*). The observation that the catalytic activity of the K109A mutant was markedly impaired in all relevant reactions suggests that mutagenesis of Lys-109 has caused global conformational changes and, thus, adversely affected the structure of the active site cleft of the mutant molecule. Despite the global effect of the mutagenesis with K109A, it was interesting to note that fVa alleviated the catalytic defect with this mutant as evidenced by a near normal amidolytic and proteolytic activity for the mutant in the presence of the cofactor. The examination of the X-ray crystal structure of the protease domain of fXa (*16*) provided a possible basis for the impaired catalytic activity of the K109A mutant. It was found that the side chain of Lys-109 is oriented toward Glu-84 and that the two residues are three dimensionally at a close distance (Glu-84A.OE − Lys-109A.NZ = 2.886 Å) to establish a salt bridge interaction (*16*). The relative three-dimensional locations of the residues under study are shown in Figure 5A. In addition to these two residues, the side chains of Glu-86 and Arg-107 are also located in close proximity (Glu-84A.OE−Arg-107A.NH = 2.487 Å) to establish ionic interactions (*16*). Both pairs of the basic and acidic residues are part of two antiparallel structures extending out of the Ca^{2+} binding 70−

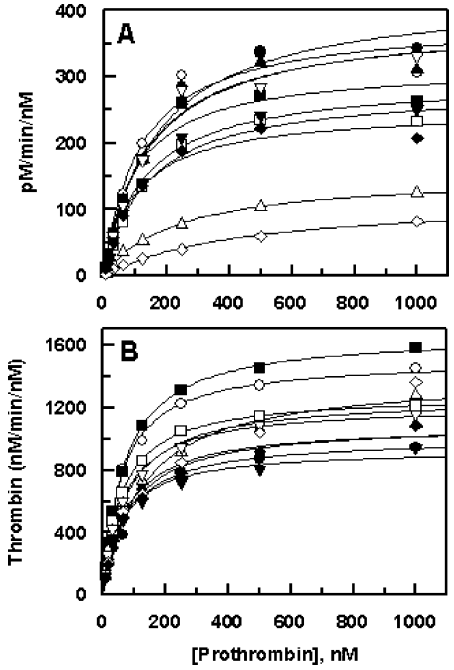

FIGURE 2: Prothrombin activation by fXa derivatives in the absence and presence of fVa. (A) In the absence of fVa, different concentrations of human prothrombin (10−1000 nM) were incubated with each fXa derivative [wild type (○), E86A (●), K90A (□), R93A (■), K96A (△), E97A (▲), D100A (▽), R107A (▼), K109A (◇), and R115A (◆) (2.5−5 nM each)] on PC/PS vesicles (10 μM) in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000. Following 15−30 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 under Materials and Methods. (B) Same as (A) except that the activation reactions by each fXa derivative (50 pM) were carried out in the presence of human fVa (25 nM) for 30 s. Solid lines in both panels are nonlinear regression fits of kinetic data to the Michaelis−Menten equation. The kinetic values are presented in Table 2.

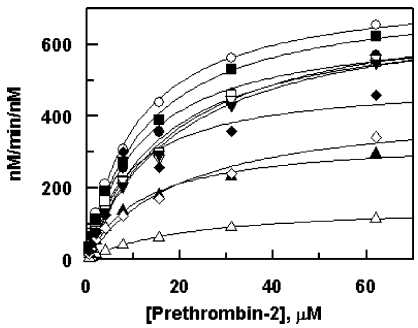

FIGURE 3: Prethrombin-2 activation by fXa derivatives in the presence of fVa. Different concentrations of recombinant prethrombin-2 (1−62 μM) were incubated with each fXa derivative [wild type (○), E86A (●), K90A (□), R93A (■), K96A (△), E97A (▲), D100A (▽), R107A (▼), K109A (◇), and R115A (◆) (0.1 nM each)] in the presence of fVa (25 nM) on PC/PS vesicles (10 μM) in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000. Following 3−4 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 under Materials and Methods. Solid lines in both panels are nonlinear regression fits of kinetic data to the Michaelis−Menten equation. The kinetic values are presented in Table 2.

80 loop of fXa (*16*). The ionic interactions of these residues appear to be critical for the stability of this loop. This may explain our repeated failure to express the E84A mutant in mammalian cells. Nevertheless, the mutagenesis of either

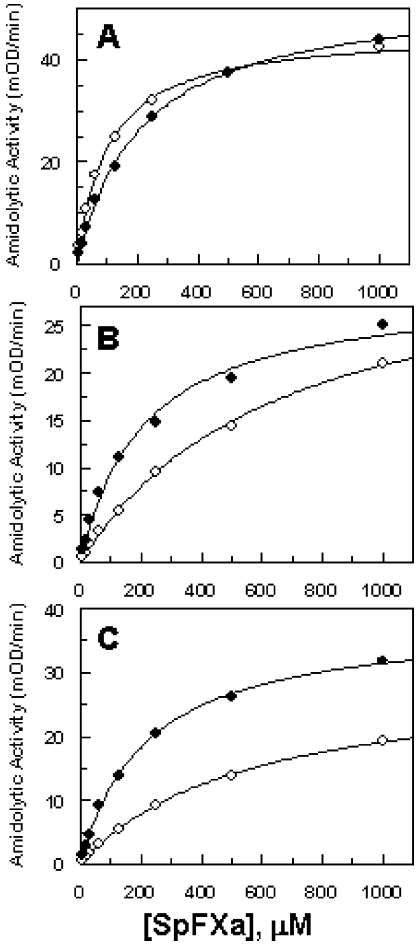

FIGURE 4: Concentration dependence of the SpFXa hydrolysis by wild-type fXa and the K109A mutant of fXa in the absence and presence of fVa. (A) The amidolytic activity of wild-type fXa (1 nM) was monitored as a function of increasing concentrations of SpFXa in the absence (○) and presence of a saturating concentration of fVa (●) on PC/PS vesicles (10 μM) at room temperature in TBS/Ca^{2+} containing 0.1 mg/mL BSA and 0.1% PEG-8000 as described under Materials and Methods. (B) Same as (A) with the exception that K109A (1.4 nM) was used in the amidolytic activity assay. (C) The amidolytic activity of K109A (1.4 nM) was monitored as a function of increasing concentrations of SpFXa in the same TBS/Ca^{2+} buffer containing either 0.1 M NaCl (○) or 0.6 M NaCl (●) under the same experimental conditions described above. The solid lines in all panels are nonlinear regression fits of kinetic data to the Michaelis−Menten equation.

Glu-86 or Arg-107 did not result in an observable negative effect in the mutant molecules, as evidenced by their normal amidolytic and proteolytic activity and reactivity with AT. It was also interesting to note that the mutagenesis of Lys-109 dramatically affected the conformation of the Na^+ binding loop which is located some 40 Å away on the other end of the molecule (*16*, *29*), suggesting that the two sites are energetically linked. This is consistent with our previous data showing that the conformation of the 70−80 loop and Na^+ binding site of fXa is allosterically linked (*24*).

Another interesting observation of this study is that, similar to interaction with Na^+, the $K_{d(app)}$ for the interaction of the Lys-109 mutant with fVa was markedly (~4-fold) impaired. However, in the presence of a saturating concentration of fVa, the fXa mutant exhibited normal activity toward prothrombin in the prothrombinase complex. Noting that Lys-109 may be involved in ionic interaction with Glu-84 as evidenced by the structural data, and the fact that all catalytic

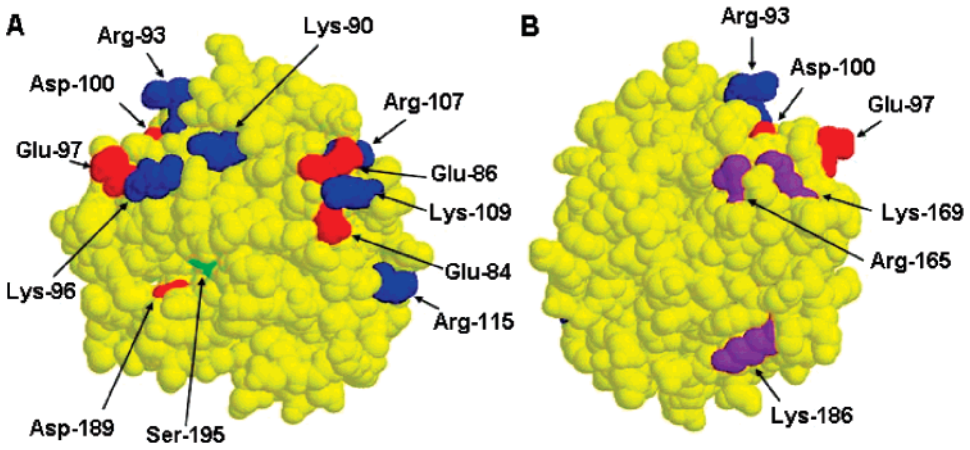

FIGURE 5: Crystal structure of the catalytic domain of fXa. (A) Space-filling representation of the catalytic domain of fXa with the basic residues under study colored in blue and acidic residues colored in red. The catalytic residue Ser-195 is colored in green, and the S1 pocket residue Asp-189 is colored in red. (B) Same as (A) with the exception that the structure is rotated by a full turn. The fVa binding residues of the 162 helix and 186 loop are colored in purple. Only three residues, Arg-93, Glu-97, and Asp-100, out of all residues under study are visible on this face of the molecule. The coordinates (Protein Data Bank code 1HCG) were used to prepare the figure (*16*).

activity of K109A was impaired in all reactions tested, the weaker affinity of the mutant for fVa cannot be construed as an indication that Lys-109 constitutes a specific binding site for the cofactor. Further support for this proposal may be provided by examining the relative positions of residues in the X-ray crystal structure of fXa which are known to interact with fVa (Figure 5B). It is known that two basic residues of the 162 helix including Arg-165 and Lys-169 (*22*, *30*) as well as Lys-186 (*23*) at the base of the S1 specificity pocket directly interact with fVa. Relative to Lys-109 and most other charged residues under study, these three known fVa binding residues are located on the opposite face of the fXa structure, and only Arg-93, Glu-97, and Asp-100 could be found on this face if the structure shown in Figure 5A was rotated by a full turn on its horizontal axis (Figure 5B). It is interesting to note that with the exception of K109A, $K_{d(app)}$ values for fVa interaction with R93A (2.7 nM) and Glu-97 (2.0 nM) were impaired the most, suggesting that both of these residues may also contribute to the high-affinity interaction of fXa with fVa. Similar to basic residues of the 162 helix, Arg-93 is part of the heparin binding exosite of fXa (*22*). We previously demonstrated that several residues of the heparin binding exosite, including Arg-165 and Arg-93, interact with fVa (*22*). On the other hand, we do not believe Lys-109 directly interacts with fVa, and the weaker affinity of this mutant for the cofactor is most likely caused by a global conformational change that has been transmitted to the specific fVa-interactive sites, the catalytic pocket and the Na^+ binding site of the mutant molecule.

The observation that both fVa and a higher concentration of Na^+ restored the impairment in the $K_{m(app)}$ for the cleavage of the chromogenic substrate by K109A suggests that, similar to Na^+, fVa binding alters the conformation of the S1 specificity pocket of the fXa mutant. These results further suggest that there is allosteric coupling between the conformations of the Na^+ and fVa binding sites and the 82−116 loop of fXa. It follows therefore that fVa binding reverses K109A-induced global conformational changes in the structure of the mutant, thereby stabilizing the Na^+ binding loop, the catalytic pocket, and the structure of the 82−116 loop of the mutant molecule. Thus, the possibility that fVa interaction with wild-type fXa is associated with subtle conformational changes in the structure of residues of the 82−116 loop that are critical for interaction with prothrombin, but do not involve any one of the charged residues under study, cannot be ruled out.

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REFERENCES

1. 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.
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. 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.
7. Dharmawardana, K. R., Olson, S. T., and Bock, P. E. (1999) Role of regulatory exosite I in binding of thrombin to human factor V, factor Va, factor Va subunits, and activation fragments, *J. Biol. Chem. 274*, 18635−18643.
8. 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.
9. Chen, L., Yang, L., and Rezaie, A. R. (2003) Proexosite on prothrombin is a factor Va-dependent recognition site for the prothrombinase complex, *J. Biol. Chem. 278*, 27564−27569.
10. Beck, D. O., Bukys, M. A., Singh, L. S., Szabo, K. A., and Kalafatis, M. (2004) The contribution of amino acid region Asp695-Tyr698 of factor V to procofactor activation and factor Va function, *J. Biol. Chem. 279*, 3084−3095.
11. Yegneswaran, S., Mesters, R. M., and Griffin, J. H. (2003) Identification of distinct sequences in human blood coagulation factor Xa and prothrombin essential for substrate and cofactor recognition in the prothrombinase complex, *J. Biol. Chem. 278*, 33312−33318.
12. Yegneswaran, S., Mesters, R. M., Fernandez, J. A., and Griffin, J. H. (2004) Prothrombin residues 473−487 contribute to factor Va binding in the prothrombinase complex, *J. Biol. Chem. 279*, 49019−49025.

13. Betz, A., and Krishnaswamy, S. (1998) Regions remote from the site of cleavage determine macromolecular substrate recognition by the prothrombinase complex, *J. Biol. Chem. 273*, 10709−10718.
14. Wilkens, M., and Krishnaswamy, S. (2002) The contribution of factor Xa to exosite-dependent substrate recognition by prothrombinase, *J. Biol. Chem. 277*, 9366−9374.
15. Krishnaswamy, S. (2004) Exosite-driven substrate specificity and function in coagulation, *J. Thromb. Haemostasis 3*, 54−67.
16. 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.
17. 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.
18. 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 chloromethyl ketone and significance of the Tyr-Pro-Pro-Trp insertion segment, *EMBO J. 8*, 3467−3475.
19. 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.
20. Rezaie, A. R., and Yang, L. (2001) Probing the molecular basis of factor Xa specificity by mutagenesis of the serpin, antithrombin, *Biochim. Biophys. Acta 1528*, 167−176.
21. 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.
22. 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.
23. 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.
24. Rezaie, A. R., and He, X. (2000) Sodium binding site of factor Xa: Role of sodium in the prothrombinase complex, *Biochemistry 39*, 1817−1825.
25. 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.
26. 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.
27. Krishnaswamy, S., and Mann, K. G. (1988) The binding of factor Va to phospholipid vesicles, *J. Biol. Chem. 263*, 5714−5723.
28. Nesheim, M. E., Eid, S., and Mann, K. G. (1981) Assembly of the prothrombinase complex in the absence of prothrombin, *J. Biol. Chem. 256*, 9874−9882.
29. 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.
30. 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.

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