Sodium Binding Site of Factor Xa: Role of Sodium in the Prothrombinase Complex†

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ABSTRACT: The nature of residue 225 on a consensus loop in serine proteases determines whether a protease can bind Na^+. Serine proteases with a Pro at this position are unable to bind Na^+, but those with a Tyr or Phe can bind Na^+. Factor Xa (FXa), the serine protease of the prothrombinase complex, contains a Tyr at this position. Na^+ is also known to stimulate the amidolytic activity of FXa toward cleavage of small synthetic substrates, but the role of Na^+ in the prothrombinase complex has not been investigated. In this study, we engineered a Gla-domainless form of FX (GDFX) in which residue Tyr^{225} was replaced with a Pro. We found that Na^+ stimulated the cleavage rate of chromogenic substrates by FXa or GDFXa ∼8−24-fold with apparent dissociation constants [$K_{d(app)}$] of 37 and 182 mM in the presence and absence of Ca^{2+}, respectively. In contrast, Na^+ minimally affected the cleavage rate of these substrates by the mutant, and no $K_{d(app)}$ for Na^+ binding to the mutant could be estimated. Unlike the wild-type enzyme, the reactivity of the mutant with antithrombin was independent of Na^+ and impaired ∼32-fold. Ca^{2+} improved the reactivity of the mutant with antithrombin ∼5-fold. Affinity of the mutant for binding to factor Va was weakened and its ability to activate prothrombin was severely impaired. Further studies with the wild-type prothrombinase complex revealed that FXa binds to factor Va with a similar $K_{d(app)}$ of 1.1−1.8 nM in the presence of Na^+, K^+, Li^+, Ch^+, and $Tris^+$ and that the catalytic efficiency of prothrombinase is enhanced less than 1.5-fold by the specific effect of Na^+ in the reaction buffer. These results suggest that (1) the loop including residue 225 (225-loop) is a Na^+ binding site in FXa, (2) the Na^+- and Ca^{2+}-binding loops of FXa are allosterically linked, and (3) the Tyr conformer of the 225-loop is critical for factor Xa function; however, both Na^+-bound and Na^+-free forms of factor Xa in the prothrombinase complex can efficiently activate prothrombin.

Factor Xa is a vitamin K-dependent coagulation serine protease that binds to factor Va on membrane surfaces in the presence of Ca^{2+} ions to rapidly activate prothrombin to thrombin in the clotting cascade (*2*, *3*). The activation complex, called the prothrombinase, activates prothrombin with a catalytic efficiency that is $>10^5$-fold greater than that of factor Xa alone (*2*, *3*). Metal ions bind to several discrete domains of factor Xa to modulate the structure and function of the protease in the activation complex. It is known that binding of the divalent cation Ca^{2+} to several of the γ-carboxyglutamic acid residues present on the N-terminus Gla[1] domain of factor Xa is required for the ability of the protease to bind factor Va on membrane surfaces (*2*, *4*). In addition to the Gla domain, two additional Ca^{2+} binding sites in factor Xa, one in the first epidermal growth factor-like (EGF-1)[1] domain and the other in the C-terminal catalytic domain, have been identified (*5*−*7*). The role of Ca^{2+} binding to the latter two domains of factor Xa in the prothrombinase complex is not completely understood. Our previous mutagenesis studies suggest that deletion of the Gla domain (GDFXa) as well as the Gla and EGF-1 domains (E2FXa) from factor Xa results in mutant molecules that can still bind factor Va in a Ca^{2+}-dependent manner, though with much lower affinity, to rapidly activate prothrombin or the Gla-domainless form of the zymogen in solution (*7*−*9*).

In addition to Ca^{2+}, several previous studies suggest that specific binding of the monovalent cation Na^+ to factor Xa stimulates the amidolytic activity of factor Xa toward cleavage of chromogenic substrates (*10*, *11*). However, the role of Na^+ or other monovalent cations in the catalytic function of factor Xa in the prothrombinase complex has

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[1] Abbreviations: FX, factor X; FXa, activated factor Xa; Gla, 4-carboxyglutamic acid; EGF, epidermal growth factor; GDFX, Gla-domainless FX from which amino-terminal residues 1−45 [in the chymotrypsin numbering system of Bode et al. (*1*)] were removed by the recombinant DNA methods; GDFX Y225P, GDFX derivative in which residue Tyr^{225} has been replaced with Pro; E2FXa, deletion mutant of factor Xa in which the Gla domain and the first EGF domain (residues 1−84) have been removed; E2FXa D70K and E2FXa E80K, E2FXa derivative in which Asp^{70} and Glu^{80}, respectively, were replaced with Lys; PEG, polyethylene glycol.

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not been studied. There are several recent reports in the literature that suggest Na^+ may play a crucial role in the catalytic activity of all coagulation serine proteases (*12*−*16*). Recent reports by Di Cera et al. (*17*) suggest that Na^+ binding to thrombin allosterically modulates the activity and specificity of thrombin. These authors demonstrated that the dissociation constant for Na^+ binding to thrombin is near the physiological concentration of Na^+ at 37 °C; as a consequence, thrombin exists in both Na^+-free (slow) and Na^+-bound (fast) forms in plasma (*15*−*17*). It is believed that the slow form of thrombin in complex with thrombomodulin functions in the anticoagulant pathway by preferentially activating protein C (*17*, *18*). In contrast, the fast form of thrombin functions in the coagulant pathway by preferentially cleaving fibrinogen to fibrin (*17*). Like that of thrombin, the catalytic function of APC is modulated by monovalent cations (*12*−*14*). In this case, however, the affinity of the protease for Na^+ is more than 20-fold higher than the physiological concentration of the cation; therefore, APC is expected to be primarily populated in its fast form in plasma (*19*). In the case of factor Xa, on the basis of the reported dissociation constant for Na^+ (K_d > 110 mM) (*10*, *16*), the protease could also exist in both slow and fast forms in plasma. It is not known if the slow and fast forms of factor Xa can bind factor Va with equal affinity to catalyze prothrombin activation in the clotting cascade.

Recently, the Na^+-binding site of thrombin was localized to the protease domain, to a consensus surface loop spanning residues 215−227 in the chymotrypsin numbering system (*16*). Molecular modeling based on the known crystal structures, as well as mutagenesis studies, indicated that residue 225 in this loop (225-loop) may be a critical residue that determines whether a serine protease can bind Na^+ (*11*, *20*). Proteases with a Pro at position 225 like trypsin, which do not require Na^+ for their catalytic function, do not bind Na^+. In contrast, proteases with a Tyr at this position like thrombin, APC, and factor Xa, which require Na^+ for their optimal catalytic function, are expected to bind Na^+. Our recent mutagenesis studies with APC supported this proposal since a $Tyr^{225} \rightarrow$ Pro substitution mutant, as well as an APC/trypsin 221−225-loop swap mutant, unlike wild-type APC, did not bind Na^+ (*19*). In this case, we discovered that an allosteric linkage between the Ca^{2+}-binding (Glu^{70}−Glu^{80}) and the Na^+-binding loops modulates the structure and function of this anticoagulant enzyme (*19*).

To determine whether the 225-loop of factor Xa is a Na^+-binding site, and if Na^+ binding to this loop is required for the catalytic function of factor Xa in the prothrombinase complex, we engineered and expressed a $Tyr^{225} \rightarrow$ Pro substitution mutant of factor X in the Gla-domainless form (GDFX Y225P) in mammalian cells. Following activation by RVV-X, the catalytic properties of wild-type and mutant GDFXa were analyzed with respect to cleavage of chromogenic substrates, activation of prothrombin, and reaction with antithrombin in both the absence and presence of Na^+ and other monovalent cations. The results suggest that like thrombin and APC, the 225-loop is a Na^+-binding site in factor Xa. Na^+ binding to this loop is critical for the catalytic function of factor Xa in cleavage of small synthetic peptide substrates and in reaction with antithrombin. However, the specific role of Na^+ in prothrombin activation is less clear since both Na^+-free and Na^+-bound forms of factor Xa in prothrombinase activated prothrombin with similar catalytic efficiencies. Finally, our results suggest that the conformations of the Ca^{2+}-binding (Asp^{70}−Glu^{80}) and the Na^+-binding 225-loops of factor Xa are allosterically linked.

MATERIALS AND METHODS

Mutagenesis, Expression, and Purification. The expression of the Gla-domainless form of factor X (GDFX) by the RSV-PL4 expression/purification vector system in human 293 cells and prethrombin-1 by the pNut-PL2 expression/purification vector system in baby hamster kidney (BHK) cells has been previously described (*9*, *21*). A $Tyr^{225} \rightarrow$ Pro substitution mutant of GDFX was prepared by the PCR mutagenesis approach, and the mutant molecule was expressed in the same vector system mentioned above. Both the wild-type and mutant zymogens were purified from the cell culture supernatants as previously described (*9*, *21*). The expression and purification of human factor X lacking both the Gla and EGF-1 domains (E2FX) and its substitution mutants $Asp^{70} \rightarrow$ Lys (E2FX D70K) and $Glu^{80} \rightarrow$ Lys (E2FX E80K) in 293 cells were previously described (*7*, *9*).

Human plasma factor X (*22*) and prothrombin (*23*) were prepared by the cited methods. Human factor Va was a generous gift from S. Krishnaswamy (Joseph Stokes Research Institute, Children's Hospital of Philadelphia, Philadelphia, PA). Some human factor Va was also purified from plasma as described previously (*24*). The active antithrombin-binding pentasaccharide was a generous gift from S. Olson (University of Illinois at Chicago, Chicago, IL). Factor X-activating enzyme from Russell's viper venom and human antithrombin [50% glycerol/H_2O (v/v)] were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Phospholipid vesicles in 5 mM Tris-HCl (pH 7.5) containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described previously (*25*). The chromogenic substrate Spectrozyme FXa (SpFXa) was purchased from American Diagnostica (Greenwich, CT), and the chromogenic substrates S2222, S2765, and S2238 were purchased from Kabi Pharmacia/Chromogenix (Franklin, OH).

Recombinant factor X derivatives were converted to their activated forms (Xa) with RVV-X as previously described (*9*). The active site concentrations of factor Xa derivatives were determined by an active site specific immunoassay using BioCap-EGR-ck (biotinyl-ε-aminocaproyl-D-glutamic acid glycylarginine chloromethyl ketone) (Haematologic Technologies, Inc.) as previously described (*26*). Prothrombin and factor Xa derivatives were desalted by passing them through PD-10 (Amersham Pharmacia Biotech) gel filtration columns equilibrated with 5 mM Tris-HCl (pH 7.5) containing 0.1% PEG 8000 and stored at −80 °C.

Steady-State Kinetics. The steady-state kinetic parameters of wild-type and Y225P mutant GDFXa toward S2765 (D-Arg-Gly-Arg-*p*-nitroanilide) were determined in 5 mM Tris-HCl (pH 7.5) containing 0.1% PEG 8000, 2.5 mM $CaCl_2$, and different monovalent chloride salts each at 0.15 M (LiCl, NaCl, KCl, and choline chloride). The rate of hydrolysis was measured at 405 nm at room temperature in a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA) as previously described (*26*). The apparent K_m and k_{cat} values for substrate hydrolysis were calculated from the Michaelis−

Menten equation. The specificity constant for each chloride salt was expressed as the ratio of k_{cat}/K_m. The specificity constant for the bulky monovalent cation choline (Ch^+) was used as a reference to determine the monovalent cation specificity as described by Dang and Di Cera (*11*). The concentration of S2765 (free of cation) ranged from 7.8 μM to 4 mM depending on the K_m values, and the concentrations of enzymes ranged from 1 to 5 nM depending on the k_{cat} values.

Dissociation Constant [$K_{d(app)}$] for Na^+. The values of $K_{d(app)}$ for Na^+ binding to each protease were determined from the effect of varying concentrations of Na^+ on the activity of the protease toward the synthetic substrate SpFXa in both the absence (10 μM EDTA) and presence of 2.5 mM $CaCl_2$. The reactions were carried out at both room temperature (22−25 °C) and 37 °C. In the past, either choline chloride or Tris-HCl has been used as the nonspecific monovalent cation to study the specific effect of monovalent cations on the catalytic function of various coagulation enzymes (*11*−*13*, *17*). In the case of factor Xa, however, we found that Na^+ stimulation of the cleavage rate of chromogenic substrates is inhibited by choline chloride. Previously, a similar effect for choline chloride on the catalytic function of factor Xa has been reported (*27*). We, therefore, used Tris-HCl to adjust the ionic strength in all experiments. The values for $K_{d(app)}$ were calculated from the hyperbolic increase in the rate of substrate hydrolysis as a function of increasing concentrations of Na^+.

Prothrombin Activation in the Presence of Factor Va. A modified prothrombinase assay was used to evaluate the effect of Na^+ ion on the catalytic efficiency of GDFXa and GDFXa Y225P toward prothrombin activation in the presence of factor Va. In this assay, human factor Va-mediated (100 nM) enhancement of the initial rate of recombinant human prethrombin-1 activation (1.5 μM) by GDFXa (2 nM) or GDFXa Y225P (100 nM) was assessed in 5 mM Tris-HCl (pH 7.5), 2.5 mM $CaCl_2$, and 0.1% PEG 8000 containing either 0.15 M sodium or choline chloride. The initial rate of activation was monitored by time course analysis at room temperature. At each time point, samples were added to 20 mM EDTA and the rate of thrombin generation was determined by an amidolytic activity assay using S2238. The affinity of each enzyme for factor Va was also evaluated by this assay. In this case, the initial rate of thrombin generation was determined in the presence of increasing concentrations of human factor Va (0.06 to 3.5 μM). The affinity of each factor Xa derivative for factor Va was inferred from the saturable dependence of the rate of thrombin generation as a function of different concentrations of factor Va.

A similar prothrombinase assay was used to evaluate the affinity of wild-type factor Xa for human factor Va in the presence of different monovalent cations. In this assay, factor Xa (0.2 nM) in complex with 35 μM PC/PS vesicles and different concentrations of human factor Va (0.3−10 nM) was incubated with 1 μM prothrombin for 1.5 min at room temperature in 5 mM Tris-HCl (pH 7.5) containing 0.1% PEG 8000, 2.5 mM $CaCl_2$, and different monovalent cations each at 0.15 M. The affinity of factor Xa for factor Va under different conditions was determined from the rate of thrombin generation. The concentration dependence of prothrombin activation by factor Xa was also studied. In this case, factor Xa (5 pM) in complex with saturating concentrations of factor Va (10 nM) was incubated with increasing concentrations of prothrombin (7.8−1000 nM) on PC/PS vesicles (35 μM) for 1.5 min at room temperature in the same buffer system. The rate of thrombin generation was determined from a standard curve prepared from the cleavage rate of S2238 (100 μM) by known concentrations of thrombin at 405 nm at room temperature in a V_{max} kinetic plate reader as described above. It was ensured that less than 10% of the prothrombin was activated at all concentrations of the substrate. The apparent K_m and k_{cat} values for prothrombin activation were calculated from the Michaelis−Menten equation. The specificity constant for each monovalent cation was expressed as the ratio of k_{cat}/K_m. The specificity constant for the bulky monovalent cation choline (Ch^+) was used as a reference to determine the monovalent cation specificity as described above for the cleavage of chromogenic substrates.

Kinetics of Inactivation by Antithrombin. The rate of inactivation of the factor Xa derivatives by antithrombin was measured under pseudo-first-order rate conditions by an end point assay method as previously described (*28*). Briefly, factor Xa derivatives (2 nM) were incubated with 1−5 μM human antithrombin in 50 μL reaction mixtures in a 96-well polystyrene plate at room temperature in 5 mM Tris-HCl (pH 7.5), 0.1% PEG 8000 in the absence and presence of 0.15 M NaCl, and 2.5 mM $CaCl_2$. In the absence of Na^+, the ionic strength of the reaction buffer was adjusted with 0.15 M Tris-HCl or choline chloride. At the end of the incubation time (5−15 min), 50 μL of 1 mM S2765 was added and the residual enzyme activities were determined by measuring the absorbance at 405 nm with a V_{max} kinetic microplate reader as described above. The second-order inactivation rate constants were calculated as previously described (*28*). The same methods were used to study the Na^+ dependence of antithrombin inactivation of factor Xa in the presence of pentasaccharide. In this case, GDFXa derivatives (1−2 nM) were incubated with antithrombin (15−100 nM) in the presence of saturating concentrations of pentasaccharide (500 nM) for 1−10 min, after which S2765 was added and the inactivation rate constants were determined as described above.

Data Analysis. The apparent K_m and k_{cat} values for substrate hydrolysis were calculated from the Michaelis−Menten equation, and the affinity of Na^+ for each factor Xa derivative [$K_{d(app)}$] was determined by nonlinear regression fits of data to a rectangular hyperbola using ENZFITTER (R. J. Leatherbarrow, Elsevier, Biosoft). All values are the average of at least three to five independent measurements ± the standard deviation.

RESULTS

Expression and Purification of Recombinant Proteins. Recombinant GDFX derivatives were expressed in human 293 cells, and prethrombin-1 was expressed in baby hamster kidney cells and isolated as described in Materials and Methods. SDS−PAGE analysis of the isolated recombinant proteins indicated that all recombinant proteins have been purified to homogeneity as evidenced by their migration as single-band molecules with the expected molecular masses under nonreducing conditions (data not shown). GDFX derivatives were activated with RVV-X, and their concentra-

Table 1: Apparent Dissociation Constants [$K_{d(app)}$] for Binding of Na^+ to Different Factor Xa Derivatives at Room Temperature and at 37 °C in both the Absence and Presence of Ca^{2+}[a]

	22−25 °C		37 °C	
	with Ca^{2+}	without Ca^{2+}	with Ca^{2+}	without Ca^{2+}
FXa	39 ± 4	220 ± 24	116 ± 14	∼638 ± 97
GDFXa	37 ± 7	182 ± 29	121 ± 14	∼586 ± 28
E2FXa	31 ± 1	151 ± 1	138 ± 21	∼689 ± 132
E2FXa D70K	147 ± 18	133 ± 5	∼575 ± 77	∼551 ± 58
E2FXa E80K	103 ± 13	120 ± 14	∼527 ± 54	∼494 ± 22

[a] The $K_{d(app)}$ values for Na^+ binding (in millimolar) to each factor Xa derivative were measured from the hyperbolic increase in the rate of cleavage of Spectrozyme Xa (75 μM) as a function of increasing concentrations of Na^+ in 5 mM Tris-HCl (pH 7.5) and 0.1% PEG 8000 in the absence (10 μM EDTA) or presence of Ca^{2+} (2.5 mM) as described in Materials and Methods. The concentration of Na^+ ranged from 0 to 720 mM. The "∼" symbol indicates that the values at 37 °C in the absence of Ca^{2+} for all factor Xa derivatives and for the D70K and E80K mutants of E2FXa in both the absence and presence of Ca^{2+} are approximate values inferred from the Michaelis−Menten equation since a complete saturation at 720 mM Na^+ was not observed. For all measurements, the total ionic strength of the reaction buffer was adjusted by addition of Tris-HCl (pH 7.5).

tions were determined by active site titration as described in Materials and Methods. The concentrations of enzymes as determined by active site titration were similar (within 90%) with the values calculated on the basis of their absorbance at 280 nm, further supporting the homogeneity of the expressed proteins.

Effect of Na^+ on Amidolytic Activity. The effect of Na^+ on the amidolytic activity of the factor Xa derivatives toward cleavage of the chromogenic substrate SpFXa was monitored as a function of increasing concentrations of Na^+ (Table 1). These studies were carried out at both room temperature and 37 °C. Since the catalytic domain of factor Xa contains a single Ca^{2+}-binding site (*7*), these studies were carried out both in the absence and in the presence of Ca^{2+}. In the absence of Ca^{2+} and Na^+, a negligible amidolytic activity was observed for GDFXa cleavage of SpFXa under the experimental conditions described in the legend of Figure 1A. The amidolytic activity of GDFXa was enhanced with increasing concentrations of Na^+ and reached to saturation with a $K_{d(app)}$ of 182 ± 29 mM at room temperature and ∼586 ± 28 mM at 37 °C (Table 1). In general, the $K_{d(app)}$ values for Na^+ interaction with factor Xa derivatives were ∼3−4-fold higher at 37 °C (Table 1). In the absence of the divalent cation Ca^{2+}, the amidolytic activity of GDFXa was enhanced ∼24-fold at a saturating concentration of Na^+ (Figure 1A). In the presence of Ca^{2+}, however, the stimulatory effect of Na^+ was ∼8-fold under the same experimental conditions (Figure 1A). The affinity of Na^+ for the protease was improved ∼5-fold in the presence of Ca^{2+} [$K_{d(app)}$ = 37 ± 7 and 121 ± 14 mM at room temperature and 37 °C, respectively]. The concerted stimulatory effect of the two metal ions on the amidolytic activity of the factor Xa derivatives was not a phenomenon related to a unique substrate, since the same results were obtained with three different substrates, SpFXa, S2222, and S2765. Only the results with SpFXa hydrolysis for GDFXa at 37 °C are presented in the figures. The Na^+ dependence of the amidolytic activity of wild-type factor Xa was similar to that of GDFXa (Table 1). Interestingly, however, the stimulatory effect of Na^+ on the amidolytic activity of prothrombinase

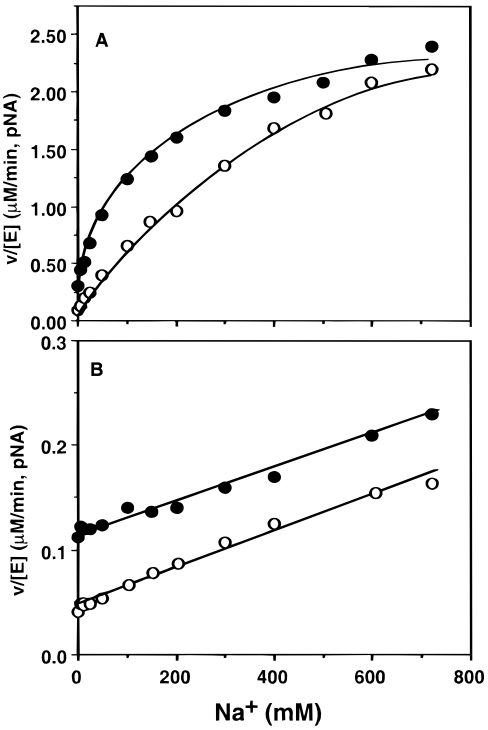

FIGURE 1: Na^+ dependence of the amidolytic activity of GDFXa and GDFXa Y225P in the absence or presence of Ca^{2+}. (A) The initial rate of hydrolysis of SpFXa (75 μM) by GDFXa (2.5 nM) in the absence (10 μM EDTA) (○) or presence (●) of 2.5 mM Ca^{2+} was measured at 37 °C as described in Materials and Methods. Solid lines are nonlinear regression fits to a rectangular hyperbola. For all measurements, the total ionic strength was adjusted to 0.72 M with Tris-HCl (pH 7.5). (B) The same as above except that the initial rate of hydrolysis of SpFXa (300 μM) by GDFXa Y225P (25 nM) was measured in the absence (○) or presence (●) of Ca^{2+}. v is the initial rate (micromolar per minute) of SpFXa cleavage. [E] is the enzyme concentration (nanomolar). pNA is *p*-nitroaniline.

(factor Xa, factor Va, PS/PS vesicles, and Ca^{2+}) was decreased to less than 2-fold (data not shown). These results appear to suggest that factor Va stabilizes the 225-loop of factor Xa in the Na^+-activated conformation, rendering the catalytic function of the protease relatively independent of Na^+.

To ensure that the high concentrations of Tris-HCl, which was used to compensate for the ionic strength of the reaction buffer, do not adversely affect the amidolytic activity of GDFXa or interfere with the Na^+ binding to the enzyme, the rate of cleavage of SpFXa by GDFXa was studied as a function of 0−720 mM Na^+ or 0−720 mM Tris-HCl alone. An essentially identical cleavage rate and $K_{d(app)}$ value for the binding of Na^+ to GDFXa were obtained if the ionic strength of the reaction buffer was not adjusted with a nonspecific monovalent cation (data not shown). With Tris-HCl alone, no amidolytic activity for GDFXa under the experimental conditions described in the legend of Figure 1A was detected in the absence of Ca^{2+}. In the presence of Ca^{2+}, the amidolytic activity of GDFXa was ∼10% of its maximal value independent of the Tris-HCl concentration (data not shown). Taken together, these results suggest that Tris-HCl and the high ionic strength of the reaction buffer do not influence the conclusions of this study.

The Na^+ concentration dependence of the amidolytic activity of the GDFXa Y225P mutant was studied in a similar fashion. Na^+ stimulated the amidolytic activity of the mutant

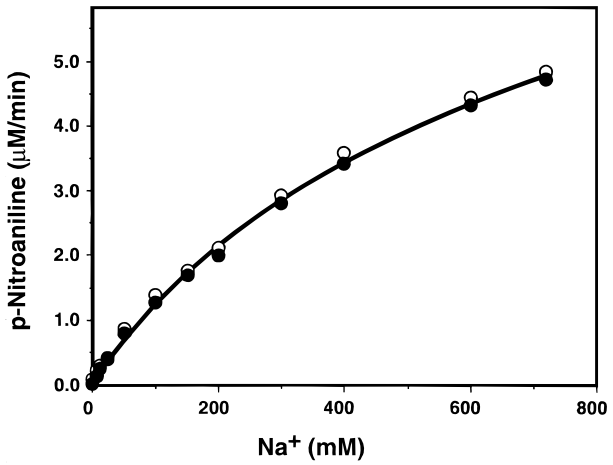

FIGURE 2: Na^+ dependence of the amidolytic activity of E2FXa D70K in the absence or presence of Ca^{2+}. The initial rate of hydrolysis of SpFXa (75 μM) by E2FXa D70K (2.5 nM) in the absence (10 μM EDTA) (○) or presence (●) of 2.5 mM Ca^{2+} was measured as a function of increasing concentrations of Na^+ (0−0.72 M) as described in Materials and Methods. The solid line is a nonlinear regression fit to a rectangular hyperbola. For all measurements, the ionic strength was adjusted to 0.72 M with Tris-HCl (pH 7.5).

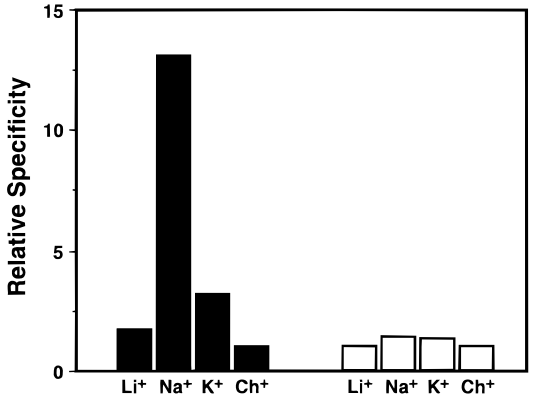

FIGURE 3: Relative specificity of GDFXa and GDFXa Y225P toward hydrolysis of the chromogenic substrate S2765 in the presence of different monovalent cations. The kinetic constants k_{cat} and K_m for the hydrolysis of S2765 by GDFXa (black bars) or GDFXa Y225P (white bars) in the presence of various monovalent cations (0.15 M) in 5 mM Tris-HCl (pH 7.5), 2.5 mM Ca^{2+}, and 0.1% PEG 8000 were calculated as described in Materials and Methods. The concentrations of the enzymes ranged from 1 to 5 nM, and the concentration of the substrate ranged from 7.8 μM to 4 mM. The specificity constant (k_{cat}/K_m) for each cation was calculated and expressed as the relative specificity using the specificity constant for choline chloride as a reference as described by Dang and Di Cera (*11*).

less than 2-fold in either the absence or presence of Ca^{2+} (Figure 1B). The $K_{d(app)}$ for the Na^+ interaction with the mutant could not be estimated since no saturation of Na^+ binding was observed if the concentration of the cation was raised to 720 mM (Figure 1B). No attempt was made to increase the concentration of Na^+ above 720 mM. The linear increase in the amidolytic activity of the mutant with the increasing concentrations of Na^+ was not an effect caused by changes in the ionic strength of the reaction buffer since a similar increase was also observed with K^+, but not with either Li^+ or Tris-HCl (data not shown). These results indicate that the mutant may still bind Na^+, but with much weaker affinity. Taken together, these results suggest that like thrombin (*16*) and activated protein C (*19*), the 225-loop is a functionally important Na^+-binding site in factor Xa.

The observation that Ca^{2+} ion increased the affinity of Na^+ for factor Xa may suggest that the two metal ion-binding loops are allosterically linked. The Ca^{2+} binding site in the catalytic domain of factor Xa is located on a loop between residues Asp^{70} and Glu^{80}, analogous to the Ca^{2+}-binding loop of trypsin (*29*). Previously, we prepared and characterized a deletion derivative of factor Xa, E2FXa D70K, in which Asp^{70} was replaced with Lys (*7*). In that study, we demonstrated that the mutant retained its function but lost its ability to bind Ca^{2+}. To further characterize the Na^+-binding site of factor Xa and identify the Ca^{2+}-binding site responsible for altering the Na^+ binding properties of the protease, the amidolytic activity of E2FXa D70K toward cleavage of SpFXa was studied as a function of different concentrations of Na^+. As shown in Figure 2, the Na^+ stimulation of the amidolytic activity of this mutant was insensitive to the absence or presence of Ca^{2+}. The same result was obtained with another E2FXa mutant in which Glu^{80} was replaced with Lys (E2FXa E80K). The $K_{d(app)}$ for Na^+ binding to the mutants was similar in both the absence and presence of Ca^{2+} (Table 1). These results support the proposal that the conformations of the Na^+- and Ca^{2+}-binding loops of factor Xa are allosterically linked. In a previous study, a similar allosteric linkage between the two metal binding loops of activated protein C was also observed (*19*).

In addition to Na^+, other monovalent alkali cations are known to stimulate the amidolytic activity of factor Xa (*10*, *11*). To determine whether the mutant can discriminate among various alkali cations, the kinetic constants for hydrolysis of S2765, S2222, and SpFXa by wild-type and mutant GDFXa were determined. While similar results were obtained with all three chromogenic substrates, the K_m of the mutant for the substrates in the presence of all cations was dramatically elevated. Due to a better solubility of S2765, detailed kinetic analysis was only carried out with this substrate. In these studies, the specificity constant (k_{cat}/K_m) for the bulky monovalent cation choline was set as a reference, and data were analyzed as described by Dang and Di Cera (*11*). As shown in Figure 3, and consistent with previous findings (*10*, *11*), Na^+ was the preferred monovalent cation for the optimal catalytic function of factor Xa. However, unlike GDFXa, the mutant lost its ability to discriminate among Li^+, Na^+, K^+, and Ch^+. The lower amidolytic activity of GDFXa in the presence of other monovalent cations was, largely, due to increased K_m values (4−8-fold) with a <2-fold effect on the k_{cat} values. Similar results were obtained with the mutant. In this case, however, relative to a K_m value of ~40 μM for the GDFXa cleavage of S2765 in the presence of Na^+ as determined in this study, this value was increased to ~1 mM for the mutant regardless of which monovalent cation was present in the reaction buffer. Similarly, relative to the k_{cat} of GDFXa in the presence of Na^+, the k_{cat} values were decreased ~2-fold with the mutant in the presence of all four cations. Thus, the catalytic efficiency of the mutant (k_{cat}/K_m) was impaired ~50-fold. These results, therefore, suggest that specific binding of Na^+ to the 225-loop induces conformational changes in the residue(s) and/or surface loop(s) surrounding the active site pocket of factor Xa which lead to improvement in the binding

of the substrate to the primary specificity pocket of the enzyme.

Role of Na^+ in Prothrombinase. Efficient prothrombin activation by factor Xa in the presence of factor Va requires an intact Gla domain for factor Xa to assemble with factor Va on the negatively charged phospholipid vesicles in the presence of Ca^{2+} (prothrombinase complex). However, factor Va is known to bind GDFXa with a lower affinity and catalyze prothrombin or prethrombin-1 activation more than 1000-fold in solution (*8*, *9*). Like cleavage of chromogenic substrates, the initial rate studies in the presence of factor Va suggested that the ability of GDFXa to activate prethrombin-1 in the absence of Na^+ and the mutant in both the absence and presence of Na^+ was dramatically impaired (Figure 4). Under the experimental conditions described in the legend of Figure 4, the rate of prethrombin-1 activation by GDFXa in the presence of Na^+ (8 mol min^{-1} mol^{-1}) was 10-fold higher than its rate in the presence of Ch^+ (0.8 mol min^{-1} mol^{-1}). Under the same conditions, the rate of activation by the mutant in the presence of Na^+ (15 μmol min^{-1} mol^{-1}) was less than 2-fold higher than that in the presence of Ch^+ (8 μmol min^{-1} mol^{-1}). It should be noted that a similar difference (<2-fold) between the effect of Na^+ and Ch^+ ions on the initial rate of prethrombin-1 activation by wild-type GDFXa was noticed, if bovine factor Va was used in the reaction (data not shown). The reason for this observation is not known, and no data with bovine factor Va are presented in this study. We next studied the factor Va concentration dependence of prethrombin-1 activation to evaluate the affinities of the GDFXa derivatives for the cofactor. In contrast to a $K_{d(app)}$ of 1.1 ± 0.1 μM for GDFXa, a $K_{d(app)}$ of 4.2 ± 0.7 μM was estimated for the binding of the mutant to factor Va (Figure 4B,C). These results suggest that the impaired catalytic efficiency of the mutant may at least be partly caused by its weaker affinity for factor Va. In the case of wild-type GDFXa, however, it is not known whether the impaired rate of prethrombin-1 activation in the absence of Na^+ (presence of Ch^+) is due to a lower affinity for factor Va or an inhibitory effect of Ch^+ on the catalytic function of factor Xa, as reported previously by others (*27*). Nevertheless, the observation that the affinity of the GDFXa mutant for binding to factor Va is impaired suggests that the Tyr conformer of the 225-loop is critical for interaction of factor Xa with its cofactor and/or substrate in this prothrombin activation system in solution.

Due to the unavailability of Na^+-free factor Va at a high concentration, the affinities of the GDFXa derivatives for factor Va could not be studied in the absence of other monovalent cations. Therefore, we evaluated the affinity of wild-type factor Xa for human factor Va in the prothrombinase complex in the presence of PC/PS vesicles and different monovalent cations. As shown in Figure 5, a similar $K_{d(app)}$ value for the factor Xa−Va interaction was observed with all monovalent cations that were tested (Na^+, 1.1 ± 0.1 nM; K^+, 1.5 ± 0.4 nM; Li^+, 1.8 ± 0.5 nM; Ch^+, 1.7 ± 0.2 nM; and $Tris^+$, 1.6 ± 0.5 nM). Further kinetic analysis suggested that unlike the significant accelerating effect of Na^+ on the proteolytic and amidolytic activity of GDFXa, the specificity constant (k_{cat}/K_m) of prothrombin activation by factor Xa in prothrombinase was minimally (<1.5-fold) affected by the presence of Na^+ in the reaction buffer (Figure 6). Comparisons of the individual kinetic constants indicated

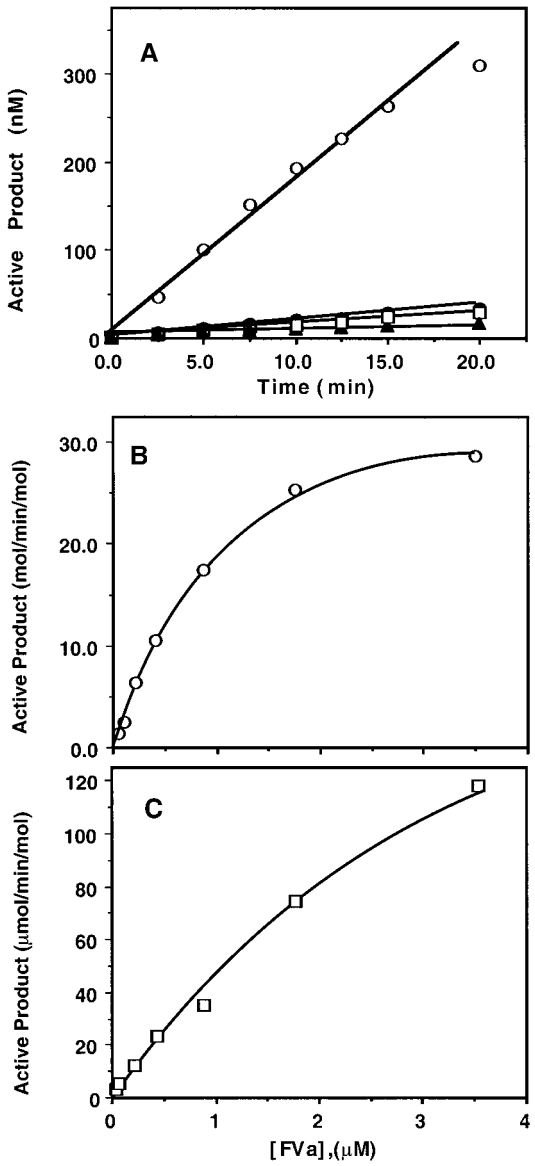

FIGURE 4: Initial rate of prethrombin-1 activation by GDFXa and GDFXa Y225P in the presence of factor Va. (A) The time course of prethrombin-1 (1.5 μM) activation by GDFXa (2 nM) or GDFXa Y225P (100 nM) was studied in the presence of human factor Va (100 nM) in 5 mM Tris-HCl (pH 7.5), 2.5 mM Ca^{2+}, and 0.1% PEG 8000 containing 0.15 M Na^+ or Ch^+. At the indicated time points, samples of the reaction mixture were removed and added to 20 mM EDTA and the rate of thrombin generation was determined by an amidolytic assay using S2238 as described in Materials and Methods. The symbols, ○ for Na^+ and ● for Ch^+, represent the initial rates of activation by GDFXa, and the symbols, □ for Na^+ and ▲ for Ch^+, represent the activation rates for GDFXa Y225P. (B and C) The reaction conditions are the same as those described for panel A except that the rate of prethrombin-1 activation by GDFXa (○) or GDFXa Y225P (□) was monitored as a function of increasing concentrations of factor Va (0.06−3.5 μM) in the presence of 0.15 M Na^+. Note that the rate of activation in panel C is given in micromoles per minute per mole. The solid lines in panel A are fits of data to a linear equation, and in panels B and C to a rectangular hyperbola.

that the minor differences in the specificity constants with all cations, except for Ch^+, are due to differences in the k_{cat} values. In the case of Ch^+, the k_{cat} value was improved ~1.5-fold, but this was compensated by ~2-fold impairment in the K_m of the reaction. These results suggest that the lower rate of prethrombin-1 activation by the GDFXa−Va complex

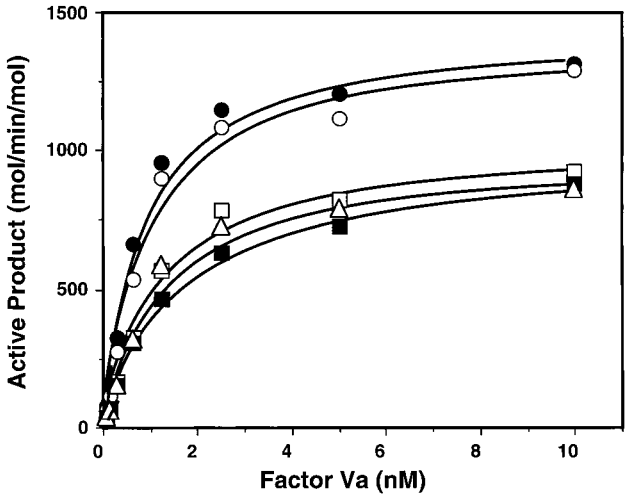

FIGURE 5: Factor Va dependence of prothrombin activation by factor Xa on PC/PS vesicles in the presence of different monovalent cations. Human factor Xa (0.2 nM) in complex with PC/PS vesicles (35 μM) and different concentrations of factor Va (0−10 nM) was incubated with human prothrombin at room temperature for 1.5 min in 5 mM Tris-HCl (pH 7.5), 2.5 mM Ca^{2+}, and 0.1% PEG 8000 in the presence of different monovalent cations each at 0.15 M: Na^+ (●), K^+ (○), Li^+ (□), Ch^+ (■), and $Tris^+$ (△). The rate of prothrombin activation was measured by a chromogenic substrate assay as described in Materials and Methods.

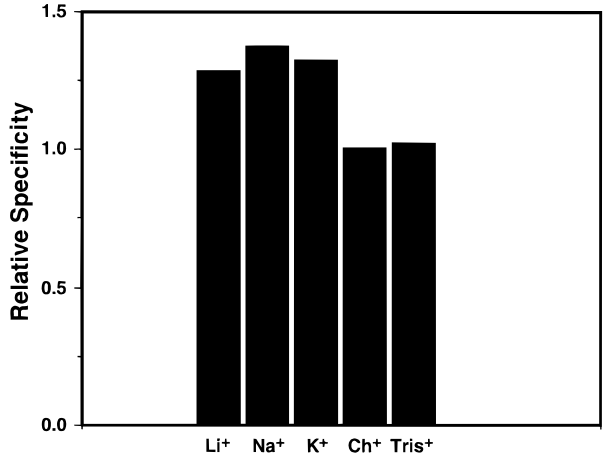

FIGURE 6: Relative specificity of prothrombin activation by prothrombinase in the presence of different monovalent cations. The kinetic constants k_{cat} and K_m for factor Va-dependent prothrombin activation by factor Xa in the presence of different monovalent cations (0.15 M) in 5 mM Tris-HCl (pH 7.5), 2.5 mM Ca^{2+}, and 0.1% PEG 8000 were calculated as described in Materials and Methods. Factor Xa (5 pM) in complex with factor Va (10 nM) on PC/PS vesicles (35 μM) was added to varying concentrations of prothrombin (7.8−1000 nM). The specificity constant (k_{cat}/K_m) for each cation was determined and expressed as the relative specificity using the specificity constant for Ch^+ as a reference as described in the legend of Figure 3.

in solution in the absence of Na^+ (Figure 4A) is most likely due to an impairment in the binding of the enzyme to the cofactor−substrate complex. Thus, the assembly of factor Xa into the prothrombinase complex on membrane surfaces corrects this defect by dramatically improving the dissociation constant of the factor Xa interaction with factor Va and/or prothrombin.

Effect of Na^+ on the Reaction of Factor Xa with Antithrombin. To determine whether Na^+ is required for the reactivity of factor Xa with antithrombin, the second-order inactivation rate constants of the factor Xa derivatives for inactivation by antithrombin were determined under pseudo-first-order rate conditions in both the absence and presence of 0.15 M Na^+, 2.5 mM Ca^{2+}, and saturating concentrations of the active pentasaccharide fragment of heparin (500 nM). In the absence of Na^+, the rate of inactivation of GDFXa by antithrombin [$(2.7 \pm 0.2) \times 10^2$ M^{-1} s^{-1}] was ~7-fold slower than the rate of inactivation in the presence of Na^+ [$(1.8 \pm 0.2) \times 10^3$ M^{-1} s^{-1}]. The inactivation rate in the absence of Na^+ was improved ~2-fold for GDFXa in the presence of Ca^{2+}. With the mutant, the inactivation rate in both the absence [$(4.0 \pm 0.6) \times 10$ M^{-1} s^{-1}] and presence of Na^+ [$k_2 = (5.6 \pm 0.4) \times 10$ M^{-1} s^{-1}] was similarly impaired. However, Ca^{2+} improved the reactivities ~5-fold in the absence [$k_2 = (1.9 \pm 0.3) \times 10^2$ M^{-1} s^{-1}] and presence of the monovalent cation [$k_2 = (2.1 \pm 0.2) \times 10^2$ M^{-1} s^{-1}]. Except for the higher activation rates, essentially identical results were obtained if the inactivation studies were carried out in the presence of the pentasaccharide. Thus, the reactivity of GDFXa with the antithrombin−cofactor complex in the presence of Na^+ was insensitive to the presence and absence of Ca^{2+} [$(3.6 \pm 0.3) \times 10^5$ M^{-1} s^{-1}]. The complex inactivated GDFXa ~4-fold slower in the absence of Na^+ [$k_2 = (9.4 \pm 0.1) \times 10^4$ M^{-1} s^{-1}]. In this case, however, most of the defect in the reactivity was corrected by Ca^{2+} [$k_2 = (2.6 \pm 0.4) \times 10^5$ M^{-1} s^{-1}]. Similarly, antithrombin inactivated the mutant at a similar rate in both the absence and presence of Na^+ [$k_2 = (1.0 \pm 0.1) \times 10^4$ M^{-1} s^{-1}]. Consistent with the results presented above, Ca^{2+} did not influence the reactivity of the mutant enzyme with the serpin in the presence of Na^+, but improved the inactivation rate 2.7-fold in the absence of Na^+ [$k_2 = (2.7 \pm 0.3) \times 10^4$ M^{-1} s^{-1}]. These results indicate that Na^+ binding to factor Xa is required for its normal reactivity with antithrombin in both the absence and presence of the cofactor. The observation that Ca^{2+} partially corrects the defect caused by mutation of the Na^+ site further supports the hypothesis that the two metal ion-binding loops of factor Xa are allosterically linked.

Next, we evaluated the effect of Na^+ ion on the reactivity of wild-type factor Xa when the enzyme is bound to factor Va on PC/PS vesicles. Relative to inactivation of free factor Xa [$k_2 = (2.2 \pm 0.1) \times 10^3$ M^{-1} s^{-1}], complex formation of factor Xa (1 nM) with human factor Va (10 nM) on PC/PS vesicles (35 μM) protected the enzyme (~2.4-fold) from inactivation by antithrombin in the presence of Na^+. In the absence of Na^+, free factor Xa or factor Xa in complex with the cofactor exhibited a similar defect in the rate of inactivation by antithrombin [$k_2 = (2.9 \pm 0.4) \times 10^2$ M^{-1} s^{-1}]. These results suggest that factor Va does not in an obvious way influence the Na^+ dependence of the factor Xa inactivation by antithrombin.

DISCUSSION

It is known that Na^+ and other monovalent cations stimulate the amidolytic activity of coagulation serine proteases toward cleavage of tripeptidyl chromogenic substrates (*10*, *12*−*15*). Recently, Dang and Di Cera localized the Na^+-binding site of thrombin to a conserved loop comprising residues 215−227 in the chymotrypsin numbering system (*16*). These authors discovered that residue 225

in this loop is a key residue that may determine whether a serine protease can bind Na^+ (*11*). They proposed that serine proteases with a Pro at this position, like trypsin, may not bind Na^+, but plasma serine proteases with a Tyr or Phe at this position, like thrombin, APC, and factor Xa, are expected to bind the monovalent cation. In agreement with this proposal, our current mutagenesis study with factor Xa and a pervious study with APC (*19*) suggest that, like thrombin, the 225-loop is a Na^+-binding site in these proteases. Our results also suggest that specific binding of Na^+ to this loop stimulates the catalytic activity of factor Xa in the reaction with small synthetic substrates as well as the natural serpin inhibitor, antithrombin. However, prothrombin activation by factor Xa in the prothrombinase complex was less sensitive to the specific effect of Na^+ in the reaction buffer. This conclusion is based on the observation that factor Xa exhibited a similar $K_{d(app)}$ for binding to factor Va and activated prothrombin with a similar catalytic efficiency (k_{cat}/K_m) in the presence of all monovalent cations tested in this study. Noting the high $K_{d(app)}$ of Na^+ for factor Xa at 37 °C, a lack of a requirement for the binding of Na^+ to the 225-loop of factor Xa in the presence of factor Va ensures that the protease functions efficiently in the prothrombinase complex in both the Na^+-bound and Na^+-free forms.

Like the impairment in the amidolytic activity, the proteolytic activity of the Y225P mutant of GDFXa toward prothrombin was dramatically impaired in both the absence and presence of factor Va. It may be thought that such a drastic effect in the catalytic function of the protease could be due to the nonconservative substitution of Tyr^{225} with Pro, which leads into serious disruptive changes in the structure and/or the correct folding of factor Xa. However, recent crystal structure determinations of three analogous mutants of thrombin, $Tyr^{225} \rightarrow$ Phe, $Tyr^{225} \rightarrow$ Pro, and $Tyr^{225} \rightarrow$ Ile, suggest that introduction of a Pro into this site of thrombin is associated with a minimal change in the structure of the molecule (*20*). Moreover, saturation mutagenesis of Tyr^{225} in thrombin in this previous study suggested that mutants with all other residues, except for Phe and His, have comparable or markedly reduced activity relative to that of the mutant containing a Pro at this position (*20*). The primary effect of mutation of residue 225 is thought to be shifting the orientation of the carbonyl oxygen atom of residue 224 (one of the Na^+ coordination ligands) and reshaping of the structure of a water channel next to the primary specificity residue, Asp^{189}, in thrombin (*11, 20*). As proposed in these previous studies, substitution of Tyr^{225} with Pro is most likely associated with similar changes in the structure of this loop in factor Xa. Therefore, in the context of these previous observations, our results suggest that the Tyr conformer of this loop is critical for the catalytic function of factor Xa. The impaired affinity of the GDFXa mutant for factor Va further suggests that adaptation of Tyr at position 225 has also played a role in specific binding of the enzyme for its target cofactor. It is not, however, known whether this loop is directly involved in binding to factor Va or its mutation is associated with long-range subtle changes in the conformation of other surface loops, critical for interaction of the enzyme with its cofactor and/or substrate.

In addition to Na^+, it is known that the catalytic activity of factor Xa and other vitamin K-dependent coagulation proteases is stimulated by the divalent cation Ca^{2+} (*7, 10, 14, 30*). The observation that in the presence of Ca^{2+}, the $K_{d(app)}$ for Na^+ binding to factor Xa was improved ~5-fold suggests that Ca^{2+} could allosterically modulate the conformation of the Na^+-binding loop of the molecule. Previously, we demonstrated that factor Xa contains a single Ca^{2+}-binding site in the catalytic domain in a loop spanning residues Asp^{70}−Glu^{80} (*7*). When we replaced Asp^{70} with Lys (D70K), the mutant protease no longer bound Ca^{2+} ions, but retained its catalytic function possibly by the stabilization of the loop by a potential salt bridge formed between Lys^{70} and Glu^{80} of the mutant, analogous to that observed in the crystal structure of thrombin (*1, 7*). Interestingly, the affinity of this mutant for Na^+ was not sensitive to the absence or presence of Ca^{2+} in the reaction buffer, supporting the hypothesis that the conformations of the monovalent and divalent cation binding loops in factor Xa are allosterically linked. The observation that the reactivity of the mutant with antithrombin in the presence of Ca^{2+} was improved ~5-fold is also consistent with this hypothesis. In a previous study, a similar interdependence between the conformation of the two metal ion-binding loops in APC was observed (*19*).

Previous studies by Di Cera et al. (*16, 17*) suggest that Na^+ binding to the 225-loop of thrombin modulates the activity and specificity of this enzyme by an allosteric mechanism. Since the concentration of Na^+ in blood is very close to the $K_{d(app)}$ of this cation for thrombin, these authors proposed that thrombin can exist in two Na^+-bound (fast) and Na^+-free (slow) forms in plasma (*15, 17*). The fast form has improved catalytic activity toward chromogenic substrates and exhibits greater specificity for fibrinogen cleavage, whereas the slow form of thrombin in complex with thrombomodulin exhibits enhanced specificity for protein C activation (*17, 18*). Since the dissociation constant for factor Xa binding to Na^+ is also near the physiological concentration of Na^+ in plasma, it is expected that factor Xa, like thrombin, can exist in both Na^+-free and Na^+-bound forms. As mentioned above, our results suggest that factor Xa can bind to factor Va on PC/PS vesicles with nearly equal affinity in the absence of Na^+. Since the Na^+-free form of factor Xa is inactivated poorly by antithrombin, it is expected that this form will activate prothrombin with an efficiency comparable to or better than that of the Na^+-bound form of factor Xa. However, it is also possible that the binding of factor Va to factor Xa stabilizes the 225-loop in the Na^+-activated conformation so that the protease becomes catalytically competent independent of Na^+. We have some support for this hypothesis in that the amidolytic activity of prothrombinase was relatively insensitive to the presence of Na^+. Alternatively, it is possible that different forms of factor Xa function in different pathways. It is known that during prothrombin activation some amount of a reaction intermediate, called meizothrombin, is generated by prothrombinase in which only the Arg^{322}−Ile^{323} peptide bond has been *cleaved* (*2, 31*). Unlike thrombin, meizothrombin is reported to function in the anticoagulant pathway of the clotting cascade (*32, 33*). It is possible that the slow or fast form of factor Xa has different catalytic properties with respect to preferential cleavage of the Arg^{322}−Ile^{323} bond with subsequent alteration in the ratio of meizothrombin to thrombin generation. Further studies will be needed to determine if any of these hypotheses are valid.

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