The Conformational Switch from the Factor X Zymogen to Protease State Mediates Exosite Expression and Prothrombinase Assembly*

Received for publication, March 19, 2008, and in revised form, April 8, 2008 Published, JBC Papers in Press, May 6, 2008, DOI 10.1074/jbc.M802205200

Raffaella Toso1, Hua Zhu1, and Rodney M. Camire1,2†

From the 1Department of Pediatrics, Division of Hematology, The Children's Hospital of Philadelphia and 2University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104

Zymogens of the chymotrypsin-like serine protease family are converted to the protease state following insertion of a newly formed, highly conserved N terminus. This transition is accompanied by active site formation and ordering of several surface loops in the catalytic domain. Here we show that disruption of this transition in factor X through mutagenesis (FXa116L and FXaV17A) not only alters active site function, but also significantly impairs Na+ and factor Va binding. Active site binding was improved in the presence of high NaCl or with saturating amounts of factor Va membranes, suggesting that allosteric linkage exists between these sites. In line with this, irreversible stabilization of FXa116L with Glu-Gly-Arg-chloromethyl ketone fully rescued FVa binding. Furthermore, the $k_m$ for prothrombin conversion with the factor Xa variants assembled into prothrombinase was unaltered, whereas the $k_{cat}$ was modestly reduced (3- to 4-fold). These findings show that intramolecular activation of factor X following the zymogen to protease transition not only drives catalytic site activation but also contributes to the formation of the Na+ and factor Va binding sites. This structural plasticity of the catalytic domain plays a key role in the regulation of exosite expression and prothrombinase assembly.

Proteolysis of zymogen proteins to their enzymatically active form is a central feature of many physiological processes, including blood coagulation (1). The paradigm for this type of activation mechanism is the zymogen to protease transition in the chymotrypsin-like serine protease family. Bond cleavage at a highly conserved site (Arg15–Ile16; the chymotrypsin numbering system is used throughout (2)) unMASKS a new N terminus, accompanied by active site formation and ordering of several surface loops. This conformational switch in factor X through mutagenesis (FXa116L and FXaV17A) not only alters active site function, but also significantly impairs Na+ and factor Va binding. Active site binding was improved in the presence of high NaCl or with saturating amounts of factor Va membranes, suggesting that allosteric linkage exists between these sites. In line with this, irreversible stabilization of FXa116L with Glu-Gly-Arg-chloromethyl ketone fully rescued FVa binding. Furthermore, the $k_m$ for prothrombin conversion with the factor Xa variants assembled into prothrombinase was unaltered, whereas the $k_{cat}$ was modestly reduced (3- to 4-fold). These findings show that intramolecular activation of factor X following the zymogen to protease transition not only drives catalytic site activation but also contributes to the formation of the Na+ and factor Va binding sites. This structural plasticity of the catalytic domain plays a key role in the regulation of exosite expression and prothrombinase assembly.

---

*This work was supported, in whole or in part, by National Institutes of Health Grants P01 HL-74124-01, Project 2 (to R. M. C.). This work was also supported by National Research Service Award T32 HL-07439-26 (to R. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Division of Hematology, 302E Abramson Research Center, The Children’s Hospital of Philadelphia, 3615 Civic Center Boulevard, Philadelphia, PA 19104. Tel.: 215-590-9968; Fax: 215-590-3660; E-mail: rcamire@mail.med.upenn.edu.

2 Nomenclature of Schechter and Berger (65).

3 The abbreviations used are: FX, factor X; FXa, activated FX; PDFX, plasmaprecipitated FX; rFX, recombinant FX; FV, factor V; FVa, activated FV; RVVX-CP, FX activator from Russell’s viper venom; pAB, 4-aminobenazmidine; EGR-CH2Cl, glutamylglycylarginyl chloromethyl ketone; SpecXa, methoxy carbonyl-cylohexylglycylglycylarginylp-nitroanilide; rTAP, recombinant tick anticoagulant peptide; PCPS, small unilamellar vesicles composed of 75% (w/w) phosphatidylcholine and 25% (w/w) phosphatidylserine; OG488-FXa, factor Xa modified with Oregon Green488; HEK293, human embryonic kidney cells; Gla, γ-carboxyglutamic acid; MES, 4-morpholineethanesulfonic acid.

---

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
183–189 and 221–225) and S1 specificity site; both of which are part of the activation domain.

In contrast to FXa, the zymogen FX is not known to bind FVa (20–22), indicating that an important step in the maturation of the protease is the exposure of the cofactor binding site. Although this could be due to removal of the large activation peptide, another possibility is that conformational activation of the protease domain following the zymogen to protease transition leads to the expression of the FVa binding site. Indirect evidence to support this comes from a recent study from our laboratory using a FXa Na⁺ binding site mutant (Y225P) (23). This mutant had markedly reduced Na⁺ binding and, surprisingly, had a destabilized Ile16–Asp194 salt bridge making the derivative “zymogen-like.” As a result, we and others have shown that the mutant has a lower affinity for both FVa and active site-directed probes (23–25). Similar results with respect to N-terminal stabilization and cofactor binding have also been found using factor IXaY225P (26).

In the current study, we have exploited the well defined mechanism of serine protease activation and prepared FXa variants with zymogen-like properties. These derivatives were employed to directly examine the relationship between the zymogen to protease transition and the expression of discrete structural determinants that govern the function of FXa.

**EXPERIMENTAL PROCEDURES**

**Materials**—The fluorophore 4-aminobenzamidine (pAB) was from Aldrich, and its concentration was determined in water using 

\[
E_{295} = 15,000 \text{ M}^{-1} \text{ cm}^{-1} \]  

(27). Glutamylglycylarginyl chloromethyl ketone (EGCH2Cl) was obtained from EMD Chemicals, Inc. (San Diego, CA). The peptide substrate methoxycarbonylcyclohexylglycylglycylglycylarginine-p-nitroanilide (SpecXa) was from American Diagnostica (Greenwich, CT). H-D-Phenylalanlylpepcocylargyline-p-nitroanilide (S-2238) and N-α-benzylcarbonyllylpeycocylargyline-p-nitroanilide (S-2765) were purchased from Diapharma Group, Inc. (West Chester, OH). Substrate solutions were prepared in water, and concentrations were verified using 

\[
E_{342} = 8270 \text{ M}^{-1} \text{ cm}^{-1} \]  

(28). All tissue culture reagents were from Invitrogen (Carlsbad, CA) except insulin-transferrin-sodium selenite, which was from Roche Applied Science. Small unilamellar phospholipid vesicles (PCPS) composed of 75% (w/w) hen egg phosphatidylcholine and 25% (w/w) porcine brain L-phosphatidylcholine was from Roche Applied Science. Small unilamellar phospholipid vesicles (PCPS) composed of 75% (w/w) hen egg phosphatidylcholine and 25% (w/w) porcine brain L-phosphatidylcholine was from Roche Applied Science.

**Proteins**—Human prothrombin, FX and factor V (FV) were isolated from plasma as described previously (30–32). Thrombin and prethrombin-1 were prepared and purified by established procedures (33, 34). The FX activator from Russell’s viper venom (RVV X.Cp) was purified as described previously (35). Human FVa was prepared by proteolytic activation of FV by thrombin and purified as described before (36). Oregon Green488-FXa (OG488-FXa) was prepared as described (30). Molecular weights and extinction coefficients (\(E^0.1%) 280 \text{ nm} \) of the various proteins used were taken as follows: RVV X.Cp, 93,000 and 1.18 (37); prethrombin, 72,000 and 1.47 (33); prethrombin-1, 49,900 and 1.78 (33); thrombin, 37,500 and 1.94 (38); FVa, 173,000 and 1.78 (39); FX, 59,000 and 1.16 (40); and FXa, 46,000 and 1.16 (40).

**Preparation of Recombinant FX**—Recombinant FX (rFX) variants were generated with the QuickChange site-directed mutagenesis kit (Stratagene) using appropriate mutagenic complementary oligonucleotides. For each variant, the entire FX cDNA was sequenced to confirm the presence of the desired mutation and to ensure that there were no polymerase-induced errors. Wild-type or mutant rFX in the mammalian expression plasmid pCMV4 were stably expressed in human embryonic kidney (HEK) 293 cells and purified as previously described (41, 42).

**Preparation of FXa**—Plasma-derived FX (PDFX) and rFX (wild-type or variants) were activated using RVV X.Cp and subsequently purified using benzamidine-Sepharose or Sephacryl S-200 as described (34, 41). Following purification, the proteins were stored in 50% glycerol at −20 °C.

**Preparation of EGR-FXa**—Recombinant FXa (7 mg) or rFXa116I (10 mg) was incubated with a 5- or 100-fold molar excess, respectively, of EGR-CH2Cl. Following modification, the proteins were applied to a Sephacryl S-200 column equilibrated in 20 mM Heps/150 mM NaCl/5 mM EDTA, pH 7.4. Fractions were pooled, precipitated with ammonium sulfate, collected by centrifugation, dissolved in 50% glycerol, and stored at −20 °C. EGR-FXa and EGR-rFXa116I have <0.1% chromogenic activity toward SpecXa.

**Characterization of FX and FXa**—Protein purity was assessed by SDS-PAGE using 4–12% gels (Invitrogen) under reducing (50 mM dithiothreitol, final) and non-reducing conditions using the MES buffer system followed by staining with Coomassie Brilliant Blue R-250. N-terminal sequence analysis was performed in the laboratory of Dr. Alex Kurosky and Steven Smith at the University of Texas Medical Branch at Galveston. Chemical y-carboxylglutamic acid (Gla) analysis was carried out in our laboratory as described (41, 42). This analysis indicates that rFX, rFX116I, and rFX117A have essentially the full complement of Gla residues (10.5–10.8 mol of Gla/mol of FX) compared with PDFX (10.7 mol of Gla/mol of FX; theoretical = 11 mol of Gla/mol of FX).

**Determination of Kinetic Parameters for Peptidyl Substrate Hydrolysis**—All kinetic measurements were performed in 20 mM Heps, 0.15 M NaCl, 0.1% (w/v) polyethylene glycol-8000, 2 mM CaCl₂, pH 7.5 (assay buffer), unless otherwise indicated. The kinetic parameters of peptidyl substrate hydrolysis (SpecXa) were measured using increasing concentrations of substrate (10–500 nM) and initiated with either free FXa (2 nM wild-type FXa; 6.0 nM mutant FXa) or FXa assembled into prothrombinase (5 nM FXa, 30 nM FVa, and 50 μM PCPS).

**Carbamoylation of Ile16 by Reaction with NaNCO**—Mixtures containing wild-type or mutated FXa (2 μM) in assay buffer were reacted with 0.2 M NaNCO essentially as described (23). The final pH of the reaction mixture upon addition of NaNCO was pH 7.45. At selected time intervals (15–300 min) an aliquot of the reaction mixture was diluted in assay buffer, and the residual enzymatic activity was determined from initial steady-state rates of SpecXa hydrolysis.

**Inhibition of FXa and Prothrombinase by Pefabloc tPA/Xa**—The inhibitory constant (Kᵢ) of Pefabloc tPA/Xa (Pefabloc, Pen-
Modulation of the FX Zymogen to Protease Transition

The initial rate of thrombin generation was measured as described (42).

Data Analysis—Data were analyzed according to the referenced equations by nonlinear least squares regression analysis using the Marquardt algorithm (45). The qualities of the fits were assessed by the criteria described (46). Reported estimates of error represent ± 2 S.D.

For competition experiments with the non-fluorescent derivatives of FXa, titration curves were analyzed as described previously (47, 48). Non-linear least square regression analysis, assuming both n and n_comp equal to 1, yielded fitted values of K_d fixed describing the binding of OG_488-FXa to FVa on the membrane surface and K_d comp describing the equivalent interaction between the competitor FXa species and FVa membranes.

Initial velocity measurements of peptidyl substrate or macromolecular substrate hydrolysis by FXa or prothrombinase were analyzed by fitting the data to the Henri-Michaelis-Menten equation (49), to yield fitted values for K_m and V_max.

Initial velocity measurements of SpecXa hydrolysis by FXa or prothrombinase using increasing concentrations of pAB or Pefabloc were analyzed according to the rate expression for classical competitive inhibition (49), to yield the fitted value for K_i.

Global Analysis of Initial Velocity Data: Effect of FXa on Peptidyl Substrate Cleavage by FXa^{16L}—The equilibrium dissociation constants for the binding of FVa to membrane-bound FXa^{16L} in the absence (K_d^{1}) and presence of peptidyl substrate (K_d^{2}), as well as the substrate dissociation constant for FXa^{16L} (K_s^{2}) and membrane-bound FXa saturated with FVa (K_s^{1}), were calculated from initial velocity measurements of SpecXa hydrolysis at different fixed concentrations of FVa, according to the system of ordinary differential equations that comprise SCHEME 1 and by using the rapid equilibrium assumption. The entire data set was globally fit using the program Dynafit (50) to extract K_s^{1}, K_d^{1}, K_{cat1}, K_s^{2}, K_d^{2}, and K_{cat2}.

RESULTS

Generation of Zymogen-like FXa Variants—To investigate the role of N-terminal insertion on the expression of various structural determinants on FXa we modified positions 16 or 17 with the intent of creating zymogen-like variants. Ile^{16} was modified to Leu, Phe, Asp, or Gly, and position Val^{17} was changed to Leu, Ala, or Gly. Variants were initially transiently transfected into HEK 293 cells, and 48 h post-transfection conditioned media was collected. Subsequently, FX antigen levels were determined by enzyme-linked immunosorbent assay, and FXa activity levels were assessed by a chromogenic-based assay.

Inhibition of FXa and Prothrombinase by pAB—The inhibitory constant (K_i) of pAB for FXa or prothrombinase was assessed assuming classic competitive inhibition. Initial velocity measurements of S-2765 hydrolysis for free FXa (1.0 or 3.0 nM, for wild-type or mutant rFXa) or prothrombinase (50 μM PCPS, 20 nM FVa, and 1.0 or 3.0 nM, for wild-type or mutant rFXa) were made in the presence of increasing concentrations of pAB (0–1.0 mM) in assay buffer.

Functional Binding Studies: The Effect of FVa on Peptidyl Substrate Cleavage by FXa^{16L}—Reaction mixtures (200 μl) containing SpecXa (10–800 μM), PCPS (50 μM), with different fixed concentrations of FVa (1, 2, 5, 8, 12, 18, and 25 nM) were prepared in the wells of a 96-well plate and allowed to incubate for 5 min at room temperature. Because the substrate stock solution was prepared in water, an appropriate volume of 10× assay buffer solution adjusted to pH 7.75 was added to each mixture to ensure that the final pH and concentration of buffer solutes was invariant. The reaction was initiated with rFXa^{16L} (10 nM, final).

Assessment of Na+ Binding—Cleavage of SpecXa (100 μM) was measured using either 2 nM wild-type or 50 nM mutant rFXa in the presence of increasing concentrations of Na+ (0–450 mM) in 50 mM Tris, 5 mM CaCl_2, 0.1% polyethylene glycol 8000, pH 7.5, at 25 °C. To adjust the ionic strength of the reaction buffer, LiCl was used as the compensating chloride salt.

Fluorescence Intensity Measurements—Reaction mixtures containing 10 nM OG_488-FXa, 50 μM PCPS, and 8 or 10 nM FVa in assay buffer were titrated with increasing concentrations of a non-fluorescent FXa competitor (i.e. rFXa^{S195A}) at 25 °C in 1×1-cm² stirred quartz cuvettes, and steady-state fluorescence intensity was measured using λ_ex = 480 and λ_em = 520 nm with a long pass filter (KV 500, Schott, Duryea, PA) in the emission beam essentially as described (30).

Kinetics of Protein Substrate Cleavage—The kinetic parameters of prothrombinase-catalyzed prothrombin activation (K_m and V_max) were determined in assay buffer by measuring the initial rate of thrombin formation at increasing concentrations of macromolecular substrate as described (23, 43, 44). Assay mixtures contained PCPS vesicles (20 μM), FVa (20 nM), and various concentrations of prothrombin (0–2.0 μM) or prethrombin-1 (0–12 μM). The reaction was initiated with FXa (0.1 nM and 0.4 nM for wild-type rFXa and mutant rFXa, respectively) for prothrombin or 0.5 nM for prethrombin-1. Prothrombin cleavage by FXa alone was measured using 1.4 μM prothrombin, 50 μM PCPS, and 10 nM rFXa or 50 nM FXa^{16L}.

tapharm, Basel, Switzerland) for FXa or prothrombinase was assessed assuming classic competitive inhibition by initial velocity measurements of chromogenic substrate (S-2765) hydrolysis. Reaction mixtures (200 μl) contained: Pefabloc (0.02–5.0 μM), FXa (3 nM wild-type or 10 nM mutant FXa), or in the case of prothrombinase, PCPS (60 μM) and FVa (20 nM or 100 nM, for wild-type and mutant rFXa, respectively). Reactions were prepared in the wells of a 96-well plate and allowed to incubate for 2 min at room temperature. The reaction was initiated with S-2765 (100 μM or 500 μM, for wild-type and mutant rFXa, respectively).

Inhibition of FXa and Prothrombinase by pAB—The inhibitory constant (K_i) of pAB for FXa or prothrombinase was assessed assuming classic competitive inhibition. Initial velocity measurements of S-2765 hydrolysis for free FXa (1.0 or 3.0 nM, for wild-type or mutant rFXa) or prothrombinase (50 μM PCPS, 20 nM FVa, and 1.0 or 3.0 nM, for wild-type or mutant rFXa) were made in the presence of increasing concentrations of pAB (0–1.0 mM) in assay buffer.

Functional Binding Studies: The Effect of FVa on Peptidyl Substrate Cleavage by FXa^{16L}—Reaction mixtures (200 μl) containing SpecXa (10–800 μM), PCPS (50 μM), with different fixed concentrations of FVa (1, 2, 5, 8, 12, 18, and 25 nM) were prepared in the wells of a 96-well plate and allowed to incubate for 5 min at room temperature. Because the substrate stock solution was prepared in water, an appropriate volume of 10× assay buffer solution adjusted to pH 7.75 was added to each mixture to ensure that the final pH and concentration of buffer solutes was invariant. The reaction was initiated with rFXa^{16L} (10 nM, final).

Assessment of Na+ Binding—Cleavage of SpecXa (100 μM) was measured using either 2 nM wild-type or 50 nM mutant rFXa in the presence of increasing concentrations of Na+ (0–450 mM) in 50 mM Tris, 5 mM CaCl_2, 0.1% polyethylene glycol 8000, pH 7.5, at 25 °C. To adjust the ionic strength of the reaction buffer, LiCl was used as the compensating chloride salt.

Fluorescence Intensity Measurements—Reaction mixtures containing 10 nM OG_488-FXa, 50 μM PCPS, and 8 or 10 nM FVa in assay buffer were titrated with increasing concentrations of a non-fluorescent FXa competitor (i.e. rFXa^{S195A}) at 25 °C in 1×1-cm² stirred quartz cuvettes, and steady-state fluorescence intensity was measured using λ_ex = 480 and λ_em = 520 nm with a long pass filter (KV 500, Schott, Duryea, PA) in the emission beam essentially as described (30).

Kinetics of Protein Substrate Cleavage—The kinetic parameters of prothrombinase-catalyzed prothrombin activation (K_m and V_max) were determined in assay buffer by measuring the initial rate of thrombin formation at increasing concentrations of macromolecular substrate as described (23, 43, 44). Assay mixtures contained PCPS vesicles (20 μM), FVa (20 nM), and various concentrations of prothrombin (0–2.0 μM) or prethrombin-1 (0–12 μM). The reaction was initiated with FXa (0.1 nM and 0.4 nM for wild-type rFXa and mutant rFXa, respectively) for prothrombin or 0.5 nM for prethrombin-1. Prothrombin cleavage by FXa alone was measured using 1.4 μM prothrombin, 50 μM PCPS, and 10 nM rFXa or 50 nM FXa^{16L}.
following activation with RVV\textsubscript{X-CP}. Whereas total antigen was comparable to wild-type FX, activity levels following processing with RVV\textsubscript{X-CP} varied among the mutants and ranged from <1% to 25% (data not shown). We speculate that these differences in activity reflect the extent to which the zymogen to protease transition was perturbed, with the lowest activity variants being more zymogen-like. Two mutants, rFX\textsubscript{A16L}, and rFX\textsubscript{V17A}, whose activities were 5 and 3% of wild-type, respectively, were chosen for further study.  

Expression and Purification of Recombinant Proteins—We established high producing stable clones expressing rFX, rFX\textsubscript{A16L}, or rFX\textsubscript{V17A} in HEK 293 cells. Each protein was purified to homogeneity and activated using established procedures (23, 42). SDS-PAGE analysis before and after disulfide bond reduction showed that the proteases were purified to homogeneity and consisted of the characteristic $\alpha$- and $\beta$-forms of FXa (Fig. 1). N-terminal sequence analysis (data not shown) and chemical Gla analysis each yielded the expected results.  

Assessment of Active Site Binding: Free FXa—To examine how changes at position 16 or 17 influence the active site, we performed kinetic experiments with several probes. Using either SpecXa, Pefabloc, or pAB, the results showed that both mutants exhibited reduced binding as the $K_m$ or $K_i$ was increased $\sim$10–25-fold relative to wild type (Table 1 and Fig. 2A). We also found that the $k_{cat}$ for Spec Xa hydrolysis was reduced 2- to 4-fold (Table 1). These data indicate that changes to the native N terminus of FXa have a dramatic effect on catalytic function. These results are consistent with the idea that active site formation and N-terminal stabilization are allosterically linked, a finding well established in the trypsinogen/trypsin system (4).

Based on the nature of the mutations, we hypothesized that N-terminal insertion during the zymogen to protease transition would be suboptimal. This could be due to the new N terminus not fully inserting into the Ile\textsubscript{16} hydrophobic pocket, or rather the new N terminus could be misaligned or not properly oriented to efficiently facilitate the necessary conformational changes. To discriminate between these possibilities, we subjected each of the proteases to chemical modification using NaN\textsubscript{C}O, which preferentially modifies the N terminus of proteases whose primary sequence is not efficiently inserting into the Ile\textsubscript{16} hydrophobic pocket, or rather would be suboptimal. This could be due to the new N terminus not fully inserting into the Ile\textsubscript{16} hydrophobic pocket, or rather the new N terminus could be misaligned or not properly oriented to efficiently facilitate the necessary conformational changes. To discriminate between these possibilities, we subjected each of the proteases to chemical modification using NaN\textsubscript{C}O, which preferentially modifies the N terminus of proteases, whose activities were 5 and 3% of wild-type, respectively, were chosen for further study.

Expression and Purification of Recombinant Proteins—We established high producing stable clones expressing rFX, rFX\textsubscript{A16L}, or rFX\textsubscript{V17A} in HEK 293 cells. Each protein was purified to homogeneity and activated using established procedures (23, 42). SDS-PAGE analysis before and after disulfide bond reduction showed that the proteases were purified to homogeneity and consisted of the characteristic $\alpha$- and $\beta$-forms of FXa (Fig. 1). N-terminal sequence analysis (data not shown) and chemical Gla analysis each yielded the expected results.

Assessment of Active Site Binding: Free FXa—To examine how changes at position 16 or 17 influence the active site, we performed kinetic experiments with several probes. Using either SpecXa, Pefabloc, or pAB, the results showed that both mutants exhibited reduced binding as the $K_m$ or $K_i$ was increased $\sim$10–25-fold relative to wild type (Table 1 and Fig. 2A). We also found that the $k_{cat}$ for Spec Xa hydrolysis was reduced 2- to 4-fold (Table 1). These data indicate that changes to the native N terminus of FXa have a dramatic effect on catalytic function. These results are consistent with the idea that active site formation and N-terminal stabilization are allosterically linked, a finding well established in the trypsinogen/trypsin system (4).

Based on the nature of the mutations, we hypothesized that N-terminal insertion during the zymogen to protease transition would be suboptimal. This could be due to the new N terminus not fully inserting into the Ile\textsubscript{16} hydrophobic pocket, or rather the new N terminus could be misaligned or not properly oriented to efficiently facilitate the necessary conformational changes. To discriminate between these possibilities, we subjected each of the proteases to chemical modification using NaN\textsubscript{C}O, which preferentially modifies the N terminus of proteases, whose activities were 5 and 3% of wild-type, respectively, were chosen for further study.

Expression and Purification of Recombinant Proteins—We established high producing stable clones expressing rFX, rFX\textsubscript{A16L}, or rFX\textsubscript{V17A} in HEK 293 cells. Each protein was purified to homogeneity and activated using established procedures (23, 42). SDS-PAGE analysis before and after disulfide bond reduction showed that the proteases were purified to homogeneity and consisted of the characteristic $\alpha$- and $\beta$-forms of FXa (Fig. 1). N-terminal sequence analysis (data not shown) and chemical Gla analysis each yielded the expected results.

Assessment of Active Site Binding: Free FXa—To examine how changes at position 16 or 17 influence the active site, we performed kinetic experiments with several probes. Using either SpecXa, Pefabloc, or pAB, the results showed that both mutants exhibited reduced binding as the $K_m$ or $K_i$ was increased $\sim$10–25-fold relative to wild type (Table 1 and Fig. 2A). We also found that the $k_{cat}$ for Spec Xa hydrolysis was reduced 2- to 4-fold (Table 1). These data indicate that changes to the native N terminus of FXa have a dramatic effect on catalytic function. These results are consistent with the idea that active site formation and N-terminal stabilization are allosterically linked, a finding well established in the trypsinogen/trypsin system (4).

Based on the nature of the mutations, we hypothesized that N-terminal insertion during the zymogen to protease transition would be suboptimal. This could be due to the new N terminus not fully inserting into the Ile\textsubscript{16} hydrophobic pocket, or rather the new N terminus could be misaligned or not properly oriented to efficiently facilitate the necessary conformational changes. To discriminate between these possibilities, we subjected each of the proteases to chemical modification using NaN\textsubscript{C}O, which preferentially modifies the N terminus of proteases, whose activities were 5 and 3% of wild-type, respectively, were chosen for further study.

Expression and Purification of Recombinant Proteins—We established high producing stable clones expressing rFX, rFX\textsubscript{A16L}, or rFX\textsubscript{V17A} in HEK 293 cells. Each protein was purified to homogeneity and activated using established procedures (23, 42). SDS-PAGE analysis before and after disulfide bond reduction showed that the proteases were purified to homogeneity and consisted of the characteristic $\alpha$- and $\beta$-forms of FXa (Fig. 1). N-terminal sequence analysis (data not shown) and chemical Gla analysis each yielded the expected results.

Assessment of Active Site Binding: Free FXa—To examine how changes at position 16 or 17 influence the active site, we performed kinetic experiments with several probes. Using either SpecXa, Pefabloc, or pAB, the results showed that both mutants exhibited reduced binding as the $K_m$ or $K_i$ was increased $\sim$10–25-fold relative to wild type (Table 1 and Fig. 2A). We also found that the $k_{cat}$ for Spec Xa hydrolysis was reduced 2- to 4-fold (Table 1). These data indicate that changes to the native N terminus of FXa have a dramatic effect on catalytic function. These results are consistent with the idea that active site formation and N-terminal stabilization are allosterically linked, a finding well established in the trypsinogen/trypsin system (4).
teins (51). The data did not show any significant difference in the rate of carbamylation between wild-type, rFXa116L, and rFXaV17A (data not shown). This indicates that the N terminus may be inserted into the protein and be protected from chemical modification. Perhaps the N terminus is suboptimally positioned and cannot properly facilitate the zymogen to protease transition in the usual way.

Assessment of Active Site Binding: Prothrombinase—In contrast to the results with free FXa, full saturation of the variants with FVa membranes to form prothrombinase resulted in almost complete rescue of active site binding (Table 1 and Fig. 2B). This was generally consistent with each of the three active site probes, including pAB, which targets the S1 specificity pocket. These data indicate that FVa somehow stabilizes the active site of each variant, thereby improving the affinity for active site-directed probes. Based on these data and our prior observations (23), one interpretation is that N-terminal insertion is allosterically linked to the expression of the FVa and S1 binding sites. To provide evidence for this, initial velocity measurements of Spec Xa cleavage by membrane-bound rFXa116L at different fixed concentrations of FVa were made (Fig. 3), followed by global analysis of all relevant equations describing the binding interactions shown in SCHEME 1. The data indicate that membrane-bound rFXa116L binds with a reduced affinity to FVa (K\textsubscript{d1} = 3.1 ± 0.6 nM) and peptidyl substrates (K\textsubscript{d1} = 1.392 ± 131 μM); however, occupation of the active site with SpecXa restored FVa binding (K\textsubscript{d2} ~ 0.42 nM) and saturating membrane-bound FXa116L with FVa restored peptidyl substrate binding (K\textsubscript{d2} = 192 ± 8.5 μM). The rates of catalysis of rFXa116L in the absence of FVa (k\textsubscript{cat1} = 70 ± 4.6 s\textsuperscript{-1}) or in the presence of saturating concentrations of FVa (k\textsubscript{cat2} = 51 ± 0.5 s\textsuperscript{-1}) are essentially the same, indicating that the binding of FVa to FXa116L does not substantially influence the rate constant for peptidyl substrate hydrolysis. Similar results were obtained with rFXaV17A (data not shown). These data provide evidence that N-terminal insertion is allosterically linked to the forma-

![FIGURE 3. Effect of FVa on peptidyl substrate cleavage by membrane bound rFXa116L. The initial rate of chromogenic substrate cleavage by membrane-bound FXa116L (10 nM; 50 μM PCPS) was determined using increasing concentrations of SpecXa in the presence of different fixed concentrations of FVa (0 nM (A), 1 nM (C), 2 nM (B), 5 nM (D), 8 nM (F), 12 nM (C), 18 nM (E), and 20 nM (F) in assay buffer. The lines are drawn following global analysis of the initial velocity data as described under “Data Analysis.” The data are representative of two similar experiments.](image-url)

![FIGURE 4. Fluorescence (F) measurements of prothrombinase assembly. Reaction mixtures containing 10 nM OG\textsubscript{abs}-FXa, 10 nM FVa, and 50 μM PCPS in assay buffer were titrated with increasing concentrations of rFX (O), rFXaS195A ( ), or rFXaI16L ( ) for panel A, or EGR-rFXa (Q) or EGR-rFXa116L ( ) for panel B. Fluorescence intensity was measured at 25 °C. The lines are drawn following analysis to independent, non-interacting sites, and the fitted values are given in Table 2. The data are representative of two to three similar experiments.](image-url)
expression of the FVα binding site is dependent upon proper stabilization of the new N terminus in the Ile16 cleft. Consistent with the notion that the zymogen to protease transition is linked to the expression of the FVα binding site and active site, we were able to fully rescue FVα binding following covalent modification of the active sites of rFXa116L and rFXaV17A with a peptidyl chloromethyl ketone (EGR-CH2Cl) (Fig. 4B and Table 2). These data show that stabilization of the active site can shift the variants from a zymogen-like state back to the protease-like state thus restoring FVα binding. These data provide evidence that N-terminal insertion is allosterically linked to both catalytic site expression and the formation of the FVα binding site.

Interaction with Na+—We have previously shown that mutation of the Na+ binding site in FXα (rFXaV223P) alters FVα binding and N-terminal insertion (23). Based on those results we speculated that rFXa116L and rFXaV17A should have reduced Na+ binding. To test this we used a chromogenic-based assay to infer binding of Na+ to FXα. To estimate the affinity of FXα and the FXα mutants for Na+, we measured the initial rate of peptidyl substrate hydrolysis at different concentrations of Na+ in the presence of saturating amounts of CaCl2. Using this method, we found that rFXa116L and rFXaV17A bound Na+ with a much lower affinity (estimated values: rFXa116L, $K_d \approx 1673 \pm 283 \text{ mm}$ and rFXaV17A, $K_d \approx 1624 \pm 152 \text{ mm}$) relative to wild-type FXα (rFXα $K_d = 114 \pm 6.6 \text{ mm}$ and PDFXα $K_d = 138 \pm 12.6 \text{ mm}$). This indicates that alteration of the zymogen to protease transition has a major influence on the structural integrity of the Na+ binding site (Fig. 5). Because the Na+ and S1 sites of FXα are known to be thermodynamically linked, proper analysis of binding data needs to take into account all relevant binding interactions (25). However, because the individual binding constants for SpecXα (in the absence of NaCl, data not shown) and Na+ are substantially altered for the variants, we were not able to perform a comprehensive linkage analysis as described by Underwood et al. (25). Thus, the binding constants derived from our experiments should be considered as estimates of the actual values. Additional studies with rFXa116L indicated that high concentrations of Na+ (500 mM) alone ($K_m = 430 \pm 16 \text{ mM}$) or together with saturating concentration of FVα membranes ($K_m = 156 \pm 14 \text{ mM}$) significantly improved binding for SpecXα.

Kinetics of Macromolecular Substrate Cleavage—To further characterize these zymogen-like variants we pursued initial velocity measurements using the macromolecular substrates prothrombin and prethrombin-1. Consistent with results using active site-directed probes, both rFXa116L and rFXaV17A exhibited a marked reduction (40-fold) in prothrombin activation in the absence of FVα relative to wild-type FXα (Fig. 6A). However, saturation of the variants with FVα membranes almost completely rescued (3-fold) the reduced rate of prothrombin activation (Fig. 6B). Further experimentation with the variants assembled in prothrombinase revealed that the variants exhibited a modest reduction in the maximal catalytic rate ($k_{cat}$ reduced $\sim$2–3-fold) with no obvious change in the $K_m$ for prothrombin (Table 3). Overall, these findings are consistent with the chromogenic substrate data as the $k_{cat}$ for Spec Xα hydrolysis was reduced $\sim$2- to 4-fold, whereas the $K_m$ was unchanged when the variants were assembled in prothrombinase. Essentially equivalent results were obtained using the alternative macromolecular substrate, prethrombin-1 (Table 3). These data indicate that the zymogen-like variants are poor enzymes.

| TABLE 2 | Equilibrium binding constants for prothrombinase assembly |
|---------|-----------------------------------------------------------|
| Competitor species | $K_d$ comp $^{ab}$ | $K_d$ fixed $^{d}$ |
| rFXa$^{S195A}$ | 1.34 ± 0.17 | 1.04 ± 0.17 |
| rFXa$^{116L}$ | 13.8 ± 1.07 | 1.22 ± 0.07 |
| rFXa$^{V17A}$ | 7.25 ± 0.65 | 1.04 ± 0.23 |

$^{a}$ Reaction mixtures containing 10 mM OG488-FXα, 50 mM PCPS and either 8 or 10 mM FVα in assay buffer were titrated with increasing concentrations of the competitor species. Fluorescence intensity was recorded as described under “Experimental Procedures.” For simplicity, the primary data in Fig. 4 is shown with only one fixed concentration of FVα.

$^{b}$ Represents the equilibrium association constant of the competitor species.

$^{c}$ Represents the equilibrium dissociation constant of the fixed species (i.e. OG488-FXα).

$^{d}$ Wild-type and rFXa were covalently modified at the active site with EGR-CH2Cl, as described under “Experimental Procedures.”

FIGURE 5. Influence of Na+ on the chromogenic activity of FXα. The initial velocity of peptidyl substrate hydrolysis (SpecXα, 10 μM) catalyzed by free FXα as a function of the NaCl concentration (0–450 mM) as described under “Experimental Procedures.” A, PDFXα (●), rFXα (○), 2 nm final. B, rFXα116L (▲), rFXaV17A (◇), 50 mM final. The solid lines were drawn following analysis of all data sets to a rectangular hyperbola; the fitted parameters are given in the text. The data are representative of two to three similar experiments.
Modulation of the FX Zymogen to Protease Transition

For serine proteases, the zymogen and protease conforma-
tional states exist in equilibrium (4). This equilibrium lies far
to the left when the proteins are unactivated and typically lies
far to the right following activation. Notable exceptions are tis-
sue type plasminogen activator, which has significant activity as
a zymogen (low zymogenicity), and factor VIIa, which fails to
attain its catalytically competent conformation after cleavage of
the Arg198–Lys200 bond (high zymogenicity) (53, 54). For trypsin
and FXa, the zymogen-like conformation is negligibly popu-
lated. In the current study, we directly examined this transition
pathway by generating FXa variants with suboptimal N-termi-
nal insertion and zymogen-like properties. We speculate that
destabilization of internal salt bridge formation by mutagenesis
at positions 16 or 17 has altered the equilibrium position
between the zymogen and the protease. Consistent with this
proposal, both FXa derivatives had altered active site pockets as
evidenced by impaired binding of small peptidyl substrates and
inhibitors. Based on the nature of the mutations it is likely that
the liberated N terminus is not positioned or oriented in the
usual way. Replacing Ile16 with Leu, and Val17 with Ala, could
have altered the electrostatic interaction between residues 16
and 194 or disrupted optimal hydrophobic contacts within the
N-terminal binding cleft, because both interactions contribute
to the transition from the inactive to the active state (12). For
the FXa variants, the reduction in their perceived activity likely
represents a change in the ratio between the zymogen-like and
protease conformations.

Structural differences between the zymogen and the protease
states are primarily found within the activation domain (4). As
a result, binding sites allosterically linked to this region should
be affected by mutations that induce a zymogen-like conforma-
tion. Our experimental findings with rFXa16L and rFXaV17A
support this, because active site binding as well as binding at the
Na+ and FVa binding sites were all substantially altered. The
catalytic site is a well known element of the activation domain,
and the Na+ binding site has been identified by structural stud-
dies and involves two loop segments (183–189 and 221–225)
within this region (55). Although the FVa binding site remains
to be completely defined, it is clear that elements of the activa-
tion domain are also involved. Several groups have shown that
the exosite II region (heparin binding site) and also an α-helix
(163–170) on the catalytic domain contribute to FVa binding
(18, 19, 56). Analysis of the FXa structure indicates that the
putative FVa binding helix is connected to the Na+ binding site
through van der Waals contacts (25). More recent studies have
shown that parts of this helical region appear linked to the S1
and Na+ binding sites (56), lending further support to the idea
that changes in the activation domain will likely impact FVa
binding. Furthermore, mutagenesis studies have shown that the
Na+ binding site and the 185–189 loop are important for FVa
binding (19, 23, 24, 57). It is unlikely, however, that all features
of the activation domain contribute to FVa binding in a direct
fashion. A clear example of this is the binding of tick anticoag-
ulant protein to FXa. Tick anticoagulant protein interacts with
in the absence of FVa membranes, but can efficiently cleave the
physiological substrate prothrombin when assembled into pro-
 thrombinase. Additional experiments were performed to eval-
uate the pathway of prothrombin activation by SDS-PAGE
using established procedures (52). Consistent with the wild-
type enzyme, cleavage of prothrombin by rFXa16L or rFXaV17A
incorporated into prothrombinase gave bands consistent with
sequential cleavage at Arg162 giving rise to meizothrombin as
the intermediate, followed by cleavage at Arg271 to yield throm-
bin (data not shown). These data show that the FXa variants in

in the absence of FVa membranes, but can efficiently cleave the
physiological substrate prothrombin when assembled into pro-
 thrombinase. Additional experiments were performed to eval-
uate the pathway of prothrombin activation by SDS-PAGE
using established procedures (52). Consistent with the wild-
type enzyme, cleavage of prothrombin by rFXa16L or rFXaV17A
incorporated into prothrombinase gave bands consistent with
sequential cleavage at Arg162 giving rise to meizothrombin as
the intermediate, followed by cleavage at Arg271 to yield throm-
bin (data not shown). These data show that the FXa variants in

TABLE 3
Kinetic constants for macromolecular substrate cleavage by
prothrombinase
Steady-state kinetic constants were derived from initial velocity studies conducted
with increasing concentrations of substrate. Concentrations of reaction compo-
ents can be found under “Experimental Procedures.” The errors in the fitted
constants represent  2 S.D. The data are representative of two to three independent
measurements.

|        | Prothrombin | Prethrombin-1 |
|--------|-------------|---------------|
|        | km          | kcat          | km          | kcat          |
|        | 10−3 M      | 10−3 M s−1   | 10−3 M      | 10−3 M s−1   |
| PDFXa  | 0.42 ± 0.02 | 2424 ± 54    | 96          | 5.4 ± 0.3    | 1088 ± 30  | 3.3     |
| rFXa16 | 0.35 ± 0.01 | 1937 ± 26    | 92          | 5.2 ± 0.4    | 1050 ± 35  | 3.4     |
| rFXaV17| 0.31 ± 0.02 | 619 ± 14     | 33          | 5.7 ± 1.8    | 399 ± 10   | 1.2     |
| rFXa   | 0.47 ± 0.03 | 887 ± 26     | 31          | 6.9 ± 0.3    | 388 ± 8.9  | 0.9     |

FIGURE 6. Activation of prothrombin. Reaction mixtures containing 1.4 μM
prothrombin and 50 μM PCPS were incubated at 25 °C in the absence (A) or in
the presence (B) of 20 nm FVa and initiated with FXa 0.1 nm (wild-type FXa) or
0.4 nm (mutant FXa). Aliquots of the reaction mixture were quenched during
the initial rate of the reaction (0, 0.5, 1, 1.5, and 2.0 min), and thrombin
generation was determined by using the chromogenic substrate S-2238. m,
PDFXa; o, rFXa; ▲, rFXa16L; and ◆, rFXaV17A. The data are representative of
two to three similar experiments.
the Na⁺ binding site and autolysis loop, yet positively influences FVa binding (58, 59). A more likely explanation is that some regions contribute directly while other regions are energetically linked; thus changes at one site can impact binding at a related site in an indirect fashion. The data of the current study provide evidence for this and indicate that interpretation of mutagenesis data, attempting to define the FVa binding site or other binding sites involving the activation domain, needs to consider these allosteric effects.

Because the equilibrium position between the zymogen and the protease states determines activity, probes that stabilize the protease conformation should realign the equilibrium position and restore activity. We were able to document this in two different ways. First, saturation of the FXa variants with FVa membranes almost completely restored binding at the active site. Second, covalent modification of rFXaI16L with a peptidyl chloromethyl ketone in the active site completely rescued FVa binding. These findings indicate that the FVa binding site is thermodynamically linked to the active site and the zymogen to protease transition. This conclusion is in line with the observation that the zymogen FX does not bind, or binds very weakly to FVa membranes as documented here and in previous work (20–22). For expression of the FVa binding site, we cannot discern whether this conformational change represents all necessary facets required for FVa binding or whether the large activation peptide present on the zymogen could also alter cofactor binding in some fashion. Nevertheless, the zymogen to protease transition clearly plays an important role in the maturation or stabilization of the FXa activation domain and contributes to the ability of the protease to bind FVa membranes.

A question that emerges from our findings is why linkage between the primary specificity site, FVa binding site, and N-terminal stabilization is not evident using wild-type FXa. We speculate that this is because wild-type FXa is already stabilized in the protease configuration while the zymogen-like FXa variants are not. Thus strong probes such as FVa will only have a minor effect on the active site of wild-type FXa; an established observation in the field (60). The interplay between these binding sites can only be revealed if the equilibrium position between the zymogen and the protease has been altered, as with FXaI16L and FVaV17A.

In addition to changes at the catalytic site and FVa binding site, both zymogen-like variants also exhibited reduced Na⁺ binding. Altered Na⁺ binding is consistent with prior observations with FXaV225P and FIXaV225P, which have destabilized N-terminal regions, altered active sites, and reduced cofactor binding (23, 26). Thus it appears that the rFXaI16L, rFXaV17A, and the Y225P variants may operate in comparable ways by destabilizing the activation domain and inducing a zymogen-like conformation. This would imply that Na⁺ binding to FXa reinforces the activation domain and thus stabilizes the protease conformation. Collectively these findings support the conclusion that the FX zymogen to protease transition drives the formation of the Na⁺ binding site, implying that the zymogen does not bind Na⁺ or does so very weakly. Structural support for this comes from the prothrombin-2-hirugen complex, and the zymogen structure of FVII, which has a Na⁺ site that is not properly formed (55, 61). Additionally, molecular modeling studies comparing FX and FXa suggest that there are major structural differences in the Na⁺ binding site (62).

The notion that the zymogen to protease transition can influence different aspects of protein function in the serine protease family is in line with several previous observations. For example, the zymogen to protease transition has been shown to influence the expression of a receptor tyrosine kinase binding site on hepatocyte growth factor (63), the expression of proexosite I on prothrombin (64), directing the prothrombin activation pathway (52), and the formation or stabilization of a Na⁺ binding site in enzymes such as thrombin (55). These findings together with results of our work indicate that the functional switch between the zymogen and protease states likely plays an important role in protease specificity and function in addition to producing a functional active site.

Although the zymogen-like FXa variants had poor catalytic activity in the absence of FVa, they could efficiently activate prothrombin once assembled in prothrombinase. Based on these differential functional states, we would expect that in a plasma environment these derivatives as free FXa would be refractory to inhibition by protease inhibitors like anti-thrombin III. Furthermore, the variants should not interfere with the initiation of coagulation following vascular damage, because they are not expected to bind tissue factor pathway inhibitor very well. However, whereas the zymogen-like variants of FXa bind FVa more weakly, they are almost completely rescued at sufficiently high cofactor concentrations and catalyze thrombin formation efficiently. Thus, zymogen-like forms of FXa with these properties may act as long-lived proteases in circulation that are otherwise inert but retain the ability to catalyze thrombin formation upon binding to FVa on the activated platelet surface. We speculate that these types of FXa variants may have the potential to serve as therapeutic procoagulants that bypass deficiencies in other clotting factors in the cascade.

In summary, altering the equilibrium position between the FX zymogen and FXa protease states has provided unique insight into macromolecular binding site expression. The allosterically linked structural changes that accompany this conformational transition play a major role in the formation of the primary specificity site as well as the Na⁺ and FVa binding sites. Because of the uniformity of the transition mechanism, our findings have broad implications for better understanding structure/function relationships related to exosite expression and protein complex assembly for coagulation factor proteases.

Acknowledgments—We are grateful to Drs. Sriram Krishnaswamy and Mettine H. A. Bos for useful suggestions and critical review of the manuscript. We also acknowledge Dr. Alex Kurosky and Steven Smith at the University of Texas Medical Branch at Galveston for N-terminal sequence analysis.

REFERENCES

1. Khan, A. M., and James, M. N. G. (1998) Prot. Sci. 7, 815–836
2. Bode, W., Mayr, I., Bauman, Y., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) EMBO J. 8, 3467–3475
3. Freer, S. T., Kraut, J., Roberts, J. D., Wright, H. T., and Xuong, N. H. (1970) Biochemistry 9, 1997–2009
4. Huber, R., and Bode, W. (1978) Acc. Chem. Res. 11, 114–122
