Prothrombinase Assembly and S1 Site Occupation Restore the Catalytic Activity of FXa Impaired by Mutation at the Sodium-binding Site

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Rodney M. Camire‡
From the Joseph Stokes, Jr. Research Institute, Division of Hematology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

Two loop segments (183–189 and 221–225) in the protease domain of factor Xa contribute to the formation of a Na\textsuperscript{+}-binding site. Studies with factor Xa indicate that binding of a single Na\textsuperscript{+} ion to this site influences its activity by altering the S1 specificity site, and substituting the 42nd Annual Meeting of the American Society of Hematology, December 1–5, 2000, San Francisco, CA (70). 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.

Factor X (FX)\textsuperscript{1} is a vitamin K-dependent two-chain glycoprotein that plays a central role in blood coagulation. This serine protease zymogen is a substrate for both the extrinsic (tissue factor/FVIIa) and intrinsic (FVIIIa/FIXa) tenase enzyme complexes, which cleave the Arg\textsuperscript{15}–Ile\textsuperscript{16} scissile bond\textsuperscript{2} in FX, releasing a 52-amino acid activation peptide generating FXa (1). Factor Xa reversibly associates with its cofactor FVa on a membrane surface in the presence of Ca\textsuperscript{2\textsuperscript{+}} ions to form prothrombinase (2). While FXa catalyzes prothrombin cleavage, the macromolecular interactions that stabilize prothrombinase lead to a profound enhancement in catalytic efficiency (2), indicating that prothrombinase, not FXa, is the physiologically relevant enzyme leading to explosive thrombin generation.

Several studies have established that small ligands such as Na\textsuperscript{+} and Ca\textsuperscript{2\textsuperscript{+}} can allosterically modulate the protease domain function of FXa (3–9). Orthner and Kosow (4) demonstrated that FXa is capable of discriminating among monovalent and divalent cations, and both Na\textsuperscript{+} and Ca\textsuperscript{2\textsuperscript{+}} were found to stimulate the cleavage of oligopeptidyl substrates. The location of the Na\textsuperscript{+} and Ca\textsuperscript{2\textsuperscript{+}}-binding sites in the protease domain has been identified through structural studies (10, 11). The Ca\textsuperscript{2\textsuperscript{+}} site in the protease domain is essentially the same as that of trypsin (12), consisting of the Asp\textsuperscript{70}–Glu\textsuperscript{80} loop. Whereas Na\textsuperscript{+} occupies a similar site in FXa as compared with thrombin, the coordinating ligands to Na\textsuperscript{+} differ, employing two loop segments (183–189 and 221–225; residues that are part of the so-called activation domain of serine proteases), including four carbonyl oxygen atoms and two water molecules (11).

It has become apparent over the past several years that metal ion binding sites in the catalytic domain of several serine proteases are allosterically linked to various structural determinants (8, 13–17). For example, two recent studies have shown that the Ca\textsuperscript{2\textsuperscript{+}}- and Na\textsuperscript{+}-binding sites in FXa appear to be allosterically linked (e.g. changes at one site influence the other and vice versa) (9, 18). In addition to linkage between the Na\textsuperscript{+} and Ca\textsuperscript{2\textsuperscript{+}}-binding sites, the S1 specificity site is also allosterically linked to the Na\textsuperscript{+}-binding site but not the Ca\textsuperscript{2\textsuperscript{+}} site (9). Similar results were also recently reported for the anticoagulant protein, activated protein C (19).

Whether Na\textsuperscript{+} binding to the catalytic domain of serine proteases has physiological relevance is open to debate, considering that the plasma concentration of Na\textsuperscript{+} is tightly regulated at \textasciitilde150 mM. It is important to consider, however, that for certain enzymes such as thrombin, the dissociation constant for Na\textsuperscript{+} is close to 150 mM (13), indicating that small changes in the cation concentration or, more likely, changes in the availability of allosteric effector molecules that are linked to the Na\textsuperscript{+}-binding site can dramatically influence enzyme activity (20).

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\textsuperscript{2}To whom correspondence may be addressed: Division of Hematology, 310B Abramson Research Center, The Children’s Hospital of Philadelphia, 34th St. and Civic Center Blvd., Philadelphia, PA 19104. Tel.: 215-590-9968; Fax: 215-590-3660; E-mail: camire@email.chop.edu.

\textsuperscript{3}The abbreviations used are: FX, factor X; FXs, activated FX; FV, factor V; FXa, activated FV; FX, FIX, factor IX; FVIIIa, factor VIII; pdFXa, plasma-derived FXa; rFX, recombinant FX; pdFXa, plasma-derived FXa; rwtFXa, recombinant wild-type FXa; RVV\textsubscript{X,CP}, FX activator from Russell’s viper venom; PAB, 4-aminobenzamidine; SpecXa, methoxycarbonyl-cyclohexylglycyl-glycyl-glycyl-arginine-\textsuperscript{7}-amido-4-methylcoumarin; PTH, phenylthiohydantoin.

\textsuperscript{4}Residue numbers in FX are based upon the chymotrypsinogen numbering system (69).
ported dissociation constant of Na⁺ for calcium-bound APC is ~23 mM, indicating that in vivo most of the APC would be bound to Na⁺ (19). The results with FXa and prothrombinase, however, are less clear. For example, the reported dissociation constant of Na⁺ for calcium-bound FXAs ranges from 43 to 280 mM (9, 18, 21). Results with prothrombinase indicate that the activity of wild-type FXa incorporated into prothrombinase using various monovalent cations was minimally (<1.5-fold) affected by the presence of Na⁺ in the reaction buffer. However, this apparently was not the case using a GLa domainless variant of FXa incorporated into prothrombinase or a GLa domainless variant (GDFXaY225P), since the rate of prethrombin-1 activation in the absence of Na⁺ was dramatically decreased (18). The Tyr²⁷⁵ to Pro variant in GDFXa and thrombin was shown to be essentially insensitive to Na⁺, and it behaves in a similar fashion to the wild-type enzymes assayed in the absence of Na⁺ (15, 18, 22). Reasons for the discrepancy between GDFXaY225P and GDFXa incorporated into prothrombinase in the absence of Na⁺ compared with full-length FXa are currently not clear, but they could relate to unanticipated effects of various monovalent cations on other constituents of prothrombinase or to the nature of GDFXa compared with full-length FXa in the assembly and function within prothrombinase under these conditions.

In the current study, three unresolved questions with respect to the FXa Na⁺-binding site were investigated: 1) does alteration of the Na⁺-binding site affect prothrombinase complex assembly; 2) what is the molecular basis for the reduced affinity of active site-directed probes for FXAs in the absence of Na⁺; and 3) is the FXa Na⁺ binding site allosterically linked to other structural determinants in the protease domain? These questions were approached using a full-length, fully γ-carboxylated derivative of FXa, rFXaY225P. This particular variant of FXa is a useful tool to study the Na⁺-free state of the enzyme, since it has a markedly reduced sensitivity toward Na⁺ (as does thrombin Y225P (22)), thus allowing for assessment of prothrombinase assembly in the presence of physiologically relevant concentrations of this monovalent cation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hepes, sodium chloride, sodium cyanate, and polyethylene glycol (average $M_w = 8000$) were from J. T. Baker Inc. Coomassie Brilliant Blue R-250, calcium chloride, EDTA, Russell’s viper venom, and IEGR-AMC were purchased from Sigma. The fluorophore 4-amino-benzamide (PAB) was from Aldrich. The concentrations of IEGR-AMC and PAB were determined in water using $E_{235} = 17,200$ $M^{-1} \text{cm}^{-1}$ (23) and $E_{222} = 15,000$ $M^{-1} \text{cm}^{-1}$ (24), respectively. The peptidyl substrate methoxy-carbonyl-cyclohexylglycyl-glycyl-arginine-$H_9267$ (S-2765) was purchased from Chromogenix (West Chester, OH). Substrate solutions were prepared in water, and concentrations were verified using $E_{235} = 8270$ $M^{-1} \text{cm}^{-1}$ (25). The affinity resin (AHV-5101-Sept) used to purify human FX from plasma and human thrombin were purchased from Hematologic Technologies (Essex Junction, VT). The calcium-dependent monoclonal human FX antibody (4G3 (26)) was obtained from Dr. Harold James (University of Texas, Tyler, TX). HEK 293 cells were obtained from ATCC (Manassas, VA). Tissue culture reagents were from Invitrogen. L-α-Phosphatidylserine (brain, sodium salt) and L-α-phosphatidylcholine (egg yolk) were purchased from Avanti Polar lipids (Alabaster, AL). Small unilamellar phospholipid vesicles were composed of 75% (w/w) phosphatidylcholine and 25% (w/w) phosphatidylethanolamine (PCPS) were prepared as described previously (27). The concentration of the phospholipid vesicles was determined by phosphorous assay (28).

**Proteins**—Human FX was isolated from fresh frozen plasma essentially as described (29). The FX activator (RVV-X-CP) was purified from chicken egg yolk viiper venom as described (30). Plasma-derived FX was isolated by immunoaffinity chromatography as described by Katzmann et al. (31) with minor modifications. Human FXa was prepared by proteolytic activation of FX by thrombin and purified as described (32). Plasma-derived human antithrombin III, recombinant tick anticoagulant peptide (rTAP) expressed in Ficha pastoris, and recombinant prethrombin-2 expressed in Chinese hamster ovary cells were generous gifts from Dr. Srima Krishnasawmy (The Joseph Stokes Jr. Research Institute, Philadelphia, PA). Molecular weights and extinction coefficients ($E_{235nm}$) of the various proteins were used as taken as follows: RVV-X-CP, 93,000 and 1.18 (33); antithrombin III, 57,500 and 0.62 (34); prethrombin-2, 37,500 and 1.95 (35); thrombin, 37,500 and 1.94 (36); factor X, 50,000 and 1.74 (37); fact X, 54,000 and 1.16 (38).

**Mutagenesis, Expression, and Purification of Recombinant FX**—The recombinant FX mutant Tyr²⁷⁵ → Pro (FXaY225P) was generated with the QuikChange site-directed mutagenesis kit (Stratagene) using two complementary oligonucleotides containing the desired mutation, the 5′-CGGAGGGGAGCCGGGATCTACACC-3′ and 5′-GGGACCGCGGGATCTACAC-3′. The entire FX cDNA was sequenced in order 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 HEK 293 cells and purified as previously described (39, 40).

**Activation of FXa to FXa and Purification on Benzamidine-Sepharose**—Plasma-derived FXa (pFX) and FXa were activated using RVV-X-CP, and subsequently purified using benzamidine-Sepharose or Sephacryl S-200 as described (39, 41). Following purification, plasma-derived and recombinant FXs were precipitated, collected by centrifugation, dissolved in 50% glycerol, and stored at −20°C.

**Characterization of Plasma-derived and rFXa/Xa**—Protein purity was assessed by sodium-dodecyl sulfate (4–12% Bis-Tris) gel electrophoresis using the MES buffer system under reducing (50 mM dithiothreitol, final concentration) and nonreducing conditions followed by staining with Coomassie Brilliant Blue R-250. Gel analysis was carried out according to the modified method of Price (42) for alkaline hydrolysis. Separation of amino acids was accomplished using a DC-4A cation exchange column on a Waters LC-1 Plus high pressure liquid chromatograph (Milford, MA), and quantitation was done by postcolumn derivatization as described by Przybsielecki (43). This analysis indicates that rwtFX and FXaY225P have essentially the full complement of GLa residues (10.5–10.8 mol of GLa/mol of FX) compared with pFX (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 mM (w/v) polyethylene glycol 8000, 2 mM CaCl₂, pH 7.5 (assay buffer) unless otherwise indicated. The kinetics of peptidyl substrate hydrolysis (SpecXa, S-2765, and S-2222) was measured using increasing concentrations of substrate and initiated with either FXa or FXa incorporated into prothrombinase.

**Inhibition of FXa and Prothrombinase by PAB**—The ability of PAB to bind to FXa was assessed by two independent methods. In the first method, the inhibitory constant ($K_i$) of PAB for FXa or prothrombinase was assessed assuming classical competitive inhibition by initial velocity measurements of SpecXa hydrolysis by either enzyme using increasing concentrations of substrate at different fixed concentrations of PAB as previously described (44). In the second method, the binding of PAB to FXa or prothrombinase was directly assayed by fluorescence. Overall dissociation constants for rwtFX binding to FXa and prothrombinase were inferred from measurements of residual enzyme amidolytic activity following incubation of the protease with increasing concentrations of the inhibitor essentially as described (46).

**Inhibition of FXa and Prothrombinase by rTAP and Determination of $K_i$**—Overall dissociation constants for rTAP binding to FXa and prothrombinase were inferred from measurements of residual enzyme amidolytic activity following incubation of the protease with increasing concentrations of the inhibitor essentially as described (46).

**Functional Binding Studies: Measurement of Rates of Thrombin Generation**—Apparent dissociation constants ($K_{app}$) for FXa binding to FVa-PCPS were inferred from initial velocity measurements generated at various FXa concentrations as described previously (47). Assay mixtures contained PCPS (60 μM), FVa (5 μM), recombinant prethrombin-2 (2 μM), and FXa at various concentrations (0.7–5.4 nM) in assay buffer. The kinetic parameters of prothrombinase-catalyzed prethrombin-2 activation ($K_{onapp}$ and $V_{max}$) were determined by measuring the initial rate of thrombin formation at increasing prethrombin-2 concentrations.
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RESULTS

Expression and Purification of Recombinant Proteins—

wrtFX and FXY225P were expressed in HEK 293 cells and purified to homogeneity. Following activation with RVVX-CP, each protein was applied to benzamidine-Sepharose. Whereas native FXa bound and was eluted from the column with 4 mM benzamidine, FXaY225P did not bind to the resin (data not shown). These findings suggest that FXaY225P has a reduced ability to bind benzamidine at the S1 specificity pocket. The mutant protein was subsequently purified by gel filtration. SDS-PAGE analysis of the purifiedzymogens (lanes 1–3) and purified proteases (lanes 4 and 5) before and after disulfide bond reduction are shown in Fig. 1.

Assessment of Binding at the Active Site—As detailed previously, the ability of GDFXaY225P to cleave small peptidyl substrates is impaired, as is that of wild-type FXa in the absence of Na+ (9, 18). Consistent with these observations, an increase in the Kcat for peptidyl substrates and a decrease in the Kcat compared with wild-type FXa was observed for full-length FXaY225P (Table I). These data indicate that the mutant protein has altered activity and suggest that the substrate binding cleft (S1–S3 site) is changed in some fashion. Similar results were obtained with rTFaY225P-bound to PCPS vesicles (data not shown; also see Fig. 3). In contrast to these results, the assembly of FXaY225P into prothrombinase restored the Kcat for peptidyl substrates to that seen with wild-type proteins, whereas the Kcat values were not significantly altered (Table I). At present, it is unclear why the Kcat was reduced by a factor of 1.5–5 for both FXaY225P and this mutant assembled in prothrombinase with the various peptidyl substrates. The data are consistent with the conclusion that prothrombinase complex assembly appears to correct defective binding of peptidyl substrates to the mutant protein.

Since changes in the Kcat for peptidyl substrates could arise from a variety of effects, the binding of a well defined S1 probe, PAB, to FXa was also investigated. Whereas the wild-type proteins bound PAB with essentially identical affinities, FXaY225P had a ~10-fold reduced affinity for PAB, indicating the S1 specificity pocket of the mutant is altered (Table II). These data are consistent with the reduced affinity of PAB observed previously for wild-type FXa in the absence of Na+ (9). In contrast, assembly of the mutant with saturating concentrations of FXa and membranes restored PAB binding to that seen with wild-type FXa assembled in prothrombinase (Table II). Similar results were also obtained with both rTFa and antithrombin III. Together, these results indicate that prothrombinase complex assembly, in the context of an altered Na+–binding site, appears to modify the affinity of active site-directed probes.

Cleavage of the Macromolecular Substrate Prethrombin-2—

Assuming that the substrate exosite is not changed in any fashion, the above data would suggest that rates of cleavage of the macromolecular substrate, prethrombin-2, by FXaY225P assembled in prothrombinase would be similar to wild-type prothrombinase. Initial velocity measurements with saturating concentrations of FXa and membranes indicate that this is indeed the case (Fig. 2). Fitting the data to the Michaelis-Menten equation indicates that the mutant assembled in prothrombinase has similar kinetic parameters for prethrombin-2 (Km = 4.35 ± 0.26 μM; kcat = 0.561 ± 0.1 s−1; kcat/Km = 0.129 μM−1 s−1) compared with wild-type prothrombinase (pdFXa, Km = 8.35 ± 0.69 μM, kcat = 1.56 ± 0.05 s−1, kcat/Km = 0.190 M−1 s−1.)
In this model, FXaY225P and FVa are membrane-bound, and S represents SpecXa. A signal to monitor the various binding interactions was provided by the difference in chromogenic activity between FXaY225P and the mutant saturated with FVa and membranes (Table I). Initial velocity measurements of SpecXa cleavage by membrane-bound FXaY225P at different fixed concentrations of FVa (Fig. 3) were made, followed by global analysis of all relevant equations describing the binding interactions depicted in Scheme I. The data indicate that membrane-bound FXaY225P binds with a decreased affinity to FVa (K_{cat} = 26.0 \pm 5.4 \text{ nm}) and peptide substrates (K_{cat} = 695 \pm 49 \text{ nm}) compared with wild-type FXa; however, occupation of the S1 site restored FVa binding (K_{cat} = 21.6 \pm 0.26 \text{ nm}), and saturating membrane-bound FXaY225P with FVa restored peptidyl substrate binding (K_{cat} = 56 \text{ nm}). These data imply that there is allosteric linkage between the Na^+", FVa, and S1 sites (i.e., the binding of FVa to FXaY225P enhances the binding of molecules that target the S1 site and vice versa). The rates of catalysis of FXaY225P in the absence of FVa (k_{cat} = 43 \pm 1.6 \text{ s}^{-1}) or in the presence of saturating concentrations of FVa (k_{cat} = 41 \pm 0.7 \text{ s}^{-1}) are essentially the same, indicating that

**TABLE I**

**Kinetic constants for the cleavage of peptidyl substrates by factor Xa and prothrombinase**

For experiments in which free factor Xa was used, 2.0 nm wild-type or 6.0 nm mutant factor Xa was incubated with increasing concentrations of substrate, and for experiments in which prothrombinase was employed 5.0 nm wild-type or mutant factor Xa was incubated with 30 nm factor Va, 50 \mu M PCPS, and increasing concentrations of substrate (10–500 \mu M) as described under "Experimental Procedures." The errors in the fitted constants represent 95% confidence limits. Data are representative of two or three similar experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme species</th>
<th>K_m (\mu M)</th>
<th>k_cat (s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free factor Xa</td>
<td>SpecXa</td>
<td>97 \pm 4.4</td>
<td>210 \pm 3.3</td>
</tr>
<tr>
<td></td>
<td>rwtFXa</td>
<td>85 \pm 6.5</td>
<td>198 \pm 18</td>
</tr>
<tr>
<td></td>
<td>rFXaY225P</td>
<td>814 \pm 154</td>
<td>54 \pm 7.1</td>
</tr>
<tr>
<td></td>
<td>S-2222</td>
<td>170 \pm 6.8</td>
<td>100 \pm 1.6</td>
</tr>
<tr>
<td></td>
<td>rwtFXa</td>
<td>148 \pm 9.9</td>
<td>98 \pm 2.4</td>
</tr>
<tr>
<td></td>
<td>rFXaY225P</td>
<td>1607 \pm 37</td>
<td>38 \pm 6.9</td>
</tr>
<tr>
<td></td>
<td>S-2765</td>
<td>50 \pm 6.2</td>
<td>202 \pm 6.8</td>
</tr>
<tr>
<td></td>
<td>rwtFXa</td>
<td>37 \pm 7.4</td>
<td>191 \pm 9.1</td>
</tr>
<tr>
<td></td>
<td>rFXaY225P</td>
<td>1102 \pm 231</td>
<td>139 \pm 22</td>
</tr>
<tr>
<td>Prothrombinase</td>
<td>SpecXa</td>
<td>192 \pm 6.7</td>
<td>220 \pm 3.3</td>
</tr>
<tr>
<td></td>
<td>rwtFXa</td>
<td>181 \pm 13</td>
<td>198 \pm 6.1</td>
</tr>
<tr>
<td></td>
<td>rFXaY225P</td>
<td>174 \pm 6.4</td>
<td>43 \pm 0.7</td>
</tr>
<tr>
<td></td>
<td>S-2222</td>
<td>301 \pm 27</td>
<td>88 \pm 4.1</td>
</tr>
<tr>
<td></td>
<td>rwtFXa</td>
<td>353 \pm 44</td>
<td>91 \pm 6.3</td>
</tr>
<tr>
<td></td>
<td>rFXaY225P</td>
<td>323 \pm 28</td>
<td>20 \pm 0.9</td>
</tr>
<tr>
<td></td>
<td>S-2765</td>
<td>145 \pm 9.9</td>
<td>258 \pm 7.1</td>
</tr>
<tr>
<td></td>
<td>rwtFXa</td>
<td>136 \pm 8.6</td>
<td>242 \pm 6.0</td>
</tr>
<tr>
<td></td>
<td>rFXaY225P</td>
<td>139 \pm 18</td>
<td>58 \pm 2.9</td>
</tr>
</tbody>
</table>

\(\mu M^{-1} s^{-1}; rwtFXa, K_m = 9.07 \pm 0.91 \mu M, k_cat = 1.54 \pm 0.06 s^{-1}, k_cat/K_m = 0.170 \mu M^{-1} s^{-1}\). Consistent with results obtained with peptidyl substrates, the k_cat for macromolecular substrate cleavage by the mutant assembled in prothrombinase was moderately reduced. These data are in contrast to those using GDFXa in the absence of Na^+ or GDFXaY225P, where the rate of prethrombin-1 activation was dramatically impaired (18).

**Assessment of Thermodynamic Linkage**—The data indicate that the binding of FVa to membrane-bound FXaY225P appears to enhance binding of molecules that target the S1 site (Tables I and II). This implies that a thermodynamic linkage exists between the FVa, S1, and Na^-binding sites. Experiments were designed such that all relevant equilibrium binding constants depicted in Scheme I could be simultaneously evaluated.

**TABLE II**

**Inhibition kinetics of synthetic peptidyl substrate cleavage by factor Xa or prothrombinase**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PAB S D</th>
<th>rTAP K_s</th>
<th>Antithrombin III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free FXa</td>
<td>55 \pm 3.3</td>
<td>0.12 \pm 0.04</td>
<td>2.76 \pm 0.13</td>
</tr>
<tr>
<td>rwtFXa</td>
<td>57 \pm 3.7</td>
<td>0.14 \pm 0.04</td>
<td>2.73 \pm 0.15</td>
</tr>
<tr>
<td>rFXaY225P</td>
<td>481 \pm 21</td>
<td>21.3 \pm 2.4</td>
<td>0.22 \pm 0.01</td>
</tr>
<tr>
<td>Prothrombinase</td>
<td>63 \pm 3.3</td>
<td>0.036 \pm 0.003</td>
<td>0.38 \pm 0.29</td>
</tr>
<tr>
<td>rwtFXa</td>
<td>59 \pm 2.4</td>
<td>0.031 \pm 0.003</td>
<td>0.36 \pm 0.15</td>
</tr>
<tr>
<td>rFXaY225P</td>
<td>87 \pm 6.9</td>
<td>0.029 \pm 0.002</td>
<td>0.29 \pm 0.20</td>
</tr>
</tbody>
</table>

\(^a\) Inhibition kinetics of factor Xa alone or prothrombinase were determined from initial velocity studies conducted with the peptidyl substrate, SpecFXa or IEGR-AMC. Details of experimental design and concentrations of reactants can be found under “Experimental Procedures.”

\(^b\) The binding of PAB to the various FXa or prothrombinase species was determined kinetically (K_i) and by fluorescence measurements (K_f). Essentially identical results were found between the two methods. The errors in the fitted constants represent 95% confidence limits. Data are representative of two or three similar experiments.

**FIG. 1**

**SDS-PAGE analysis of purified factor X and factor Xa.** Purified proteins (3 \mu g/lane) were subjected to SDS-PAGE without (A) or with disulfide bond reduction (B) (50 mM dithiothreitol) and visualized by staining with Coomassie Brilliant Blue R-250. Lane 1, human pdFXa; lane 2, rwtFXa; lane 3, FXaY225P; lane 4, human pdFXa; lane 5, rwtFXa; lane 6, FXaY225P. The apparent molecular weights of the standards are indicated on the left.
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**FIG. 2.** Determination of kinetic constants for cleavage of prethrombin-2 by prothrombinase. The initial velocity of thrombin generation was determined at increasing concentrations of prethrombin-2 with 5.0 nM prothrombinase (5.0 nM FXa, 30 nM FVa, and 60 μM PCPS) in assay buffer (pdFXa (●), rwtFXa (▲), or FXaY225P (○)). The data points represent the average of duplicate measurements (S.D. < 10%), and the data are representative of two similar experiments. The lines were drawn following analysis of all data sets to the Henri-Michaelis-Menten equation using the fitted parameters: pdFXa, $K_m = 8.35 \pm 0.69 \mu M$, $k_{cat} = 1.56 \pm 0.05 s^{-1}$; rwtFXa, $K_m = 9.07 \pm 0.91 \mu M$, $k_{cat} = 1.54 \pm 0.06 s^{-1}$; and FXaY225P, $K_m = 4.35 \pm 0.26 \mu M$, $k_{cat} = 0.561 \pm 0.01 s^{-1}$.

**FIG. 3.** Effect of factor Va on peptidyl substrate cleavage by membrane-bound factor XaY225P. The initial velocity of peptide substrate hydrolysis catalyzed by membrane-bound FXaY225P (3 nM; 60 μM PCPS) was determined using increasing concentrations of SpecXa in the presence of different fixed concentrations of FVa (0 nM (●), 0.5 nM (▲), 1.0 nM (Johnson), 2.0 nM (●), 3.0 nM (▲), 5.0 nM (▲), 10.0 nM (●), and 20.0 nM (◇)) in assay buffer. The data are representative of two similar experiments. The lines are drawn following global analysis of initial velocity data as described under “Data Analysis” using the fitted parameters (see Scheme I): $K_{d1} = 26.0 \pm 5.4 nM$, $K_{d2} = 69.5 \pm 49.4 nM$, $K_{d3} = 2.1 \pm 0.32 nM$, $K_{d4} = 56 \pm 19 nM$, $k_{cat1} = 43 \pm 1.6 s^{-1}$; and $k_{cat2} = 41 \pm 0.7 s^{-1}$.

the binding of FVa to FXaY225P does not influence the rate constant for peptidyl substrate hydrolysis.

Additional experiments were also performed aimed at assessing the FXaY225P-FVa interaction using the macromolecular substrate, prethrombin-2. Measurements of the conversion of a single fixed concentration of prethrombin-2 to thrombin were conducted using increasing concentrations of FVa at a single, fixed concentration of FXa and PCPS (Fig. 4). Because of the experimental conditions chosen and the very high $K_m$ of membrane-bound FXa for prethrombin-2 (47), the equilibrium dissociation constant measured in the following experiment is expected to be analogous to $K_{d1}$ depicted in Scheme I. The inferred equilibrium dissociation constant for membrane-bound FVa binding to FXaY225P ($K_{d1} = 17.3 \pm 1.1 nM$) was ∼3-fold greater than that observed with the wild-type proteins (pdFXa, $K_{d1} = 6.2 \pm 0.4 nM$; rwtFXa, $K_{d1} = 6.3 \pm 0.5 nM$; and FXaY225P, $K_{d1} = 17.3 \pm 1.1 nM$), indicating that the loss in activity observed with wild-type FXa (second-order rate constants, 45.0 mM s$^{-1}$ min$^{-1}$ versus 7.50 mM$^{-1}$ min$^{-1}$; Fig. 5, closed symbols) was 6-fold faster compared with wild-type FXa. braces, the rate of disappearance of PTH-Ile 16 decreased by factors 33 compared with wild-type FXa. braces, and sequencing methods (56, 57), it is possible that the reduction in activity of FXaY225P may be unrelated to modification of the N terminus of heavy chain was monitored over time. To control for internal inconsistencies in the sequencing set-up and reaction, an internal protein standard (rTAP) was included, and the values of PTH-Ile$^{16}$ (N terminus of heavy chain of Fxa) to PTH-Tyr (N terminus of rTAP) was expressed as a ratio. Consistent with activity measurements, the rate of carbamylation of FXaY225P was 6.8-fold faster compared with wild-type FXa using the sequencing method (second-order rate constants, 41.0 mM$^{-1}$ min$^{-1}$ versus 6.01 mM$^{-1}$ min$^{-1}$; Fig. 5, open symbols), indicating that the loss in activity correlates very well with modification of the N-terminal Ile$^{16}$.

It is well documented for trypsin that formation of the Ile$^{16}$-Asp$^{194}$ internal salt-bridge is allosterically linked to the S1 specificity site (58). Since FXaY225P appears to have a partially destabilized N-terminal insertion, occupation of the S1 site of FXaY225P should stabilize the Ile$^{16}$-Asp$^{194}$ salt bridge. FXaY225P in the presence of several fixed concentrations of PAB...
Alteration of the FXa Sodium-binding Site

The zymogen to protease transition in the trypsin-like serine protease family of proteins is initiated following proteolytic liberation of a highly conserved N terminus and removal of an activation peptide. As shown for the trypsinogen/trypsin system, a new free α-amino group (Ile16) forms a salt bridge with the Asp194 carboxylate group following activation that either results in or is associated with a conformational change in the so-called “activation domain” (residues comprising positions 16–19, 142–152, 184–194, and 216–223) (59, 60). Since N-terminal insertion is energetically linked to the maturation of the activation domain, alteration of any of these structural elements including the S1 specificity pocket should positively or negatively influence the others.

The results of the current study indicate that alteration of the FXa Na- binding site by changing residue 225 from a Tyr to a Phe results in the transformation of this serine protease from a zymogen-like state to a zymogen-like state, providing a reasonable explanation for the observations of reduced activity (this study and Ref. 18). This shift in favor of the zymogen-like state results from destabilization of the N terminus (Ile16) of FXaY225F and adversely affects FVa binding and binding of substrates to the S1 specificity pocket. Consistent with thermodynamic principles, protection of the N-terminal Ile16 from modification was achieved by saturation of the S1 specificity site with PAB, thereby favoring the active or protease-like conformation (Fig. 6). Additionally, saturation of membrane-bound FXaY225F with FVa also assisted the variant to adopt a more protease-like state, suggesting that the FVa and Na- binding sites are allosterically linked (Figs. 2–4). These findings are consistent with the well-documented zymogen to protease transition in trypsinogen/trypsin (61) (i.e., the zymogen state and the protease state exist in an equilibrium that can be shifted depending on environmental conditions (e.g., various ligands)).

At present, it is not clear whether the activity of free FXa is modulated by Na+ in vivo, since there are conflicting reports on the dissociation constant of Na+ for calcium-bound FXa (9, 18, 21). Additionally, it is unlikely that the levels of Na+ would change significantly enough to influence the activity of FXa, since the Na+ concentration is tightly regulated. However, if Na+-bound and Na- free forms of FXa are equally populated in vivo, then these forms of the enzyme would be sensitive to changes in the availability of allosteric effector molecules that are linked to the Na- binding site. These changes could influence a variety of reactions in which FXa participates (e.g., activation of FV, FVII, cellular receptors, and interactions with TFPI and ATIII). With respect to prothrombin activation, the current data indicate that once membrane-bound FXaY225F is saturated with FVa, the rate of macromolecular substrate cleavage is similar to native prothrombinase, calling into question the physiological significance of Na+ in influencing this reaction. However, as the catalytic activity of prothrombinase appears to be insensitive to changes at the FXa Na- binding site (Fig. 2), the binding of membrane-bound FXaY225F to FVa is adversely affected (Figs. 3 and 4). Thus, whereas the overall rate of thrombin generation by prothrombinase may be influenced by changes at the FXa Na- binding site, the degree of this change would depend upon the amount of FVa available and possibly other factors that could influence the equilibrium between the two forms of the enzyme.

The FXa Na+ and S1 specificity sites are part of the so-called activation domain and would presumably be disordered in the

FIG. 5. Carbamylation of FXa and FXaY225F by NaNCO. Reaction mixtures containing 2.0 μM of plasma-derived FXa (squares) or FXaY225F (circles) in assay buffer adjusted to pH 7.0 were reacted with 0.2 mM NaNCO. At selected time intervals (0, 15, 30, 60, 90, 120, 150, 210, 240, 270, and 300 min), an aliquot of the reaction mixture was either mixed with assay buffer and the remaining enzymatic activity was determined from initial steady state rates of SpecXa hydrolysis (closed symbols) or aliquots were mixed with 1.5 mM hydroxylamine, pH 8.0, and then frozen at −80 °C for N-terminal sequence analysis as described under “Experimental Procedures” (open symbols). The percentage of residual activity or signal remaining was plotted as a function of time upon incubation with NaNCO. Data derived from activity measurements (closed symbols) are representative of four similar experiments, whereas data derived from the sequence analysis (open symbols) represent a single experiment. The lines are drawn according to rate expressions described under “Data Analysis” using the fitted parameters: pdFXa, 7.50 ± 1.49 min−1; FXaY225F, 45.0 ± 1.58 min−1; and pdFXa, 6.01 ± 0.58 min−1; and FXaY225F, 41.0 ± 7.3 min−1 (open symbols).

was reacted with NaNCO over an extended time course (Fig. 6A). Global analysis of all data sets to equations describing all relevant interactions depicted in Scheme II (where P represents PAB) permitted the determination of the equilibrium dissociation constant (Kd) for PAB-FXaY225F and the second order rate constants for the modification of FXaY225F by NaNCO in the absence (k0) and presence (k1) of saturating PAB (“FXaY225F indicates that it has been covalently modified at the N terminus with NaNCO). Inspection of the observed rate constants (kobs, derived from Fig. 6A) as a function of the PAB concentration (Fig. 6B) indicates that the interaction of FXaY225F with PAB is relatively weak (Kd = 726 ± 53 μM), results that are in agreement with direct binding fluorescence and kinetic measurements (Table II). Additionally, saturation of FXaY225F by PAB afforded complete protection of the enzyme from carboxylation, as evident by an extremely small second order rate constant (k1 = <1 × 10−6 min−1) compared with FXaY225F in the absence of PAB (k0 = 39.0 ± 1.28 min−1). These data indicate that occupation of the S1 site of the variant results in stabilization of the N-terminal insertion and resistance of the enzyme to carboxylation. These results also support the idea that the reduction in activity observed with FXaY225F in the presence of cyanate is related to modification of the N terminus, since the loss in activity could be reversed upon occupation of the S1 site (Fig. 6).

**Scheme 2**

\[
\text{FXaY225F} + P + \text{NaNCO} \xrightarrow{k_4} \text{FXaY225F-P + NaNCO} \\
\xrightarrow{k_0} \text{*FXaY225F} + \text{NaNCO + P} \\
\xrightarrow{k_1} \text{*FXaY225F-P-NaNCO}
\]
zymogen and become ordered upon N-terminal insertion. A recently described model of the zymogen FX suggests that this may be the case (62). Comparison of the zymogen model with the active enzyme reveals that residues making up the calcium (Asp70–Glu150), sodium (Ala206–Asp214; Gly219–Gly226) and autolysis loops (Thr144–Arg150) undergo major changes in their backbone positions upon the zymogen to protease transition. These results suggest that these regions in FX are at least influenced by this transition. Since it is already well documented, at least for trypsinogen/trypsin, that the S1 specificity site and formation of Ile16–Asp194 are allosterically linked, it is reasonable to hypothesize that other elements of the activation domain are also linked to the zymogen to protease transition. By definition, this means that alterations at the Na+–binding site, for example, will influence the formation of the Ile16–Asp194 salt bridge, consistent with observations of the current study (Figs. 5 and 6).

The idea that changes at the Na+–binding site can influence ion pair formation is in agreement with recent findings indicating that an increase in the pH results in a decrease in the Na+ affinity for several serine proteases, including FXa (63). It was hypothesized that deprotonation of an ion pair (pK_a ~ 9.2; assumed, but not proven to be Ile16–Asp194) by changes in pH will result in a break of the salt bridge and yield an internal disruption of the Na+–binding site. Whereas it is clear that large changes in the pH capable of disrupting ion pair formation cannot be a mechanism by which the FXa Na+–binding site is modulated in vivo, the study nevertheless provides evidence that formation of the Ile16–Asp194 salt bridge appears crucial in the stabilization of the architecture of the Na+–binding site.

Thus, a key aspect of the zymogen to protease transition for serine proteases that are sensitive to Na+ may be the formation of a functional Na+–binding site. This model would predict that the Na+–site of the zymogen binds the cation with a very weak affinity; high affinity binding would follow activation of the zymogen and ordering of the Na+–site. Support for this idea comes from the current study as well as other studies. For example, inspection of the crystal structure of the homologous zymogen prethrombin-2 in complex with hirugen reveals that, in contrast to thrombin, there are several key movements of loop segments and critical groups in the vicinity of the Na+–site that would prevent Na+ binding to the zymogen, consistent with its apparent absence in the prethrombin-2 structure (11, 64). The same also appears to be true with the recently described FVII zymogen structure, which demonstrates that loop regions making up the Na+–binding site (184–193 and 215–224) are shifted up to 12 Å compared with the tissue factor/FVIIIa structure (65). Additionally, using a direct fluorescence binding assay, De Cristofaro et al. (16) were not able to detect Na+ binding to protein C, suggesting that the Na+ site in this zymogen is exposed only after its activation.

In addition to disruption of the N-terminal insertion and S1 specificity pocket, mutation at the Na+–site also results in decreased cofactor binding, suggesting that the FVAs binding site is allosterically linked to elements in the activation domain. The data indicate that occupation of the S1 specificity site of membrane-bound FXaY225P enhances binding at the FVAs binding site and vice versa, explaining why these two enzymes (FXaY225P and mutant prothrombinase) have different reactivities toward active site-directed probes (Tables I and II; see Scheme I). In contrast to these observations, the binding of membrane-bound FXa to wild-type FXa is accompanied by a minor change in active site function (66, 67). A possible explanation for this is that linkage between the FVAs and S1 binding sites is obscured in wild-type FXa, because the protease-like conformation is highly favored. In contrast, FXaY225P, with an altered Na+–binding site, exists in a zymogen-like conformation, thereby making linkage between the FVAs and S1 sites more discernible. This explanation would appear consistent with the observation that the zymogen FX does not detectably bind FVAs or S1-directed probes.

A possible alternative explanation to allosteric linkage is that steric factors are influencing the affinity of FVAs for membrane-bound FXaY225P. For example, disruption of the Ile16–Asp194 salt bridge in FXaY225P could result in movement of the N-terminal segment such that it sterically hinders the binding of FVAs to membrane-bound FXaY225P. Implicit in this argument is that the enzyme exists in two states, one in which the N terminus blocks the FVAs binding site and one in which the site is accessible. Any molecule that facilitates the formation of the Ile16–Asp194 salt bridge (e.g., binding of S1 probes) would shift the equilibrium between the two states, removing this
steric factor and restoring FVa binding. At present, this scenario cannot be formally ruled out, and future studies are needed to distinguish between the two possibilities.

Examination of the FXa crystal structure in the vicinity of the putative FVa binding helix (68), Na⁺ site, and S1 specificity site may provide, however, some insight into how these elements could be allosterically linked at the structural level. As previously noted (9, 11), the Na⁺ site in FXa is linked to the S1 specificity site (specifically Asp189) via two water molecules. The FVa binding helix (residues 162–169) is connected to the Na⁺-binding site through van der Waals’ contacts between Tyr225 and Val163 and between the OH group of Tyr225 and (via two water molecules) Ser167. When the FXa Tyr225 to Pro mutation was modeled (9) the results indicate that its carbonyl oxygen atom would point away from the Na⁺-binding site and that its side chain would be unable to make contacts with the FVa binding helix. The data of the current study are consistent with this model. Future experiments aimed at preventing internal salt bridge formation should provide further evidence that the FVAs, S1, and Na⁺-binding sites consist of a structural network allosterically linked to the zymogen to protease transition.

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