The Critical Role of the 185–189-Loop in the Factor Xa Interaction with Na\(^+\) and Factor Va in the Prothrombinase Complex*

Received for publication, August 30, 2004, and in revised form, September 2, 2004
Published, JBC Papers in Press, September 3, 2004, DOI 10.1074/jbc.M409964200

Aliereza R. Rezaie\(\text{*}\) and Farooq Ahmed S. Kittur

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

Factor X is a vitamin-K-dependent serine protease zymogen in plasma that upon activation to factor Xa (fXa) forms a high affinity complex with other components of the prothrombinase complex (factor Va, negatively charged phospholipid vesicles, and calcium) to activate prothrombin to thrombin during the blood coagulation process (1–5). The catalytic efficiency of fXa in the prothrombinase complex is five orders of magnitude higher than that of fXa alone (1, 2). Most of the accelerating effect of the prothrombinase complex assembly is attributed to the cofactor function of factor Va (fVa), which can bind to and alter the kinetic properties of fXa by inducing conformational changes in the structure of the protease and also by providing secondary binding sites for the substrate prothrombin in the activation complex (6–10). The molecular details of the fXa interaction with fVa have not been fully elucidated. However, recent results have indicated that the mutagenesis of the basic residues of the heparin binding 162-helix on the protease domain (especially Arg\(^{165}\) in the chymotrypsinogen numbering system (11)) dramatically impairs the ability of fXa to interact with fVa in the prothrombinase complex (12). In addition to the 162-helix, other basic residues of the heparin binding site of fXa have been implicated in fVa binding (12, 13); however, the exact contribution of these residues to the recognition mechanism of the protease-cofactor interaction has not been studied in detail.

Similar to other coagulation proteases, fXa is an allosteric enzyme, and thus the binding of metal ions and protein cofactors to several solvent exposed surface loops near or remote from the catalytic pocket can modulate the activity and specificity of the protease. For instance, the binding of both Ca\(^{2+}\) to the 70-loop and Na\(^{+}\) to the 225-loop is known to markedly promote the activity of fXa toward synthetic substrates and natural target inhibitors (14, 15). Recent thermodynamic linkage analyses have indicated that both of these metal ion binding sites are energetically linked in fXa (15–17). Moreover, the same studies have demonstrated that the Na\(^{+}\) and the S1 sites of fXa are also allosterically linked (16, 17). The S1 site of fXa and other coagulation proteases is a conserved Asp residue at position 189 that is known to interact with a basic residue of all substrates and inhibitors and thus is responsible for determining the P1-Arg specificity of these proteases (11, 18). The S1-Asp\(^{189}\) of coagulation proteases is located on an exposed surface loop (185–189-loop) immediately below the Na\(^{+}\)-binding 225-loop (18, 19). This loop has several non-conserved residues that are unique for each coagulation protease. In the case of thrombin, a recent mutagenesis study showed that the charged residues of this loop are critical for the catalytic activity and the monovalent cation binding specificity of the protease (20). The same loop in fXa has three charged residues, Asp\(^{185}\), Lys\(^{186}\), and Glu\(^{188}\), with unknown functions (18). In this study, we substituted these residues with Ala in three separate constructs and expressed the mutants in mammalian cells. The characterization of these mutants in appropriate kinetic assays revealed that both Asp\(^{185}\) and Lys\(^{186}\) alter the Na\(^{+}\) affinity of fXa, suggesting that they are allosterically linked to the metal ion binding site of the protease. The ability of the Lys\(^{186}\) mutant to bind to the S1 site specific probe p-aminobenzamidine was also dramatically impaired. Moreover, both kinetic and equilibrium binding data suggested that Lys\(^{186}\) is a binding site for fVa, as shown by an ~6-fold impaired affinity of the...
mutant for the cofactor. The Lys^{186} mutant also exhibited a dramatic impairment in amidoletic and proteolytic activity and reactivity with antithrombin (AT), tissue factor pathway inhibitor (TFPI), and recombinant tick anticoagulant peptide (rTAP). Interestingly, IVa restored the proteolytic defect of the K186A mutant in both prothrombin activation and rTAP binding assays; however, the cofactor did not influence other activities of the mutant. The defective activity of the K186A mutant was also partially corrected at 0.5 mM NaCl. Taken together, analysis of these results suggests that IVa, Na^+, and S1 sites of fXa are allosterically linked. Moreover, there appears to be a similarity between the fXa-fVa interaction with prothrombin and rTAP. Because the crystal structure of the fXa-rTAP complex is known (21), the interaction of the fXa mutant with the inhibitor can be used as a model system to understand how the cofactor may function in the prothrombinase complex during prothrombin activation.

EXPERIMENTAL PROCEDURES

\textbf{Mutagenesis and Expression of Recombinant Proteins—}The expression of wild-type and conditions of K293 cells described previously (22). The factor X mutants in the chymotrypsinogen numbering (11) Asp^{186} → Ala (D185A), Lys^{186} → Ala (K186A), and Glu^{188} → Ala (E188A) corresponding to residues Asp^{408}, Lys^{411}, and Glu^{12} in the factor X cDNA numbering (23) were generated in the same expression/purification vector system by standard PCR mutagenesis methods as described (22). After confirmation of the accuracy of the mutagenesis by DNA sequencing, the constructs were introduced into HEK293 cells and the mutant proteins were isolated from 20 liters of cell culture supernatants by a combination of immunoaffinity and ion exchange chromatography using the HPC4 monoclonal antibody and a Mono Q ion exchange column as described (22, 24). The fully glycosylated proteins were eluted from the ion exchange column at 0.4 mM NaCl as described previously (24).

Active-site-directed serine protease probe, PAB, was determined by DNA sequencing, the constructs were introduced into HEK293 cells and the mutant proteins were isolated from 20 liters of cell culture supernatants by a combination of immunoaffinity and ion exchange chromatography using the HPC4 monoclonal antibody and a Mono Q ion exchange column as described (22, 24). The fully glycosylated proteins were eluted from the ion exchange column at 0.4 mM NaCl as described previously (24).

\textbf{Interaction with TFPI and rTAP—}The ability of fXa derivatives to bind either TFPI or rTAP was determined by incubating each fXa (1–2 nm) with different concentrations of TFPI or rTAP (0.2–25 nm) in TBS/Ca^2+ in 80 μl volumes in 96-well polystyrene assay plates at room temperature. The dissociation constants were estimated by nonlinear regression analysis of data using the quadratic equation describing the tight binding interactions as described (27).

\textbf{Inhibition by PAB—}The affinity of fXa derivatives for interaction with the active-site directed serine probe protease, PAB, was determined by incubating each protease (1–3 nm) with different concentrations of PAB (0–6.4 mM) in the presence of different fixed concentrations of S2765 (50 μM) as described (23). The dissociation constants were measured from the cleavage rate of the chromogenic substrate as described above, and the K_i values were determined by global fitting of data to a competitive binding equation as described (28).

\textbf{Activation of Prothrombin—}The initial rate of prothrombin activation by the wild-type and mutant fXa derivatives was measured in both the absence and presence of factor Va as described (22, 24). In the absence of the cofactor, the concentration dependence of prothrombin activation was studied by incubating each fXa derivative (5–10 nm) with increasing concentrations of the zymogen (20–2500 nM) on PC/PS vesicles (25 μm) at room temperature. The initial rate of thrombin generation was monitored at 405 nm by a V_max kinetic microplate reader (Nalge Nunc International, Rochester, NY). The rate of thrombin generation in each activation reaction was determined from a standard curve prepared from the cleavage rate of S2238 by known concentrations of thrombin under exactly the same conditions.

In the presence of the cofactor, first the apparent affinity of fXa derivatives for binding fVa was evaluated in a prothrombinase assay as described (22, 24). Briefly, fXa (0.1 nm) was mixed with varying concentrations of human fVα (0–20 nm) on PC/PS vesicles (10 μm) in TBS/Ca^{2+} at room temperature. Following 5–10 min incubation, small aliquots of the activation reactions were transferred into wells of a 96-well assay plate containing S2305 (100 μM in TBS/Ca^{2+} at room temperature). The rate of hydrolysis was measured at 405 nm by a V_max kinetic microplate reader. The concentration of thrombin generated in each activation reaction was determined from a standard curve prepared from the cleavage rate of S2238 by known concentrations of thrombin under exactly the same conditions.

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was added to a final concentration of 20 mM, and the concentrations of thrombin generated were determined by an amidolytic activity assay as described above. It was ensured that less than 15% of prothrombin was activated at all concentrations of the substrate.

**Fluorescence Anisotropy Measurements**—The affinities of fXa derivatives for interaction with fVa were evaluated by their ability to displace OG₄₈₈-EGR-fXa from the bound fVa at equilibrium as monitored by a decrease in the fluorescence anisotropy of the labeled fXa-fVa complex using an Aminco-Bowman series 2 spectrophotometer (Spectronic Unicam, Rochester, NY). First, the equilibrium dissociation constant (Kᵰ) for the interaction of OG₄₈₈-EGR-fXas with factor Va was determined by titrating increasing concentrations of fVa (1–50 nM) with a fixed concentration of the labeled fXa (10 nM) on PC/PS vesicles (10 μM) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, containing 0.1% PEG 8000 and 5 mM CaCl₂. The excitation and emission wavelengths were set at 490 and 520 nm, respectively. The bandwidths were set at 8 nm for both excitation and emission. The titration was performed by the addition of 1–2 μl of stock solution of fVa (1–4 μM) to the labeled fXa in a quartz cuvette in 1-ml volumes at 25 °C. The titration volume did not exceed 2% of the total volume of the reaction. The increase in the anisotropy of OG₄₈₈-EGR-fXas upon interaction with fVa was recorded and plotted as a function of the cofactor concentrations. The Kᵰ value for the interaction of the labeled fXa for fVa was calculated by nonlinear least-squares computer fitting of the data by the quadratic binding equation as described (29).

The affinity of the mutants for interaction with fVa was then evaluated by their ability to decrease the anisotropy of the OG₄₈₈-EGR-fXa-fVa complex in a competitive titration assay. The competitive assay was carried out by the addition of small aliquots of increasing concentrations of the wild-type or mutant fXa derivatives to a fixed concentration of OG₄₈₈-EGR-fXa (10 nM) and factor Va (25 nM) in a 1-ml volume, monitoring the decrease in the fluorescence intensity of the reaction upon the displacement of the labeled fXa from the cofactor. Data were collected for up to 50 nM fXa, and the Kᵰ values were estimated using a cubic equation as described (30).

**RESULTS**

**Amidolytic Activities**—The initial characterization of the fXa mutants indicated that with the exception of E188A, both D185A and K186A exhibit impaired amidolytic activities toward three chromogenic substrates (SpFXa, S2765 and S2222) in TBS/CA²⁺. Noting that previous mutagenesis of the homologous loop of thrombin altered the monovalent cation specificity of the protease (20), we decided to determine the kinetic parameters for the hydrolysis of SpFXa in a buffer containing three different monovalent cations. The results presented in Table I suggested that among the three monovalent cations tested, all fXa mutants retained their preference for Na⁺, as judged by the higher specificity constants (kₐp/Kᵰ) for all three mutants in the presence of Na⁺. However, in the presence of all three metal ions, the amidolytic activities of both D185A and K186A mutants were impaired. Thus, in the Na⁺-containing buffer, a less than 2-fold decrease in both the Kᵰ and kₐp values of the substrate hydrolysis was observed with the D185A mutant, and a dramatic decrease of ~51-fold in the specificity constant was observed with the K186A mutant (Table I). The primary defect in substrate hydrolysis by K186A was in the Kᵰ parameter, suggesting that the chromogenic substrate cannot effectively bind to the active site pocket of the mutant protease. A close examination of the kinetic data presented in Table I further suggested that the 3-fold impairment in the specificity constant of D185A in the presence of Na⁺ was decreased to a less than 2-fold decrease in the presence of K⁺ and nearly no difference was observed in the presence of Li⁺. This is derived from the observation that both wild-type and D185A fXa cleaved the chromogenic substrate with comparable specificity constant in the presence of Li⁺ (Table I). Similarly, the ability of the K186A mutant to discriminate between the monovalent cations was some how diminished.

**Kₐp(app) for Interaction with Na⁺**—Previous results have indicated that the 185–189-loop and the Na⁺-binding 225-loop of thrombin are energetically linked (20). This may also be true in fXa, and thus the underlying cause of the defect of the amidolytic activity of fXa mutants may be caused by their altered interaction with the Na⁺ ion. To test this possibility, the apparent dissociation constants for the interaction of the metal ion with fXa mutants were evaluated by an amidolytic activity assay using three different chromogenic substrates (Fig. 1). Indeed, both D185A and K186A mutants exhibited impaired affinity for Na⁺. Thus, in contrast to a Kₐp(app) value of ~120–165 mM for the Na⁺ interaction with wild-type fXa, the corresponding value for the D185A mutant (~275–365 mM) was impaired ~2-fold (Fig. 1 and Table II). The impairment in the ability of the K186A mutant to interact with Na⁺ was so dramatic that no Kₐp(app) value could be estimated for this mutant because the amidolytic activity of the mutant remained linear for up to 800 mM Na⁺ with all three chromogenic substrates (Fig. 1). These results suggest that similar to thrombin, the conformations of the 186-loop and the 225-loop are allosterically coupled, and thus the dramatic impairment in the amidolytic activity of the K186A mutant may also be caused by its inability to effectively interact with Na⁺. These results are also in agreement with previous thermodynamic linkage analysis that demonstrated that the binding of Na⁺ to the 225-loop of fXa allosterically modulates the specificity of the S1 site (Asp¹⁸⁶) interaction with the P1-Arg of substrates (16, 17). To determine whether the mutagenesis of the 186-loop influences the S1 site specificity of the mutants, their ability to bind to the S1 site specific probe of the trypsin-like serine proteases PAB was examined. As shown in Table III, the inhibition profiles of the mutants closely paralleled their amidolytic activities. Thus, the Kᵰ value for the D185A mutant interaction with PAB was impaired ~2-fold, and the corresponding value with the K186A mutant was impaired ~32-fold, suggesting that the mutagenesis has altered the conformation of the S1 site of the mutant proteases.

**Inhibition by AT and TFPi**—Similar to the amidolytic activity and the interaction with Na⁺, the K186A mutant exhibited marked impairments in its reactivity with AT and affinity for TFPi (Table III). The effect of the mutagenesis in the reactivity of the K186A mutant with AT and TFPi also closely paralleled the decrease in the specificity constant of the mutant toward SpFXa (Table I) and PAB (Table III) further suggesting that the altered interaction of both inhibitors with the S1 site of fXa

### Table I

| Substrate | Kᵰ (μM) | kₐp (10⁻⁶ M⁻¹ s⁻¹) | kₐp/Kᵰ (10⁻⁶ M⁻¹ s⁻¹) |
|-----------|---------|---------------------|-----------------------|
| SpFXa     | Na⁺     | 69.0 ± 4.0          | 141.6 ± 2.5           | 2.05 ± 0.16          |
|           | K⁺      | 244.9 ± 6.0         | 137.8 ± 1.2           | 0.56 ± 0.02          |
|           | Li⁺     | 442.6 ± 22.4        | 101.2 ± 2.2           | 0.23 ± 0.06          |
| D185A     | Na⁺     | 130.0 ± 4.6         | 92.5 ± 1.0            | 0.71 ± 0.03          |
|           | K⁺      | 289.4 ± 10.7        | 94.9 ± 1.3            | 0.33 ± 0.02          |
|           | Li⁺     | 428.1 ± 22.7        | 81.9 ± 2.0            | 0.19 ± 0.01          |
| K186A     | Na⁺     | 2200 ± 200          | 89.0 ± 5.5            | 0.04 ± 0.006         |
|           | K⁺      | 3500 ± 300          | 53.1 ± 3.0            | 0.02 ± 0.003         |
|           | Li⁺     | 4700 ± 200          | 57.8 ± 2.2            | 0.01 ± 0.001         |
| E188A     | Na⁺     | 60.6 ± 3.9          | 145.6 ± 2.5           | 2.4 ± 0.02           |
|           | K⁺      | 2347 ± 6.3          | 137.7 ± 1.4           | 0.59 ± 0.02          |
|           | Li⁺     | 462.4 ± 32.9        | 115.2 ± 3.9           | 0.27 ± 0.03          |
may primarily be responsible for the observed defect in the activity of the fXa mutant.

Interaction with Factor Va and Prothrombinase Activity—The $K_{d(app)}$ value for the interaction of the fXa mutants with fVa was evaluated in a prothrombinase assay. It was found that the ability of the mutants to interact with fVa in the prothrombinase complex has been altered. Although the alteration in the observed affinity for the cofactor was minimal for both D185A and E188A mutants, there was a significant ~6-fold impairment in the $K_{d(app)}$ value of the K186A interaction with the cofactor (Table IV). The catalytic activity of fXa derivatives toward prothrombin in both the absence and presence of a saturating concentration of fVa is presented in Fig. 2 and Table IV. Both D185A and E188A mutants activated prothrombin with a $K_{m(app)}$ value that was similar to that observed for the wild-type fXa; however, the corresponding value for the K186A mutant was elevated by an order of magnitude (Fig. 2A and Table IV). Moreover, the $k_{cat}$ values with both D185A and K186A mutants were impaired ~3-fold (Table IV). Interestingly, the catalytic defect of K186A toward prothrombin was completely restored in the presence of a saturating concentration of fVa, as shown by the comparable kinetic parameters obtained for both wild-type and mutant fXa in the presence of the cofactor (Fig. 2B and Table IV). On the other hand, the $k_{cat}$ for the activation of prothrombin by D185A remained ~2-fold lower than wild-type fXa in the presence of the cofactor. Prothrombin activation by E188A was normal in both the absence and presence of fVa (Fig. 2 and Table IV).

In light of the ability of fVa to restore the catalytic activity of the K186A mutant toward prothrombin, the ability of the cofactor to overcome the catalytic defect of the mutant in other fXa reactions was also examined. It is known that rTAP binds to both the active-site and secondary exosites of fXa to inhibit the protease with a $K_i$ value of ~0.2 nM (31). Moreover, fVa is shown to function as a cofactor to improve the affinity of rTAP for binding to fXa (32). Similar to all other reactions, the affinity of rTAP for interaction with the K186A mutant was dramatically impaired. Thus, in contrast to a $K_i$ value of 242 pm for wild-type fXa, the corresponding value was 8.0 nM for K186A, suggesting a 33-fold impairment in the affinity of the mutant for interaction with the inhibitor. Interestingly, the mutant also interacted with rTAP in the presence of fVa with a normal $K_i$ value of 268 pm suggesting a 30-fold cofactor effect for fVa in the interaction (Fig. 3A). In the case of the wild-type fXa, the cofactor enhanced the affinity of rTAP ~5-fold, yielding a $K_i$ value of 52 pm for the protease-inhibitor interaction (data not shown). Surprisingly, fVa did not restore the catalytic defect in the activity of the K186A mutant in any other fXa reactions described above, suggesting that certain aspects of the recognition mechanism of prothrombin and rTAP by the fXa-fVa complex are shared. It should be noted that the affinity of D185A and E188A for interaction with rTAP was not impaired but improved 2~3-fold, and the effect of fVa on the inhibitor interaction with these mutants was not evaluated.

To examine whether (similar to fVa) higher concentrations of Na$^+$ can restore the catalytic defect of the K186A interaction with rTAP, the $K_i$ value for the inhibitor was also determined in 0.5 M NaCl. Interestingly, relative to that in 0.1 M NaCl, the $K_i$ value for the interaction of the mutant in 0.5 M Na$^+$ (~2 nM) was improved ~4-fold (Fig. 3B). These results confirm the hypothesis that the 186-loop is energetically linked to the Na$^+$ site of fXa and that the disruption of the Na$^+$ site is responsible.

### Table II

| Substrate | SpFXa | S2765 | S2222 |
|-----------|-------|-------|-------|
| fXa       | 163.8 ± 15.0 | 134.7 ± 20.2 | 120.7 ± 10.2 |
| D185A     | 363.0 ± 25.4 | 290.5 ± 19.2 | 274.3 ± 18.5 |
| K186A     | ND     | ND    | ND    |
| E188A     | 161.3 ± 10.6 | 123.7 ± 8.0 | 116.1 ± 8.8 |

The $K_{d(app)}$ values (in mM) for the interaction of fXa derivatives (1–5 nM) with Na$^+$ were determined from the saturable increase in the rate of substrate hydrolysis as a function of increasing concentrations of Na$^+$ (0–800 mM) using three different chromogenic substrates with final concentrations near their $K_m$ values for fXa (100 μM SpFXa, 50 μM S2765, and 200 μM S2222). The background activities at the zero Na$^+$ were deducted, and the data were analyzed by the Langmuir isotherm equation. All values are derived from the kinetic data presented in Fig. 1. All values are the average of 3 measurements ± standard errors. ND, not determinable as the amidolytic activities with K186A remained linear for up to 800 mM Na$^+$ (Fig. 1).
for the defective function of the mutant. In interaction with TFPi, increasing the Na⁺ concentration did not improve the affinity of the K186A mutant for the inhibitor but rather impaired it ~2-fold, suggesting that the higher concentration of NaCl interferes with possible ionic interactions of TFPi with the protease. Thus, the opposing effects of Na⁺ did not allow to assign the effect of mutation to the Na⁺ site in the TFPi interaction. However, the $K_f$ value for the interaction of K186A with PAB was also improved ~5.5-fold in high salt. Thus, in contrast to a $K_f$ value of ~1802 μM for the inhibitor in 0.1 M NaCl, as shown in Table III, the $K_f$ value for the K186A mutant was improved to 324.9 ± 63.9 μM in 0.5 M NaCl. Relative to the $K_m$ value for the hydrolysis of S2765 by the mutant in 0.1 M NaCl (1.54 ± 0.1 mM, legend of Table III), the corresponding value in 0.5 M NaCl (0.3 ± 0.06 mM) was also improved ~4.5-fold. A similar ~4-fold improvement in the reactivity of the K186A mutant with AT was also observed (data not shown). It should be noted that wild-type FXa did not exhibit a significant improvement in its affinity for PAB in 0.5 M NaCl, and an ~1.5-fold improvement in the wild-type FXa reaction with AT and rTAP was observed (data not shown). The beneficial effect of Na⁺ in interaction of the mutant with IVa and reaction with prothrombin could not be evaluated because higher concentrations of the metal ion interfered with the interaction of the Gla domain with PC/PS vesicles. Taken together these results suggest that the catalytic defect of the mutant is primarily mediated by disruption of the Na⁺ site and that this site is allosterically linked to the 186-loop of the protease.

Equilibrium Binding Measurements—Previous results have indicated that fluorescence anisotropy of OG₄₈₅-EGR-FXa is increased upon interaction with IVa (6). Thus, we decided to evaluate the affinity of the FXa derivatives for binding to IVa by a competitive binding assay at equilibrium. As presented in Fig. 4A, the interaction of the active-site-labeled FXa with IVa was associated with a saturable increase in the fluorescence of OG₄₈₅ with a $K_D$ value of ~1.6 nM, which is similar to the $K_D$ value of 1.55 nM determined for the FXa-IVa interaction by this method in a previous study (33). The ability of the FXa derivatives to compete with OG₄₈₅-EGR-FXa for binding to IVa was evaluated from a decrease in the fluorescence of the labeled FXa-IVa complex as a function of increasing concentrations of wild-type or mutant proteases (Fig. 4B). Analysis of the binding data suggested that wild-type FXa competes with OG₄₈₅-EGR-FXa for interaction with IVa with a $K_D$ value of 2.3 nM that is similar to the same value obtained for the labeled FXa interaction with IVa in this assay, and thus yielded a $K_D$ value of 11.7 nM. A $K_P$ value of 2.6 nM for the E188A interaction with IVa was obtained by this competitive titration assay, supporting the kinetic data that Glu₁⁸⁸ minimally contributes to the specificity of the prothrombinase reaction. In contrast to a ~1.5-fold difference in the $K_{d_{app}}$ of wild-type and D185A interaction with IVa as determined by the prothrombinase assay (Table III), the difference in $K_{p}$ values was elevated to ~3-fold ($K_P = 6.6$ nM), suggesting that Asp₁⁸⁵ may also contribute to the affinity of the IVa interaction (Fig. 4 and legend).

| TABLE III | Kinetic constants for the inhibition of FXa derivatives by AT, TFPI, and PAB |
|-----------|--------------------------------------------------------------------------------------------------|
| FXa       | $k_{2,mon}$ (AT) $K_m$ (TFPI) $K_p$ (PAB) | $k_{2,mon}$ (AT) $K_m$ (TFPI) $K_p$ (PAB) |
| D185A     | $2.3 \pm 0.5 \times 10^6$ $1.2 \pm 0.1 \times 10^4$ $55.7 \pm 4.3$ | $2.3 \pm 0.5 \times 10^6$ $1.2 \pm 0.1 \times 10^4$ $55.7 \pm 4.3$ |
| K186A     | $6.2 \pm 0.7 \times 10^4$ $1.2 \pm 0.1 \times 10^4$ $60.7 \pm 25.8$ | $6.2 \pm 0.7 \times 10^4$ $1.2 \pm 0.1 \times 10^4$ $60.7 \pm 25.8$ |
| E188A     | $4.1 \pm 0.1 \times 10^4$ $1.2 \pm 0.1 \times 10^4$ $55.7 \pm 4.3$ | $4.1 \pm 0.1 \times 10^4$ $1.2 \pm 0.1 \times 10^4$ $55.7 \pm 4.3$ |

$K_{d_{app}}$, $k_{cat}$, and $K_{d_{app}}$ values were determined for binding to TFPI listed in the last column, and $k_{cat}$, $K_m$, and $K_I$ values of 31.9 ± 1.7 μM and 145.7 ± 1.9 s⁻¹ for FXa, 50.0 ± 2.2 μM and 69.3 ± 0.9 s⁻¹ for D185A, 1.34 ± 0.1 μM and 80.0 ± 3.3 s⁻¹ for K186A, and 42.5 ± 2.5 μM and 160.9 ± 2.6 s⁻¹ for E188A. All values are the average of at least 2–3 measurements ± standard errors.

TABLE IV | Kinetic constants for the activation of prothrombin by FXa derivatives in the absence and presence of IVa and the apparent dissociation constants ($K_{d_{app}}$) for their interaction with the cofactor |
|------------|--------------------------------------------------------------------------------------------------|
| FXa        | $K_{d_{app}}$ (nM) $k_{cat}$ (nM/min/nM) $K_{d_{app}}$ (nM) | $K_{d_{app}}$ (nM) $k_{cat}$ (nM/min/nM) $K_{d_{app}}$ (nM) |
| Prothrombin, PC/PS, Ca²⁺ | 156.1 ± 33.5 | 0.77 ± 0.05 | 55.7 ± 0.03 |
| Prothrombin, PC/PS, fVa, Ca²⁺ | 60.3 ± 4.0 | 1520.1 ± 27.5 | 0.57 ± 0.03 |
| D185A      | 195.8 ± 38.1 | 0.25 ± 0.01 | 829.0 ± 19.6 | 0.84 ± 0.04 |
| K186A      | 53.9 ± 4.8 | 1254.3 ± 134.4 | 0.28 ± 0.01 | 3.1 ± 0.2 |
| E188A      | 1254.3 ± 134.4 | 0.28 ± 0.01 | 1253.1 ± 36.9 | 3.1 ± 0.2 |
| Prothrombin, PC/PS, fVa, Ca²⁺ | 63.4 ± 4.6 | 1418.3 ± 28.3 | 0.87 ± 0.06 |

The second-order rate constants for the AT inhibition of FXa derivatives (1–5 nM) in the absence of cofactor ($k_{2,mon}$) were determined from the residual activities of the proteases after their incubation at room temperature with AT (0.5–4.0 μM) for 10–30 min in TBS/NaCl as described under “Experimental Procedures.” The $k_{2,mon}$ values were determined by the same procedures except that AT (25–200 nM) was in complex with 1 μM pentasaccharide. The $K_f$ values for binding to TFPi were determined by incubating each FXa derivative (1 nM for all and 5 nM for K186A) with TFPi (0.75–25 nM) in the same buffer. Following 30 min of incubation at room temperature SpFXa was added to a final concentration of 200 μM and the $K_f$ values were estimated by nonlinear regression analysis of data using a quadratic equation describing the tight binding interactions. The $K_p$ values for PAB (0–6.4 mM) were measured from the decrease in the initial rate of varying concentrations of S2765 (0.01–4.25 mM) hydrolysis produced by the competitive inhibitor. Global fitting of data yielded the $K_f$ values for PAB listed in the last column, and $k_{cat}$, $K_m$, and $k_{cat}$ values of 31.9 ± 1.7 μM and 145.7 ± 1.9 s⁻¹ for FXa, 50.0 ± 2.2 μM and 69.3 ± 0.9 s⁻¹ for D185A, 1.34 ± 0.1 μM and 80.0 ± 3.3 s⁻¹ for K186A, and 42.5 ± 2.5 μM and 160.9 ± 2.6 s⁻¹ for E188A. All values are the average of at least 2–3 measurements ± standard errors.
Concentrations of human prothrombin (20–2500 nM) was incubated with sense and presence of fVa.

The kinetic values are presented in Table IV.

Taken together, both kinetic and direct binding data suggest that Lys186 (and perhaps also Asp185) interact with fVa in the prothrombinase complex. Although a saturating concentration of fVa restored the catalytic defect with the K186A mutant, the effect of Na⁺ was dramatically weakened so that no Ki value for the interaction of the mutant with the metal ion could be determined. The impairment in the Na⁺ affinity of K186A paralleled the dramatic decrease in the specificity constant of the mutant in hydrolysis of chromogenic substrates, reaction with AT, interaction with PAR, TFPI, and rTAP, and activation of prothrombin. It is unlikely that Lys186 is a direct interactive site with the 162-helix but through the fVa interaction with the 186-loop of the protease.

DISCUSSION

We have demonstrated in this study that the 185–189-loop of fXa is energetically linked to the Na⁺ site of the protease. This is derived from the observation that the apparent affinity of both D185A and K186A mutants for interaction with Na⁺ was impaired ~2-fold, which correlated well with a similar extent of impairment in both the amidolytic and proteolytic activity of the mutant. Interestingly, however, the affinity of the D185A mutant for interaction with rTAP was improved ~2-fold. Previous data have indicated that the Na⁺ and S1 sites are not mediated through the fVa interaction with the 162-helix but through the fVa interaction with the 186-loop of the protease.
The 185–189-Loop of Factor Xa

The 185–189-loop of FXa with Asp185, Lys186, and Glu188 are shown in green. The side chain of the S1-Asp185 at the end of the loop is shown in yellow. The Na⁺ binding loop from residues 221–225 is colored in red, and the FVa binding 162-helix is shown in yellow. The catalytic triad residues (from top to bottom) Asp102, His57, and Ser195 are shown in yellow. The coordinates (Protein Data Bank accession code 1HCG) were used to prepare the figure (18).

The observation that a saturating concentration of FVa completely restored the catalytic defect of the K186A mutant toward prothrombin in the prothrombinase assay suggests that FVa through interaction with the 185–189-loop optimizes the conformations of the Na⁺ and/or S1 sites for interaction with the substrate in the activation complex. However, the observation that the cofactor affected neither the activity of the K186A mutant with small chromogenic substrates nor its affinity for interaction with TFPI and PAB suggests that the optimization of the S1 subsite may not significantly contribute to the cofactor function of FVa in the prothrombinase complex. Thus, FVa overcomes the catalytic defect of K186A, most likely through the modulation of the Na⁺ binding loop of the mutant protease. A possible scenario is that the basic residues of the Na⁺-binding loop of FXa (Arg221, Lys222, and Lys224) specifically interact with prothrombin, and the mutagenesis-mediated conformational change in the loop impairs their interaction with prothrombin. Thus, an allosteric modulation of this loop by FVa could account for the restoration of the catalytic activity of the mutant in the prothrombinase complex. Further support for this hypothesis is provided by the observation that similar to the activation of prothrombin FVa also restored the catalytic defect of the K186A interaction with rTAP. Noting the existence of an allosteric linkage between the 185–189-loop and the Na⁺ site of FXa and the importance of the basic residues of the Na⁺ loop for the protease interaction with rTAP (21, 27), the most likely explanation for the lower affinity of K186A for rTAP is the altered conformation of the Na⁺ loop in the mutant protease. It follows therefore that FVa restores the catalytic defect of K186A in the prothrombinase complex through modulation of the Na⁺ loop. Thus, similar to rTAP, it is likely that the basic residues of the Na⁺ loop of FXa interact with prothrombin in the prothrombinase complex. This is the most

are energetically linked (16, 17). Thus, it is possible that the mutagenesis has decreased the flexibility of the Na⁺-binding loop with the D185A mutant so that the cofactor effect of the metal ion is not relayed properly to the S1 site of the mutant protease. Structural and mutagenesis data have indicated that rTAP tightly binds to FXa with the N-terminal P1-Tyr of the inhibitor interacting with the S1 site and an acidic region interacting with the basic residues of the Na⁺ site (21, 31). Thus, the altered conformation of the Na⁺ loop in D185A appears to be favored for the inhibitor interaction. The S1 site interaction with rTAP is not impaired, likely because the P1-Tyr of the inhibitor would not interact with this site by a mechanism similar to the P1-Arg interaction of other substrates and inhibitors of FXa. Taken together, these results suggest that the 185–189-loop influences the affinity of FXa for the Na⁺ site and that the two sites are energetically linked.

Both kinetic and equilibrium binding data suggested that Lys186 (perhaps also Asp185) interacts with FVa in the prothrombinase complex. We and others have previously demonstrated that the basic residues of the 162-helix of FXa (in particular Arg165) also interact with FVa (12, 13). The examination of the three-dimensional location of the 185–189-loop of FXa in the x-ray crystal structure of FXa suggests that the side chains of both Lys186 and Asp185 are exposed to the solvent molecules, and they are located in the proximity of the 162-helix on the same side of the protease (18) (Fig. 5). Thus, FVa can interact with both of these loops to modulate to specificity of FXa in the prothrombinase complex. A recent thermodynamic linkage analysis noted an allosteric coupling between FVa and Na⁺ sites in FXa, which was attributed to the interaction of the cofactor with the 162-helix of the protease (16). However, the observation of the current study that R165A has both normal amidolytic activity and apparent affinity for interaction with Na⁺ suggests that the allosteric modulation of the Na⁺ site by FVa is caused by the cofactor interaction with Lys186, not with the 162-helix of FXa.

The observation that a saturating concentration of FVa completely restored the catalytic defect of the K186A mutant toward prothrombin in the prothrombinase assay suggests that FVa through interaction with the 185–189-loop optimizes the conformations of the Na⁺ and/or S1 sites for interaction with the substrate in the activation complex. However, the observation that the cofactor affected neither the activity of the K186A mutant with small chromogenic substrates nor its affinity for interaction with TFPI and PAB suggests that the optimization of the S1 subsite may not significantly contribute to the cofactor function of FVa in the prothrombinase complex. Thus, FVa overcomes the catalytic defect of K186A, most likely through the modulation of the Na⁺ binding loop of the mutant protease. A possible scenario is that the basic residues of the Na⁺-binding loop of FXa (Arg221, Lys222, and Lys224) specifically interact with prothrombin, and the mutagenesis-mediated conformational change in the loop impairs their interaction with prothrombin. Thus, an allosteric modulation of this loop by FVa could account for the restoration of the catalytic activity of the mutant in the prothrombinase complex. Further support for this hypothesis is provided by the observation that similar to the activation of prothrombin FVa also restored the catalytic defect of the K186A interaction with rTAP. Noting the existence of an allosteric linkage between the 185–189-loop and the Na⁺ site of FXa and the importance of the basic residues of the Na⁺ loop for the protease interaction with rTAP (21, 27), the most likely explanation for the lower affinity of K186A for rTAP is the altered conformation of the Na⁺ loop in the mutant protease. It follows therefore that FVa restores the catalytic defect of K186A in the prothrombinase complex through modulation of the Na⁺ loop. Thus, similar to rTAP, it is likely that the basic residues of the Na⁺ loop of FXa interact with prothrombin in the prothrombinase complex. This is the most
logical explanation to account for the similarities in the cofactor function of fVa in two systems. It is known that the basic residues of the autolysis loop also interact with rTAP (21, 27); however, these residues may not be a target for the cofactor function of fVa because we have already demonstrated that their mutagenesis do not significantly affect the catalytic function of the mutants in the prothrombinase complex (22). The hypothesis that the Na⁺/H11001 loop of fXa may interact with prothrombin needs to be tested by a mutagenesis study. In a Gla-domainless factor X expression system, our previous attempt to study this question was not successful because mutagenesis of these residues resulted in “loss of function” mutants exhibiting dramatically impaired activity in all fXa related kinetic assays (27, 34). This phenomenon is likely related to the existence of intricate allosteric linkages between the S1, Na⁺, and fVa site of fXa as clearly demonstrated in this study.

Acknowledgement—We thank Dr. Chandrashekhara Manithody for assistance with the construction and purification of the fXa mutants.

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Alireza R. Rezaie and Farooqaheem S. Kittur

J. Biol. Chem. 2004, 279:48262-48269.
doi: 10.1074/jbc.M409964200 originally published online September 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409964200

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