Thermodynamic Linkage between the S1 Site, the Na\(^+\) Site, and the Ca\(^{2+}\) Site in the Protease Domain of Human Coagulation Factor Xa

STUDIES ON CATALYTIC EFFICIENCY AND INHIBITOR BINDING*

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The serine protease domain of factor Xa (FXa) contains a sodium as well as a calcium-binding site. Here, we investigated the functional significance of these two cation-binding sites and their thermodynamic links to the S1 site. Kinetic data reveal that Na\(^+\) binds to the substrate bound FXa with \(K_d\) ~39 mM in the absence and ~9.5 mM in the presence of Ca\(^{2+}\). Sodium-bound FXa (sodium-Xa) has ~18-fold increased catalytic efficiency (~4.5-fold decrease in \(K_m\) and ~4-fold increase in \(k_{cat}\)) in hydrolyzing S-2222 (benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide), and Ca\(^{2+}\) further increases this \(k_{cat}\) ~4-fold. Ca\(^{2+}\) binds to the protease domain of substrate bound FXa with \(K_{d}\) ~705 \(\mu\)M in the absence and ~175 \(\mu\)M in the presence of Na\(^+\). Ca\(^{2+}\) binding to the protease domain of FXa (Xa-calcium) has no effect on the \(K_m\) but increases the \(k_{cat}\) ~4-fold in hydrolyzing S-2222, and Na\(^+\) further increases this \(k_{cat}\) ~1.4-fold. In agreement with the \(K_m\) data, sodium-Xa has ~5-fold increased affinity in its interaction with p-aminobenzamidine (S1 site probe) and ~4-fold increased rate in binding to the two-domain tissue factor pathway inhibitor; Ca\(^{2+}\) (±Na\(^+\)) has no effect on these interactions. Antithrombin binds to Xa-calcium with a ~4-fold faster rate, to sodium-Xa with a ~24-fold faster rate and to sodium-Xa-calcium with a ~28-fold faster rate. Thus, Ca\(^{2+}\) and Na\(^+\) together increase the catalytic efficiency of FXa ~28-fold. Na\(^+\) enhances Ca\(^{2+}\) binding, and Ca\(^{2+}\) enhances Na\(^+\) binding. Further, Na\(^+\) enhances S1 site occupancy, and S1 site occupancy enhances Na\(^+\) binding. Therefore, Na\(^+\) site is thermodynamically linked to the S1 site as well as to the protease domain Ca\(^{2+}\) site, whereas Ca\(^{2+}\) site is only linked to the Na\(^+\) site. The significance of these findings is that during physiologic coagulation, most of the FXa formed will circulate as a zymogen with a molecular weight of ~59,000 and consists of a light chain (amino acids 1–139) and a heavy chain (amino acids 143–448) held together by a single disulfide bond between Cys-132 and Cys-302 (1). Upon activation by factor VIIa, Ca\(^{2+}\), and tissue factor or by factor IXa, Ca\(^{2+}\), phospholipid, and factor VIIIa, a single peptide bond in factor X between residues Arg-194(c15) and Ile-195(c16) is cleaved with resultant formation of a serine protease, factor Xa (FXa), and release of a 52-residue activation peptide (1, 2). FXa is converted to its β-form where a ~4-kDa peptide is cleaved off from the COOH terminus of the heavy chain; this, however, does not result in a loss of coagulant activity (3). FXa converts prothrombin to thrombin (IIa) in a reaction requiring Ca\(^{2+}\), phospholipid membrane, and factor Va (FVa) (2, 4). Through its active site Ser-397(c195), FXa also binds to the serpin antithrombin (AT) (5) and to the second Kunitz domain of tissue factor pathway inhibitor (TFPI) (6).

The NH\(_2\) terminus light chain of human FXa contains 11 \(\gamma\)-carboxyglutamic acid (Gla) residues and represents the Gla domain (residues 1–39); the Gla domain is followed by a few aromatic residues (hydrophobic stack; residues 40–45), and two epidermal growth factor (EGF)-like domains (EGF1 residues 46–84, EGF2 residues 85–128). The heavy chain contains the serine protease domain essential for catalysis and features the active site triad of His-236(c57), Asp-282(c102), and Ser-397(c195) (1, 5). The Gla domain possesses four intermediate and several low affinity Ca\(^{2+}\)-binding sites that appear to be essential for binding of the Gla domain to phospholipid membrane (8). The protease and the EGF1 domains each contain one high affinity Ca\(^{2+}\)-binding site, which is essential for optimal FXa binding to FVa (8–12).

It has been reported that serine proteases, like IIa, that contain Tyr or Phe at position c225 possess a sodium binding site, whereas proteases like trypsin with a Pro at this position do not contain such a site (13). IIa binds to Na\(^+\) via two carbonyl O atoms of residues Arg-c221A and Lys-c224 and four water molecules (14, 15). When residue at c225 is a Pro, as in trypsin, or in Yc225P mutant of IIa, the carbonyl O atom of the residue c224 points away from the Na\(^+\) coordination shell and
The fold change in specificity constant is given in parentheses.

To keep the ionic strength constant, the concentration of Na\(^+\) was 500 mM as measured by a conductivity meter as outlined by Wells and Di Cera (28). The proteins were prepared using insolubilized Russell’s viper venom as outlined by Wells and Di Cera (28). The NH\(_2\)-terminal sequence analysis of Des-44-X revealed two sequences of approximately equimolar amounts, one corresponding to the modified light chain (Lys-Asp-Gly-Asp-Gln). This indicates that Des-44-X does not contain the Gla domain. SDS Gel Electrophoresis—FXa gel electrophoresis was performed using the Laemmli buffer system (26). The acrylamide concentration was 15%, and the gels were stained with Coomassie Brilliant Blue dye. All proteins used in the present study were \(\sim 98\%\) pure.

Measurement of S-2222 Amidolytic Activity of FXa Proteins—The concentration of FXa and Des-44-Xa was used between 1 and 5 nM each. The S-2222 concentration ranged from 20 \(\mu\)M to 3 mM. The buffer used was 50 mM Tris, pH 7.4, containing 0.1% PEG and various salt combinations. The p-nitroaniline release was measured continuously (3\(\Delta_{405}\) nm/min) for up to 30 min using a Beckman DU65 spectrophotometer equipped with a Soft-Pac kinetics module. An extinction coefficient of 9.9 mM\(^{-1}\) cm\(^{-1}\) at 405 nm was used in calculating the amount of p-nitroaniline released (30). All reactions were performed in triplicate. The \(K_m\) and \(k_{cat}\) values were obtained using the Enzyme Kinetics program from Erithacus Software.

PABA Binding—Binding of PABA was measured by increase in its intrinsic fluorescence upon binding to the active site of FXa using an SLM 8000C fluorescence spectrophotometer. The concentration of FXa used was 1.7 \(\mu\)M, and the excitation wavelength was 336 nm (31). A titration of the protein solution (300 \(\mu\)l) was performed by adding small increments (1–2 \(\mu\)l) of 1 or 2 mM stock solution of PABA, and the resulting fluorescence at 376 nm was measured at each point after the attainment of equilibrium conditions (usually 1 min). Excitation bandwidth was set at 4 nm, and emission bandwidth was set at 2 nm. Because binding of PABA to FXa is expected to be relatively weak (32), high concentrations of PABA are needed to study this interaction. This leads to a significant absorption of the exciting light, and the data therefore require corrections for the inner filter effect (32, 33). First, the nonlinear dependence of fluorescence on PABA concentration in the presence of PABA was measured continuously (3\(\Delta_{405}\) nm/min) for up to 30 min using a Beckman DU65 spectrophotometer equipped with a Soft-Pac kinetics module. An extinction coefficient of 9.9 mM\(^{-1}\) cm\(^{-1}\) at 405 nm was used in calculating the amount of p-nitroaniline released (30). All reactions were performed in triplicate. The \(K_m\) and \(k_{cat}\) values were obtained using the Enzyme Kinetics program from Erithacus Software.

The corrected fluorescence (\(F_{corr}\)) was then obtained using Equation 2 (32).

\[
F_{obs} = (Pm + i)/i^{1/2} \quad \text{(Eq. 1)}
\]

where \(P\) is the concentration of PABA, \(m\) and \(i\) are, respectively, the slope and intercept of the expected linear dependence on PABA concentration in buffer, \(e\) is the extinction coefficient of PABA at 336 nm excitation wavelength (experimentally determined to be 1426 m\(^{-1}\) cm\(^{-1}\)), and \(L\) is effective path length for the fluorescence cell. The value of \(L\) determined using Equation 1 was 0.4 ± 0.2. The corrected fluorescence signal (\(F_{corr}\)) was then obtained using Equation 2 (32).

\[
F_{corr} = F_{obs} \times 10^{ec} \quad \text{(Eq. 2)}
\]

Binding data were then fitted to a single ligand binding site with a defined background using the following equation.

\[
y = b + [(e - b)x]/(K_{d,app} + x) \quad \text{(Eq. 3)}
\]

where \(K_{d,app}\) is the apparent dissociation constant, \(y\) is the inner filter corrected fluorescence at a given PABA concentration depicted by \(x\), \(b\) is the corrected background fluorescence in the absence of protein, and \(e\) is the corrected fluorescence at saturating concentrations of PABA.

### Table I

| Conditions | \(K_m\) (mM) | \(k_{cat}\) (s\(^{-1}\)) | \(k_{cat}/K_m\) |
|-----------|-------------|----------------|---------------|
| Na\(^+\) | Ca\(^2+\) | Na\(^+\) | Ca\(^2+\) |
| - | - | 514 ± 21 | 16 ± 2 | 31 (1) |
| - | + | 154 ± 15 | 73 ± 8 | 640 (4) |
| + | - | 154 ± 22 | 69 ± 7 | 118 (4) |
| + | + | 132 ± 15 | 107 ± 10 | 810 (26) |

*The fold change in specificity constant is given in parentheses.*

Therefore, calcium is a potent inhibitor of FXa catalytic activity. However, in the absence of calcium and 485 mM in the presence of 5 mM Ca\(^{2+}\). The results presented are the average of five experiments. The \(K_m\) and intermediate concentrations of Na\(^+\) are listed in the legends to Fig. 2.
Role of Na⁺ Site in Factor Xa Function

**Fig. 1.** Effects of monovalent cations on the amidolytic activity of FXa. A, no compensation for ionic strength. Reaction mixtures (150 μl) contained 100 μM S-2222 and 2.5 nM FXa in 50 mM Tris, pH 7.4, 0.1% PEG 8000, and various concentrations of cations. The ions used are K⁺ (open circles), Rb⁺ (closed circles), Li⁺ (open triangles), and Ch⁺ (closed triangles). Chloride salt of each ion was used. B, effect of Na⁺ on the amidolytic activity of FXA. Reaction conditions are the same as in A. Monovalent cation concentration was maintained constant at 0.2 mM by the addition of Rb⁺ (closed circles) or Ch⁺ (closed triangles). Open circles represent control experiments where no compensating ion was added.

**Interaction of FXa with Two-domain TFPI—**These reactions were carried out in Dynatech microtiter well plates. Each reaction mixture (150 μl) contained 400 μM S-2222, constant FXa, which varied from 50 to 1600 pmol in different sets of experiments and a constant predetermined concentration of TFPI. 55 μl of S-2222/TFPI mixture was added to each well containing 95 μl of FXA. Starting at time 0, absorbance at 405 nm (p-nitroaniline release) was recorded for up to 6 h in Bio-Rad microtiter Manager PC software. Reaction conditions are provided in the legend to Fig. 8. The data were analyzed based upon the slow tight binding mechanism established for FXa-TFPI as follows,

\[
\begin{align*}
\text{Xa} + \text{TFPI} & \rightleftharpoons \text{Xa:TFPI} \\
\text{Xa:TFPI} & \rightleftharpoons \text{Xa:TFPI*}
\end{align*}
\]

(Eq. 4)

where Xa:TFPI is the steady state complex, which then isomerizes and results in the formation of Xa:TFPI*, k₁ and k₂ are the forward and reverse rates for the initial binding, and k₃ and k₄ are the forward and reverse rates for the isomerization step. To calculate the binding parameters, first the initial (v₀) and steady state (vₛ) velocities were calculated using Equation 5, as described by Morrison and Walsh (34),

\[
[P] = v_0 + [(v_0 + v_S)(k_{obs})^{-1} - 1] \cdot \exp(-k_a t)
\]

(Eq. 5)

where [P] represents the concentration of p-NA formed at time t, v₀ and vₛ are respectively the rates of substrate hydrolysis before and after the steady state is achieved, and kₐ is the rate of conversion of v₀ to vₛ. For these reactions, TFPI concentrations were in large excess of FXa. Thus, the free TFPI concentration did not change significantly during the course of the reaction. Further, the rates in the control experiments performed in the absence of TFPI did not change over the course of measurement. Thus, substrate depletion did not contribute to vₛ.

The values of kₐ were obtained above were fitted to Equation 3 to resolve the individual parameters. In this case in Equation 3, y represents kₐ when a given concentration of two-domain TFPI depicted by x, whereas b and c, respectively, represent k₁ and (k₃ + k₄), and Kₒₙₐₚ represents k₂/k₄ (35).

The value of Kₒₚ (k₉/k₈) was obtained from Kₙₐₚ using the following equation.

\[
K_k = K_{napp} / (1 + [S]/K_i)
\]

(Eq. 6)

where [S] is the S-2222 concentration. The Kᵣ values obtained under different conditions (listed in Table I) were used to obtain Kₙₐₚ.

**Binding of AT to FXa—**Serpentin AT binds FXa irreversibly and should displace PABA bound at the active site. Thus, the AT binding to FXa was monitored by measuring the decrease in fluorescence of FXa-PABA solution. The wavelengths for excitation (bandwidth, 4 nm) and emission (bandwidth, 2 nm) were set at 336 and 376 nm, respectively. For each salt condition, FXa at 1.7 mM was mixed with PABA at the respective Kᵣ concentration (Table II) in the presence of 100 ng/ml (found to be

**Fig. 2. Na⁺-mediated potentiation of S-2222 hydrolysis in the absence of Ca²⁺.** A, effect of Na⁺ on the Kᵣₚ and Vᵢₚ of S-2222 hydrolysis. Monovalent ion concentrations are: 0 mM Na⁺/500 mM Rb⁺ (closed triangles), 15 mM Na⁺/470 mM Rb⁺ (open squares), 50 mM Na⁺/450 mM Rb⁺ (closed squares), 80 mM Na⁺/420 mM Rb⁺ (closed triangles), 100 mM Na⁺/400 mM Rb⁺ (open triangles), 150 mM Na⁺/350 mM Rb⁺ (open diamonds), 200 mM Na⁺/300 mM Rb⁺ (closed diamonds), 300 mM Na⁺/200 mM Rb⁺ (closed hexagons), 400 mM Na⁺/100 mM Rb⁺ (open hexagons), and 500 mM Na⁺/0 mM Rb⁺ (crosses). Kᵣₚ and Vᵢₚ values were calculated using the enzyme kinetics program from Erithacus Software; for clarity, higher concentrations of substrate used are not shown in the graph. The concentration of FXA was 2.5 nm. The value of Kᵣₚ (μM) at 0 mM Na⁺ was 514 ± 21, at 15 mM Na⁺ it was 509 ± 18, at 30 mM Na⁺ it was 454 ± 41, at 50 mM Na⁺ it was 381 ± 36, at 80 mM Na⁺ it was 306 ± 32, at 100 mM Na⁺ it was 280 ± 28, at 150 mM Na⁺ it was 276 ± 29, at 200 mM Na⁺ it was 240 ± 31, at 300 mM Na⁺ it was 146 ± 24, at 400 mM Na⁺ it was 108 ± 21, and at 500 mM Na⁺ it was 114 ± 18 mM. For these experiments, two stock buffers were made. One buffer was 50 mM Trisoma base, 0.1% PEG 8000, 1 mM EDTA (acid form), and 500 mM RbCl adjusted to pH 7.4 with HCl. In the second buffer 500 mM RbCl was replaced by 500 mM NaCl, and the pH was again adjusted to 7.4 with HCl. These two buffers were mixed in appropriate proportions to yield the desired concentration of Na⁺ and Rb⁺. Thus, the monovalent ion concentration was always 500 mM, and the buffer containing 500 mM RbCl had no added source of Na⁺. B, Vᵢₚ as a function of Na⁺. Data were fitted to Equation 3. Here, y represents maximum velocity at saturating concentrations of Na⁺, b is the offset (maximum velocity at zero concentration of Na⁺), x is the concentration of Na⁺, and Kₒₚ is the Kᵣ of Na⁺ for Xa(S) in the absence of Ca²⁺. The values of b and y (converted to Kᵣₑₐₚ) are given in Table I.
saturation) low molecular weight heparin. AT was then added, and the decrease in the inner filter corrected fluorescence was determined at 1–5 s intervals for up to 30 min. The data were fitted to the equation below.

\[ F_t = F_0 \exp(-k_{obs}t) \]  

(Eq. 7)

where \( F_0 \) and \( F_t \) are the inner filter corrected fluorescence values at time \( t \) and 0 seconds, respectively, and \( k_{obs} \) is the observed pseudo first order rate constant. Under the conditions of experiment, the value of \( k_{obs} \) is related to the \( k'_{obs} \) as follows,

\[ k_{obs} = k'_{obs} [K_{PABA} + [PABA]] \]  

(Eq. 8)

where \( k'_{obs} \) is pseudo first order rate constant extrapolated to 0 concentration of PABA. As the PABA concentration was fixed at the \( K_{PABA} \) in each case, the values of \( k_{obs} \) were multiplied by a factor of 2 to obtain the values of \( k'_{obs} \). The values of \( k_{obs} \) were then plotted against the AT concentration to obtain \( k_{cat} \) values.

Molecular Modeling—X-ray structures of FXa (7), Ila (14, 15), and trypsin (36) were analyzed using the program O (37). Residue Tyr-c225 in FXa was mutated to Pro and manually adjusted based upon the coordinates of trypsin (36) and Ila Yc225P mutant (15). Local region was energy minimized using the program O (37).

RESULTS

Effects of Monovalent Cations on the Amidolytic Activity of FXa—Hydrolysis of S-2222 by FXa was measured at various concentrations of Li\(^+\), Na\(^+\), K\(^+\), Rb\(^+\), and choline (Ch\(^+\)). These data are presented in Fig. 1. In one set of experiments, the activity of FXa was measured at increasing cation concentrations from 0 to 0.2 mM (Fig. 1A). FXa hydrolyzed S-2222 (100 \( \mu \)M) in the absence of any ion (in 50 mM Tris, pH 7.4, 0.1% PEG 8000) at \(-0.1 \mu \text{mol/min/nM FXa}\). K\(^+\) increased this rate \(-1.7\)-fold, whereas Rb\(^+\) and Li\(^+\) had no significant effect. On the other hand, Ch\(^-\) decreased this rate \(-3.5\)-fold. Further, Na\(^+\)

Although changes in ionic strength in the range of 0–0.2 M Na\(^-\) did not affect the amidolytic activity of FXa (Fig. 1A), yet its effects on macromolecular recognition cannot be predicted. Therefore, we standardized a system to study the effects of Na\(^+\) at a constant monovalent ion concentration. In several previous studies with coagulation enzymes, including FXa, Ch\(^+\) has been employed as an inert ion (13, 19, 23). Based upon these reports and the data presented in Fig. 1A, we wished to further examine the effects of Ch\(^-\) and Rb\(^+\) on FXa activity. FXa activity was measured in reaction mixtures containing various
concentrations of Na$^+$ and appropriate concentrations of Ch$^+$ or Rb$^+$ to yield a monovalent cation concentration of 0.2 M. The results of these studies are presented in Fig. 1B. Data obtained in the absence of any compensating ion could be fitted to a single site ligand binding equation (Equation 3). However, use of Ch$^+$ to compensate for ionic strength skewed the curve form a regular hyperbola toward being sigmoidal in nature. This can be explained by a combination of the potentiating effect of Na$^+$ and the inhibitory effect of Ch$^+$ operating simultaneously. On the other hand, the response curve obtained using Rb$^+$ as an inert ion resembled the curve obtained in the absence of any compensating ion. These data establish that Ch$^+$ is not an inert ion but inhibits FXa activity, whereas Rb$^+$ is the ion of choice to compensate for ionic strength in studying FXa function. Data showing that Ch$^+$ inhibits FXa activity are consistent with previous observations (38, 39).

Because 200 mM Na$^+$ does not appear to saturate the Na$^+$ site in FXa, we opt to test whether or not we could use 500 mM NaCl as the highest salt concentration without affecting the catalytic activity of FXa. Thus when we performed experiments with buffers containing 500 mM RbCl, the rate of S-2222 hydrolysis (at 100 mM or 200 mM) was the same as with 200 mM RbCl. Similarly, when reaction buffer contained 200 mM Na$^+$ and 300 mM Rb$^+$, the rate of hydrolysis was identical to that obtained with 200 mM Na$^+$ without Rb$^+$. Thus, it appears that we could use up to 500 mM NaCl in our system to study the role of Na$^+$ in FXa function.

Na$^+$ Potentiation of S-2222 Hydrolysis by FXa in the Absence of Na$^+$—These data are presented in Fig. 3. The kinetic data indicate that Ca$^{2+}$ does not change the $K_{d,app}$ but increases the $k_{cat}$ by 4-fold. Importantly, the $K_{a}$ of Ca$^{2+}$ in its interactions with Xa(S) (Xa saturated with S-2222) in the absence of Ca$^{2+}$; this value was calculated to be 39 ± 4 mM.

Ca$^{2+}$ Potentiation of S-2222 Hydrolysis by FXa in the Presence of Na$^+$—These data are presented in Fig. 4. The kinetic data indicate that Na$^+$ decreases the $K_{m,app}$ by 4.5-fold and increases the $k_{cat}$ by 1.4-fold. Importantly, Ca$^{2+}$ decreases the $K_{d}$ of Na$^+$ interaction with Xa(S) to 9.5 mM as compared with 39 mM (Fig. 2) when Ca$^{2+}$ is absent. Thus Ca$^{2+}$ site is thermodynamically linked to the Na$^+$ site.

Ca$^{2+}$ Potentiation of S-2222 Hydrolysis by FXa in the Presence of Na$^+$—These data are presented in Fig. 5. As noted previously (Fig. 3), Ca$^{2+}$ does not change the $K_{m,app}$ but increases the $k_{cat}$ by 1.4-fold in the presence of Na$^+$ as well. Furthermore, Na$^+$ decreases the $K_{d}$ of Ca$^{2+}$ interaction with Xa(S) to 170 mM as compared with 700 mM (Fig. 3) when Na$^+$ is absent. Thus, as noted above, Na$^+$ site is linked to the Ca$^{2+}$ site.
corrected data were fitted by nonlinear regression to Equation 3 to clearly establish the interdependence of the binding of Na in the presence and absence of Na (8). The Kd values for Xa(S) in the absence and presence of NaCl concentrations in the absence (open circles) and presence (filled circles) of 5 mM Ca2+

The above experiments were repeated using Des-44-Xa, which has only one high affinity Ca2+-binding site in the presence of Na+. This Ca2+-binding site is localized to the protease domain of FXa (8). The Kd Na values for Xa(S) in the absence and presence of Ca2+ were ~45 and ~11 mM, respectively. Similarly, the Kd Ca values for Xa(S) in the absence and presence of Na+ were ~700 and ~170 μM, respectively. These data demonstrate that it is the protease domain Ca2+-binding site that influences the Kd for Na+ binding and vice versa. Moreover, when trypsin (400 pM) that lacks Na+ site was used to hydrolyze S-2222 (100 μM), the rates in the presence and absence of Na+ were the same (1.1 μM/min), and Ca2+ only minimally affected this rate (1.8 μM/min).

**Linkage between the Substrate Binding Site, the Na+ Site, and the Protease Domain Ca2+ Site of FXa**—The above data clearly establish the interdependence of the binding of Na+ and Ca2+ in the protease domain of FXa. Fig. 6 depicts this as a thermodynamic cycle. Xa(S) can be converted to the state with both ions bound (sodium-Xa(S)-calcium) by acquiring either Na+ (via Xa(S)-calcium) or Ca2+ (via sodium-Xa(S)). The ratio of the Kd Na in the presence and absence of Ca2+ is 4.1, and that of Kd Ca in the presence and absence of Na+ is 4.2. Therefore, the net sum of binding energy over the cycle appears to be 0. This establishes the thermodynamic linkage between the Na+ site and the protease domain Ca2+-binding site of FXa.

Because Na+ affects the Km of S-2222 hydrolysis, there appears to be a link between the substrate binding and the Na+ -binding site. Both in the absence and presence of Ca2+,
Role of Na\(^{+}\) Site in Factor Xa Function

**FIG. 9.** Effects of Na\(^{+}\) and Ca\(^{2+}\) on the inhibition of FXa with AT. The binding of AT was studied by its ability to displace PABA from the S1 site of FXa. Reaction mixtures (300 μl) contained 1.7 μM FXa, 100 ng/ml low molecular weight heparin, and various concentrations of AT in 50 mM Tris, pH 7.4, and 0.1% PEG 8000. The corrected pseudo first order rate constants (k_{obs}) are presented as a function of AT. Various salt combination used are: 500 mM Rb\(^{+}\), 1 mM EDTA (open circles); 500 mM Na\(^{+}\), 1 mM EDTA (open triangles); 5 mM Ca\(^{2+}\), 485 mM Rb\(^{+}\) (closed circles); and 5 mM Ca\(^{2+}\), 485 mM Na\(^{+}\) (closed triangles). The concentration of PABA used in each case was fixed at the K_d PABA (Table II). Experiments were initiated by the addition of AT to an equilibrated mixture of FXa-PABA, and the decrease in fluorescence was recorded up to 30 min. Data were fitted to Equation 7 to obtain k_{obs}. Corrected k_{obs} (k_{obs}^\text{corr}) were obtained as per Equation 8 and were then used to obtain the second order rate constant, k_{on}.

**TABLE IV**

Effects of calcium and sodium on the second order bimolecular rate constants (k_{on}) for inhibition of FXa by AT

| Conditions | Na\(^{+}\) | Ca\(^{2+}\) | k_{on} | Fold increase (k_{on}) |
|------------|-----------|-----------|-------|----------------------|
|            | mm⁻¹ s⁻¹ |           |       |                      |
| −          | 3.12 ± 0.22 | 24                   |       |
| +          | 3.67 ± 0.31  | 28                   |       |

Effects of Na\(^{+}\) and Ca\(^{2+}\) on the Interaction of PABA with FXa.—We next investigated whether Na\(^{+}\) and/or Ca\(^{2+}\) affect the S1 site of the active site in FXa. We used the S1 site probe PABA for these studies. The interaction of PABA with FXa was determined under each of the four salt conditions. These data are presented in Fig. 7 and summarized in Table II. Under saturating concentration of Na\(^{+}\), the affinity of PABA for FXa was increased ~5-fold as compared with that in its absence. Further, Ca\(^{2+}\) had no effect on the K_d PABA in the absence or presence of Na\(^{+}\). These K_d PABA data agree well with the K_m data presented in the previous section where Na\(^{+}\) decreased the K_m ~4-fold and Ca\(^{2+}\) had no effect.

Effects of Na\(^{+}\) and Ca\(^{2+}\) on the Binding of Two-domain TFPI and AT with FXa.—In this section we investigated the role of Na\(^{+}\) and Ca\(^{2+}\) in binding of FXa to its two physiologic inhibitors, namely, TFPI and AT. TFPI consists of three tandem Kunitz-type domains, and the second domain is responsible for binding to FXa with an overall K_{d} in the nanomolar range (41). In this study we used two-domain TFPI, which contains the second FXa-binding domain. The data obtained were treated according to the tight binding inhibitory mechanism (34, 35) and analyzed using Equations 5, 3, and 6, respectively. The kinetic parameters were obtained for all four forms of FXa depicted on the left side in Fig. 6. Representative data are presented in Fig. 8, and the kinetic parameters for all four conditions used are listed in Table III. The affinity for the first step of FXa-TFPI complex formation (Equation 4) was increased ~4-fold in the presence of saturating concentration of Na\(^{+}\); however, the slow isomerization step leading to the formation of FXa-TFPI* was not affected. Interestingly, Ca\(^{2+}\) in the presence or absence of Na\(^{+}\) had no effect on any of these kinetic parameters.

The binding data for AT, which belongs to the serpin family of inhibitors are presented in Fig. 9 and the second order rate constants for all four forms of FXa (left side of Fig. 6) are listed in Table IV. Na\(^{+}\) increased the k_{on} by ~24-fold in the absence of Ca\(^{2+}\), and addition of Ca\(^{2+}\) had minimal further effect. Ca\(^{2+}\) increased the k_{on} by ~4-fold in the absence of Na\(^{+}\), and addition of Na\(^{+}\) further increased this rate constant by ~7-fold. These AT binding data are consistent with the specificity constant data presented in Table I.

**DISCUSSION**

Recently, Dang and Di Cera (13) have reported that several serine proteases including those in coagulation possess a functional Na\(^{+}\) site. X-ray crystal structures of IIa (14, 15) and FXa (7, 14) are reported where the Na\(^{+}\) site in these molecules in defined. The Na\(^{+}\) site in IIa uses a single loop involving the carboxyl O atoms of residues c221A and c224 as well as four water molecules, whereas the Na\(^{+}\) site in FXa uses two loops involving the carboxyl O atoms of residues c185, c185A, c222, and c224 as well as two water molecules. The nature of the residue c225 plays an important role in orienting the carboxyl O atom of c224 toward the Na\(^{+}\) coordination shell (7, 14, 15).

Further, FXa also possesses a Ca\(^{2+}\) site in the protease domain involving the carboxyl groups of Asp-c70 and Glu-c80 as well as carboxyl O atoms of residues c72 and c75. The occupancy of Ca\(^{2+}\) at this site prevents proteolysis in the autolysis loop, the integrity of which is essential for FXa biologic activity (8, 42). The protease domain of FXa also contains a FVa-binding site that involves the c162-c169 helix (12), and residues Arg-c164 and Arg-c165 are important for this interaction (12, 43). Fig. 10A presents a schematic view of the spatial relationships of the Ca\(^{2+}\) site, autolysis loop, Asp-c189 S1 site, Na\(^{+}\) site, FVa-binding helix, and the catalytic triad. The objective of the present study was to examine the role of binding of Ca\(^{2+}\) and Na\(^{+}\) in regulating the FXa catalytic efficiency.

In the absence Na\(^{+}\), Ca\(^{2+}\) increased the k_{cat} of S-2222 hydrolysis ~4-fold (Table I). Similarly, it increased the AT binding rate ~4-fold (Table IV), which in all probability represents formation of the acyl intermediate. In the presence of Na\(^{+}\), however, Ca\(^{2+}\) had minimal effect (~1.4-fold) on the hydrolysis of S-2222 or AT binding. Of interest, Ca\(^{2+}\) in the presence or absence of Na\(^{+}\) had no effect on the k_{m} of S-2222 hydrolysis as well as on the PABA binding and a tight binding reversible inhibitor, TFPI. Thus, Ca\(^{2+}\) does not affect the ground state binding of substrates/inhibitors to FXa. The ~4-fold increase in k_{cat} for S-2222 hydrolysis and in the formation of acyl covalent FXa-AT complex may be due to lowering of the transition state energy. In FXa, Ca\(^{2+}\) loop is linked to the carboxyl O atoms of Gly-c193 and Asp-c194 through four water molecules (7). These two residues play an important role in positioning the Ser-c195 active site side chain for attacking the peptide bond in substrates and serpins (44). Further, the amide N atoms of c193 and c195 make H bonds with the oxyanion, which develops during the formation of the transition state tetrahedral intermediate (44, 45). The Ca\(^{2+}\) binding may stabilize the proper orientation of these two amide N atoms. In trypsin, the interaction of Ca\(^{2+}\) loop with the carboxyl O atoms of c193 and c194

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3 D. Zhong, K. Padmanabhan, and S. P. Bajaj, unpublished data.
is absent (36), which may explain the lack of significant effect of Ca$^{2+}$ on its activity. In the presence or absence of Ca$^{2+}$, Na$^{+}$ increased the affinity of S-2222 (Table I), PABA (Table II), or TFPI (Table III) binding to FXa; 4.5-fold. Because Ca$^{2+}$ does not influence the $K_m$ value of S-2222 and has no effect on the PABA or TFPI binding, it would appear that these effects are primarily mediated through the S1 site in FXa. These results can be readily rationalized, because the Na$^+$ site in FXa is directly linked to the S1 specificity pocket residue Asp-c189 (Fig. 10B). Occupancy of the Na$^+$ site could rigidify the c189 side chain for optimal interaction with the P1 residue (Arg) of substrates and inhibitors. Furthermore, the FVa binding helix in FXa is connected to the Tyr-c225 of the Na$^+$-binding loop via H bonds and van der Waals’ contacts (Fig. 10B). These interactions may or may not be interrupted in the absence of Na$^+$.

**FIG. 10.** Schematic representation of the structure of the protease domain of human FXa. **A,** overall fold of the protease domain. Coordinates (PDB code 1HCG) used are those of Padmanabhan et al. (7). β-Sheets are in red, α-helices are in blue, Ca$^{2+}$-binding loop is in white, the two Na$^+$-binding loops are in yellow and white, respectively, and the autolysis loop is in magenta. The active site residues (Asp-c102, His-c57, and Ser-c195) are labeled D, H, and S, respectively. The amino- and carboxyl termini are marked N and C, respectively. Position of the FVa binding helix is indicated, and the Arg-c165 side chain, which is important for FXa-FVa interaction, is shown. Tyr-c225 side chain thought to be an important determinant for Na$^+$ binding is also depicted. Sodium is shown as a white sphere, and the positions of four residues, namely, c185, c185A, c222B, and c224, which provide the carboxyl O atoms for coordinating to the Na$^+$, are indicated (14). Ca$^{2+}$-binding site involves carbonyl groups of c72 and c75, and the carboxyl groups of c70 and c80. Binding of Ca$^{2+}$ prevents the cleavage of Arg-c150/Gln-c151 in the autolysis loop (8). Residue numbers are based upon chymotrypsin numbering. Ca, calcium; Na, sodium. **B,** relationships of the Na$^+$-binding site to the S1 site and FVa binding helix in FXa. The two Na$^+$-binding loops, c183-c189, colored red, and c221-c225, colored magenta, and the FVa binding helix colored by atom type are shown. Na$^+$ is shown as a blue sphere, and water molecules are shown as red spheres. Dotted lines are H bonds, and dashed lines are van der Waals’ interactions. The Na$^+$ is coordinated by the carbonyl O atoms of c185, c185A, c222, and c224 and two water molecules. The benzene ring of Tyr-c225 makes van der Waals’ contacts with Val-c163, and the OH group of Tyr-c225 is connected via two water molecules to Ser-c167 of the FVa binding helix. Tyr-c225 was mutated to Pro-c225 using the structure of trypsin (36) and thrombin mutant Yc225P (15). When residue c225 is proline, its carbonyl O atom points away from the Na$^+$ site, and its side chain is unable to make contacts with the FVa binding helix. Residue numbers are based upon chymotrypsin numbering.
While this manuscript was in review, a study suggesting that FVa binding may not be affected by the Na⁺ site appeared in press (46). However, further critical data are needed to fully support this conclusion.

Of significance is the observation that Na⁺ site and the protease domain Ca²⁺ site are thermodynamically linked. Thus Ca²⁺ increases the affinity of Na⁺ by ~4·1-fold, and Na⁺ increases the affinity of protease domain Ca²⁺ site by a similar fold (Fig. 6). The thermodynamic linkage between the Na⁺ site and the protease domain Ca²⁺ site of FXa is schematically depicted in Fig. 6. This linkage is also supported by our extensive kinetic data. Thus, although the effects of Na⁺ and Ca²⁺ at individual steps of the thermodynamic cycle depicted in Fig. 6 are different, the overall total change in individual steps of the thermodynamic cycle depicted in Fig. 6. Thus during physiological clotting, FXa formed will mostly exist in sodium-Xa-calcium form in the presence of Ca²⁺ and expressed this mutant. This mutant-Xa(S) bound Na⁺ with caution. A Ec80K mutant of FXa, which is thought to support this conclusion.

Press (46). However, further critical data are needed to fully eliminate the Na⁺ that has maximum biological activity. In an earlier study (23), Tyr-c225 was mutated to Pro to the results obtained with this mutant should be interpreted not totally impaired in binding to Na⁺ but has ~5-fold reduced affinity. This is in contrast to the results reported for Yc225P mutant of IIa, which does not bind Na⁺ (13, 15). These observations could be explained on the basis of difference in the nature of Na⁺ site in IIa (15) and FXa (14). As stated earlier, the Na⁺ site in IIa involves only two carbonyl O atoms from the protein (14, 15), whereas in FXa it involves four carbonyl O atoms (14). This is shown in Fig. 10B. A change of Tyr-c225 to Pro will result in the loss of one ligand out of two in IIa and one out of four in FXa (Fig. 10B). Thus, three ligands from the protein in Yc225P FXa may still be available to bind Na⁺, and the results obtained with this mutant should be interpreted with caution. A Ec80K mutant of FXa, which is thought to mimic the Ca²⁺-binding site has also been used to study the role of Ca²⁺ on Na⁺ binding (23). This mutant-Xa(S) in our hands binds Na⁺ with a dissociation constant of ~25 mM in the presence of absence of Ca²⁺. This dissociation constant of ~25 mM is very close to the 40 mM Kd,Na obtains in the absence of Ca²⁺ for wild-type FXa. Thus, Ec80K FXa mutant may not completely mimic the protease domain Ca²⁺ occupied wild-type Xa(S). Our data with the Ec80K FXa mutant are consistent with our previous studies on Ec70K factor IX mutant, which did not completely mimic the properties of Ca²⁺-occupied form of factor IXa (47). We are currently in the process of expressing several mutants of FX to further understand the nature of the Na⁺ site as well as the protease domain Ca²⁺ site in this important coagulation protease.