doi:10.1016/j.jmb.2005.04.052 J. Mol. Biol. (2005) 350, 78–91

JMB

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Na^+ Site in Blood Coagulation Factor IXa: Effect on Catalysis and Factor VIIIa Binding

Amy E. Schmidt[1,2], Jonathan E. Stewart[2], Akash Mathur[2] Sriram Krishnaswamy[3] and S. Paul Bajaj[1,2]*

[1]*UCLA/Orthopaedic Hospital Department of Orthopaedic Surgery and Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA*

[2]*Department of Pharmacological & Physiological Sciences, Saint Louis University School of Medicine, St Louis, MO 63104 USA*

[3]*Joseph Stokes Research Institute, Children's Hospital of Philadelphia, and Department of Pediatrics, University of Pennsylvania, Philadelphia PA 19104, USA*

**Corresponding author*

During blood coagulation, factor IXa (FIXa) activates factor X (FX) requiring Ca^{2+}, phospholipid, and factor VIIIa (FVIIIa). The serine protease domain of FIXa contains a Ca^{2+} site and is predicted to contain a Na^+ site. Comparative homology analysis revealed that Na^+ in FIXa coordinates to the carbonyl groups of residues 184A, 185, 221A, and 224 (chymotrypsin numbering). Kinetic data obtained at several concentrations of Na^+ and Ca^{2+} with increasing concentrations of a synthetic substrate (CH_3-SO_2-D-Leu-Gly-Arg-*p*-nitroanilide) were fit globally, assuming rapid equilibrium conditions. Occupancy by Na^+ increased the affinity of FIXa for the synthetic substrate, whereas occupancy by Ca^{2+} decreased this affinity but increased k_{cat} dramatically. Thus, Na^+-FIXa-Ca^{2+} is catalytically more active than free FIXa. $FIXa_{Y225P}$, a Na^+ site mutant, was severely impaired in Na^+ potentiation of its catalytic activity and in binding to *p*-aminobenzamidine (S1 site probe) validating that substrate binding in FIXa is linked positively to Na^+ binding. Moreover, the rate of carbamylation of NH_2 of Val16, which forms a salt-bridge with Asp194 in serine proteases, was faster for $FIXa_{Y225P}$ and addition of Ca^{2+} overcame this impairment only partially. Further studies were aimed at delineating the role of the FIXa Na^+ site in macromolecular catalysis. In the presence of Ca^{2+} and phospholipid, with or without saturating FVIIIa, $FIXa_{Y225P}$ activated FX with similar K_m but threefold reduced k_{cat}. Further, interaction of FVIIIa:$FIXa_{Y225P}$ was impaired fourfold. Our previous data revealed that Ca^{2+} binding to the protease domain increases the affinity of FIXa for FVIIIa ~15-fold. The present data indicate that occupancy of the Na^+ site further increases the affinity of FIXa for FVIIIa fourfold and k_{cat} threefold. Thus, in the presence of Ca^{2+}, phospholipid, and FVIIIa, binding of Na^+ to FIXa increases its biologic activity by ~12-fold, implicating its role in physiologic coagulation.

Keywords: serine protease; factor IXa; sodium; calcium; factor VIIIa

Introduction

Serine proteases are a class of enzymes that are involved in diverse physiologic processes including digestion, fertilization, coagulation, fibrinolysis, ossification, and complement fixation.[1–3] The serine protease domains of enzymes belonging to the clan SA are comprised of two antiparallel β-barrel subdomains with the active site residing in a cleft between the two subdomains.[4] These enzymes

Abbreviations used: pAB, *p*-aminobenzamidine; Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; PL, phospholipid; FIX_{WT}, wild-type factor IX; FIX_{NP}, normal plasma factor IX; FIX_{Y225P}, factor IX with Tyr225 [395]→Pro mutation; FIX_{E80V}, factor IX with Glu80 [245]→Val mutation; FXIa, factor XIa; FX, factor X; TBS, 50 mM Tris–HCl, 150 mM NaCl, pH 7.5; *p*NA, *p*-niroaniline; BSA, bovine serum albumin; PEG, polyethylene glycol; HBSP, 20 mM Hepes, 0.15 M NaCl, 0.1% PEG, pH 7.5; DFP, diisopropylfluorophosphate; dEGRck, dansyl-Glu-Gly-Arg-chloromethyl ketone; dEGR-FIXa, factor IXa inhibited with dEGRck; CBS 31.39, CH_3SO_2-D-Leu-Gly-Arg-*p*-nitroaniline; S-2288, H-D-Ile-Pro-Arg-*p*-nitroaniline; S-2222, benzoyl-Ile-Glu-Gly-Arg-*p*-nitroaniline.

E-mail address of the corresponding author: pbajaj@mednet.ucla.edu

contain a catalytic triad composed of His57, Asp102, and Ser195.[1] Ser195 is hydrogen bonded to $N^{\varepsilon 2}$ of His57 whereas $N^{\delta 1}$ of His57 is hydrogen bonded to the buried side-chain of Asp102.[5] This arrangement makes Ser195 an unusually strong nucleophile and permits peptide bond hydrolysis. The substrate-binding sites that are involved in precise interactions are referred to as S*n*,… S3, S2, S1, S1′, S2′, S3′,… S*n*′ sites, and the amino acid residues of the substrate that occupy these sites are referred to as P*n*,… P3, P2, P1, P1′, P2′, P3′,… P*n*′, respectively.[6] In the trypsin family of clan SA, Asp189 is at the bottom of the S1 specificity pocket, and interacts with the positively charged side-chains of the P1 residue Lys or Arg in the substrate.[7] The peptide bond in the substrate that is cleaved is between residues P1 and P1′.

A review of the literature indicates that, on the basis of Ca^{2+} and Na^+-binding properties, the trypsin family of proteases may be further divided into four subgroups: (1) enzymes that do not have a Ca^{2+} site or a Na^+ site, such as plasmin, factor XIa, and factor XIIa;[8] (2) enzymes that have only a Ca^{2+} site, such as bovine trypsin and pig elastase;[8–10] (3) enzymes that have only a Na^+ site, such as thrombin;[11,12] and (4) enzymes that have both a Ca^{2+} site and a Na^+ site such as factor Xa (FXa), activated protein C (APC), FVIIa, and FIXa†.[12–17] Notably, the Ca^{2+} site is located in the N-terminal subdomain 1 and the Na^+ site is located in the C-terminal subdomain 2.[8–18]

FIX is a 415 residue vitamin K-dependent protein that circulates in the blood as an inactive zymogen (57,000 Da) of the serine protease FIXa.[19,20] During physiologic coagulation, it is activated by FXIa/Ca^{2+} and FVIIa/tissue factor/Ca^{2+}.[20,21] Activation of FIX by either enzyme occurs in two sequential steps to produce an active serine protease and release of a 35 residue activation peptide of amino acid residues 146–180.[20,22] Upon activation, the newly generated amino group of Val16 [181]‡ then turns inward and forms a salt-bridge with the carboxylate group of Asp194 [364];[19,20] this interaction is a defining characteristic of conversion of the zymogen to the active enzyme.[2,7] The N-terminal light chain (residues 1–145) of FIXa contains the γ-carboxyglutamic acid (Gla) domain followed by two epidermal growth factor-like domains (EGF1 and EGF2), whereas the C-terminal heavy chain (residues 181–415) contains the serine protease domain.[19,20]

FIXa activates FX to FXa in the coagulation cascade, which requires three known cofactors for physiologic hemostasis, namely, Ca^{2+}, phospholipid (PL) provided by platelets, and FVIIIa.[24] The Gla domain of FIX/FIXa possesses several Ca^{2+}-binding sites,[25,26] while the EGF1 and protease domains each have one high-affinity Ca^{2+}-binding site.[23,27,28] The Gla domain of FIX/FIXa is essential for its binding to PL or platelets, and the EGF1 domain of FIX is required for activation by FVIIa/tissue factor/Ca^{2+}.[29–32] The Gla domain, EGF1 domain, EGF2 domain, and the protease domain of FIXa have all been implicated in binding to FVIIIa.[30,33–37]

Here, a comparative analysis of homologous proteins reveals that the Na^+ site in FIXa is similar to that in FXa, APC, and FVIIa but not to that in thrombin. Functional characterization of the Na^+ site in FIXa indicates that, like in APC[14] and FXa,[38] it is linked thermodynamically to the substrate-binding site primarily through the S1 site. However, unlike in APC[14] and FXa,[38] the protease domain Ca^{2+} site in FIXa is also linked to the substrate-binding site. Further, in contrast to FXa and APC where the Na^+ site and Ca^{2+} site are strongly linked, this linkage is weak in FIXa. Importantly, Na^+-binding in the absence or in the presence of Ca^{2+} shifts the zymogen–enzyme equilibrium of FIXa further towards a more enzyme-like conformation, such that the catalytic activity and FVIIIa binding are improved. The importance of the functional significance of the Na^+-binding site is manifested in two naturally occurring mutations in FIXa that disrupt the Na^+ site region in hemophilia B patients§.[39] An initial account of this work has been presented.[40]

Results

Relationship of the putative Na^+ site, protease domain Ca^{2+} site and active site in FIXa

Figure 1 illustrates the position of the putative Na^+ site‖ in FIXa and its spatial relation to the protease domain Ca^{2+} site, active site, and the FVIIIa binding 162-helix [330-helix]. Based upon the structures of FXa, FVIIa, and APC, it involves the carbonyl groups of residues 184A [353], 185 [354], 221A [391], and 224 [394]. It is predicted that the solvent water molecules will provide the other two ligands, as is the case in FXa, FVIIa, and APC. In modeling the Na^+ site, no adjustments in the main chain of FIXa (PDB code 1rfn) were necessary and all protein coordinating ligands were within

† For comparison, the chymotrypsin numbering system is used throughout. Residue 225 in chymotrypsin corresponds to residue 395 in FIX. Where necessary, the amino acid corresponding to the numbering in chymotrypsin is given followed by the FIX numbering in square brackets e.g. 225 [395].

‡ FIXa is used to refer to both $FIXa_{NP}$ and $FIXa_{WT}$ as there were no observable kinetic differences between them in the experiments performed in this study.

§ The 13th edition (2004) is available at: http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html.

‖ Since the FIXa protein and solvent ligands involved in coordination to Na^+ have not been structurally determined, the proposed Na^+ site in this work should be viewed as a putative site. Identification of the precise ligands involved in precise Na^+ coordination must await solving the crystal structure of FIXa at a higher resolution than currently known.

2.8–3.1 Å. Next, we directed effort towards understanding the role of Na^+ in synthetic substrate hydrolysis and in modulating the FIXa zymogen–enzyme equilibrium, as well as in macromolecular catalysis and FVIIIa binding.

Effects of monovalent cations on the amidolytic activity of FIXa

We first wished to find an inert monovalent cation that could be used to keep the ionic strength constant during the kinetic experiments. In separate experiments, hydrolysis of CBS 31.39 (CH_3SO_2-D-Leu-Gly-Arg-*p*-nitroaniline) by FIXa was measured at various concentrations of three monovalent cations, namely, choline (Ch^+), Li^+, or Rb^+. As shown in Figure 2(a), Li^+ and Rb^+ did not inhibit or potentiate the amidolytic activity of FIXa; whereas, Ch^+ had an inhibitory effect. Further, Na^+ potentiated the amidolytic activity of FIXa to similar extent whether or not Li^+ or Rb^+ was present (Figure 2(b)). In contrast, Ch^+ inhibited the potentiation of FIXa activity by Na^+ (Figure 2(b)). On the basis of these experiments, Rb^+ was chosen as the compensatory ion for further studies.

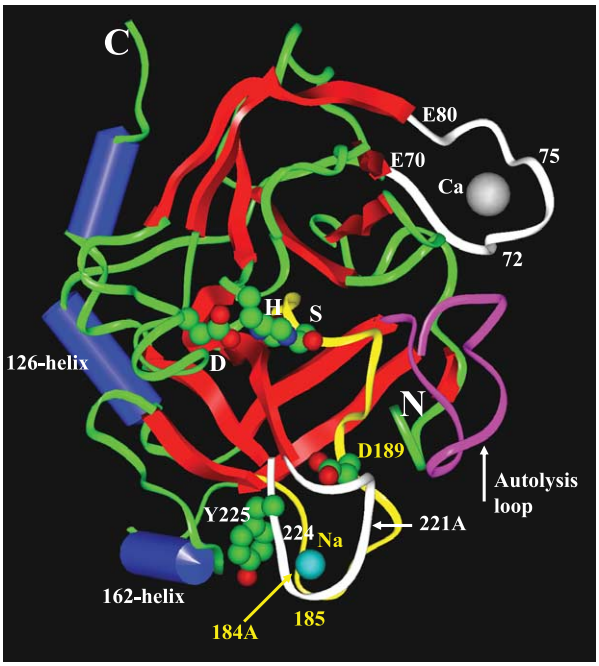


Figure 1. Location of the putative Na^+ site and the Ca^{2+} site in the protease domain of human FIXa. The β-sheets are in red, α-helices are in blue, the Ca^{2+}-binding loop is in white, the two Na^+-binding loops are in yellow (183–189 [353–359]) and white (221–225 [390–395]), and the autolysis loop is in magenta. The amino and carboxyl termini are marked *N* and *C*, respectively. Oxygen is red, nitrogen is blue, carbon is green, Na^+ is cyan, and Ca^{2+} is white. The S1 site specificity residue, Asp189 [359] that is most likely linked to the Na^+ site *via* water molecules is colored by atom type. The active site residues (His57 [221], Asp102 [269], and Ser195 [365]) are labeled H, D, and S, respectively. The two helices, 126 helix [293 helix] and 162 helix [330 helix], implicated in binding to FVIIIa are also shown. Residues 184A [353], 185 [354], 221A [391], and 224 [394] that are important in binding to Na^+, and residues 70 [235], 72 [237], 75 [240], and 80 [245] that are involved in binding to Ca^{2+} are labeled. Tyr225 [395], an important determinant for Na^+-binding, is depicted.[11,17]

Effect of Na^+ and Ca^{2+} on the potentiation of CBS 31.39 hydrolysis by FIXa

The enhancement of substrate hydrolysis using various concentrations of Na^+ or Ca^{2+} at different concentrations of CBS 31.39 is shown in Figure 3(a) and (b), respectively. Similarly, the enhancement of

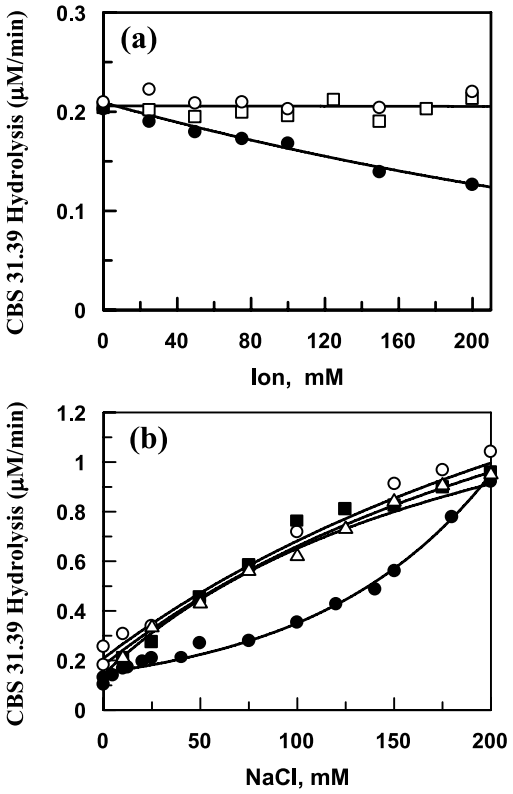


Figure 2. Effect of monovalent cations on the amidolytic activity of FIXa. (a) The effect of Rb^+, Ch^+, and Li^+ on the amidolytic activity of FIXa. Reaction mixtures contained 1 mM CBS 31.39 and 125 nM FIXa in 50 mM Tris (pH 7.4), 0.1% (w/v) PEG 8000, 1 mM EDTA, and various concentrations of either Rb^+ (open circles), Ch^+ (filled circles), or Li^+ (open squares). The chloride salt of each ion was used. (b) The effect of Na^+ on the amidolytic activity of FIXa. The buffer used is the same as that used in (a). Filled squares represent an experiment where increasing concentrations of Na^+ were used with no compensating ion. In the other experiments, the concentration of monovalent cation was kept constant at 0.2 M by the addition of Rb^+ (open circles), Ch^+ (filled circles), or Li^+ (open triangles) as compensating ions. For example, when the concentration of Na^+ used was 50 mM, the concentration of Rb^+, Li^+, or Ch^+ was 150 mM. Similarly, when the concentration of Na^+ used was 150 mM, the concentration of Rb^+, Li^+, or Ch^+ was reduced to 50 mM. Thus, at any point, the combined total concentration of Na^+ and the compensating ion (Rb^+, Li^+, or Ch^+) was 200 mM.

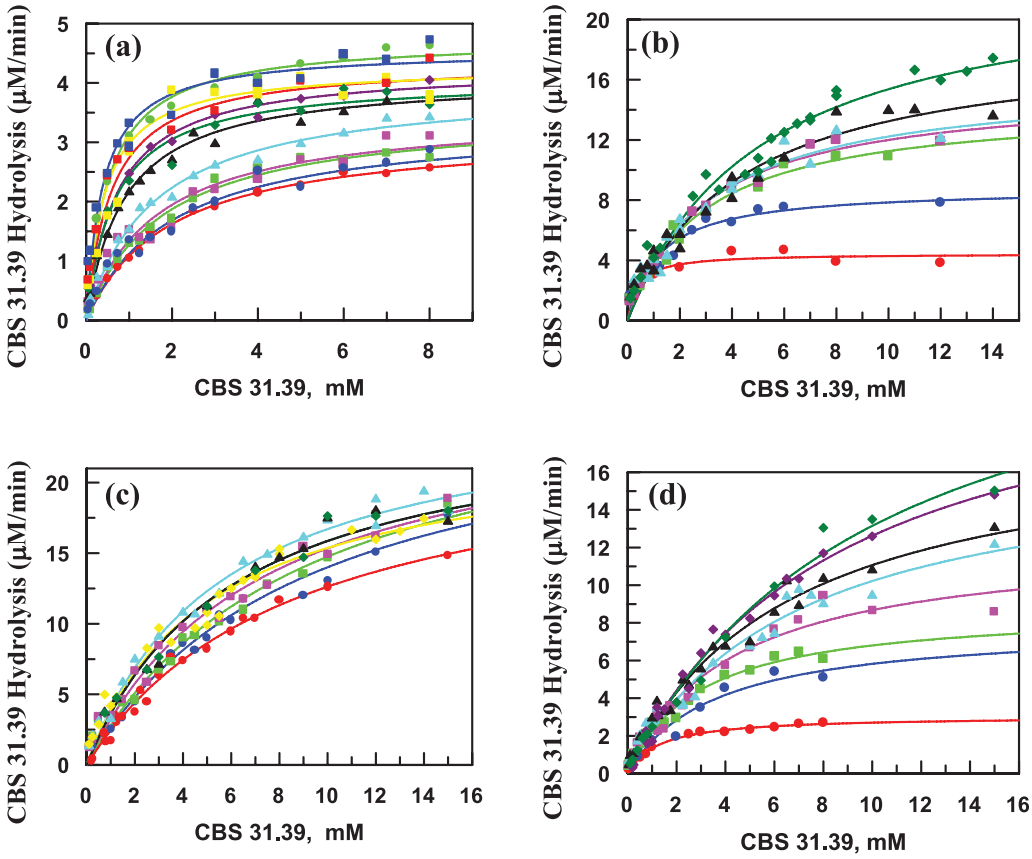
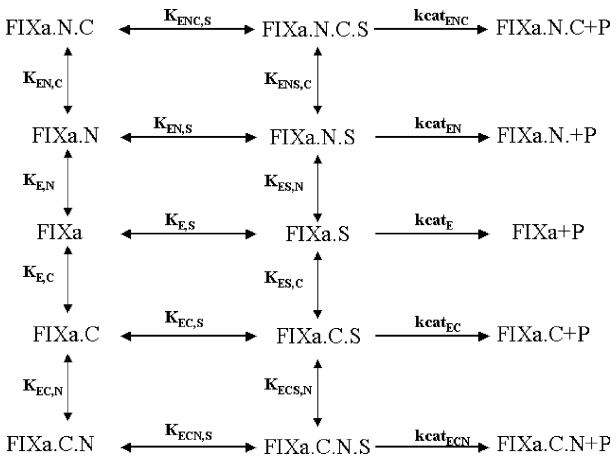


Figure 3. Sodium and calcium-mediated potentiation of CBS 31.39 hydrolysis by FIXa. (a) The Na^+-mediated potentiation of CBS 31.39 hydrolysis in the absence of Ca^{2+}. The concentrations of Na^+ are: 0 mM (red circles), 5 mM (blue circles), 10 mM (green squares), 15 mM (magenta squares), 20 mM (cyan triangles), 50 mM (black triangles), 75 mM (dark green diamonds), 100 mM (purple diamonds), 125 mM (yellow squares), 150 mM (red squares), 190 mM (blue squares), and 200 mM (green circles). (b) The Ca^{2+}-mediated potentiation of CBS 31.39 hydrolysis in the absence of Na^+. The concentrations of Ca^{2+} are: 0 mM (red circles), 0.2 mM (blue circles), 0.5 mM (green squares), 0.75 mM (magenta squares), 1 mM (cyan triangles), 3 mM (black triangles), and 5 mM (dark green diamonds). (c) The Na^+-mediated potentiation of CBS 31.39 hydrolysis in the presence of 5 mM Ca^{2+}. The concentrations of Na^+ are: 0 mM (red circles), 10 mM (blue circles), 20 mM (green squares), 50 mM (magenta squares), 100 mM (cyan triangles), 125 mM (black triangles), 150 mM (yellow diamonds), and 185 mM (dark green diamonds). (d) The Ca^{2+}-mediated potentiation of CBS 31.39 hydrolysis in the presence of 200 mM Na^+.The concentrations of Ca^{2+} are: 0 mM (red circles), 0.05 mM (blue circles), 0.1 mM (green squares), 0.25 mM (magenta squares), 0.5 mM (cyan triangles), 1 mM (black triangles), 3 mM (purple diamonds), and 5 mM (dark green diamonds). All data are normalized to 125 nM FIXa, and Rb^+ was used as the compensatory ion to keep the ionic strength at 0.2 M. All lines are drawn following global analysis according to Scheme 1, using the fitted values in Table 1.

substrate hydrolysis using various concentrations of Na^+ in the presence of Ca^{2+} or various concentrations of Ca^{2+} in the presence of Na^+ is depicted in Figure 3(c) and (d), respectively. The entire data set was fit globally using the program Dynafit according to Scheme 1, and the calculated parameters are given in Table 1. In this scheme, N represents Na^+, C represents Ca^{2+}, E represents enzyme, and S and P represent substrate and product, respectively. Here, $K_{ENC,S}$ equals $K_{ECN,S}$ and k_{catENC} equals k_{catECN}.

The binding of Na^+ to FIXa increased the affinity of the substrate by 6.5-fold, with only a small increase in k_{cat} (see Table 1). Conversely, binding of Ca^{2+} to FIXa decreased the affinity of the substrate by 4.7-fold and increased k_{cat} by 8.6-fold. Na^+ binding to Ca^{2+}-bound FIXa increased the affinity of the substrate by 1.9-fold without affecting k_{cat}. Further, Ca^{2+} binding to Na^+-bound FIXa decreased the affinity of the substrate by 16.3-fold and increased k_{cat} by 6.3-fold. Of interest is the observation that in the absence of substrate, the Ca^{2+} and Na^+ sites are linked positively, whereas in the presence of substrate these sites are linked negatively; however, the linkage is weak in each case. Thus, Na^+ increases the affinity; whereas Ca^{2+} increases k_{cat} and decreases the affinity of FIXa for the substrate. Conclusions from the global fitting approach were verified independently by initial velocity studies of peptidyl substrate cleavage at near saturating concentrations of Na^+ and/or Ca^{2+}. There was good agreement between steady-state kinetic constants determined under these conditions (Table 2) with those inferred from the global analysis (Table 1). Overall, the catalytic efficiency of Na^+-FIXa is 5.6-fold higher, that of



Scheme 1.

FIXa-Ca^{2+} is 1.7-fold higher, and that of Na^+-FIXa-Ca^{2+} is 3.5-fold higher than for free FIXa (Table 2).

Effect of Na^+ and Ca^{2+} on the potentiation of CBS 31.39 hydrolysis by $FIXa_{Y225P}$ and $FIXa_{E80V}$

Dang and Di Cera have postulated that a change of residue Tyr225 [395] to Pro would impair the Na^+ site in FIXa.[17] Thus, $FIXa_{Y225P}$ was used to verify the contribution of Na^+ binding to the catalytic activity of FIXa towards a synthetic substrate. K_m and k_{cat} data for $FIXa_{Y225P}$ hydrolysis of CBS 31.39 are given in Table 2. In the absence of both Na^+ and Ca^{2+}, the rates of hydrolysis of CBS 31.39 were slow and only approximate values could be obtained. Further, Na^+ increased the catalytic activity of $FIXa_{Y225P}$, suggesting that the Na^+ site in the mutant is not abolished but is severely impaired. This is consistent with modeling of the Na^+ site in FIXa, where three of the four protein ligands may still be available to coordinate with Na^+ (Figure 1).

Addition of 5 mM Ca^{2+} increased K_m to 7.8 mM for $FIXa_{Y225P}$, a value that is similar to that obtained with FIXa in the presence of Ca^{2+} but in the absence of Na^+. However, in contrast to FIXa, this K_m value did not decrease further upon addition of Na^+. Importantly, as seen for FIXa, Ca^{2+} was able to increase the k_{cat} values of $FIXa_{Y225P}$ substantially (Table 2). However, it was still fourfold lower than Na^+-FIXa-Ca^{2+}. Thus, Ca^{2+} cannot fully overcome the structural impairment caused by Na^+ site disruption.

$FIXa_{E80V}$, a mutant defective in binding Ca^{2+} in the protease domain,[41] hydrolyzed CBS 31.39 (5 mM) poorly and the rate was $\sim$0.5 μM min^{-1} in the absence of Na^+ and Ca^{2+} at an enzyme concentration of 375 nM. Addition of 10 mM Ca^{2+} or 200 mM Na^+ did not result in an increase the hydrolytic rate of synthetic substrate by $FIXa_{E80V}$. The lack of potentiation by Ca^{2+} is expected, since the mutant is defective in binding to Ca^{2+}. Since the change in V_{max} by Na^+ is only 1.4-fold for FIXa (Table 1), it is to be expected that the activity of the Ca^{2+} mutant was not improved enough to be measured accurately. These observations support the premise that the Ca^{2+} effects observed for FIXa in the present study are due to occupancy of the protease domain Ca^{2+} site.

Effects of Na^+ and Ca^{2+} on the interaction of *p*AB with FIXa and $FIXa_{Y225P}$

In the previous section, Na^+ binding to FIXa

Table 1. Scheme 1 parameters determined by global fit analysis

Parameters	Fitted value
$K_{E,S}$ (mM)	2.09±0.48
$K_{EN,S}$ (mM)	0.32±0.09
$K_{EC,S}$ (mM)	9.86±0.67
$K_{ENC,S}$ (mM)	5.22±0.33
$K_{E,N}$ (mM)	89.9±40.7
$K_{ES,N}$ (mM)[a]	13.8±2.15
$K_{EC,N}$ (mM)[b]	54.8±4.3
$K_{ECS,N}$ (mM) [c]	28.8±4.0
$K_{E,C}$ (μM)	69.4±14.5
$K_{ES,C}$ (μM)[d]	330±120
$K_{EN,C}$ (μM)	42.3±12.6
$K_{ENS,C}$[e] (μM)	690±340
k_{catE} (min^{-1})	25.9±2.7
k_{catEN} (min^{-1})	33.7±1.2
k_{catEC} (min^{-1})	215±8.7
k_{catENC} (min^{-1})	212±5.5

Note that the value of $K_{ENC,S}$ is the same as that of $K_{ECN,S}$ and the value of k_{catECN} is the same as that of k_{catENC}.
[a] $K_{ES,N}=K_{E,N}$ ($K_{EN,S}/K_{E,S}$).
[b] $K_{EC,N}=K_{E,N}$ ($K_{EN,C}/K_{E,C}$).
[c] $K_{ECS,N}=K_{ES,N}$ ($K_{ENS,C}/K_{ES,C}$).
[d] $K_{ES,C}=K_{E,C}$ ($K_{EC,S}/K_{E,S}$).
[e] $K_{ENS,C}=K_{EN,C}$ ($K_{ENC,S}/K_{EN,S}$).

Table 2. Effect of Na^+ and Ca^{2+} on the Hydrolysis of CBS 31.39 by FIXa and $FIXa_{Y225P}$

Conditions				
Na^+	Ca^{2+}	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
FIXa				
−	−	1.9±0.1	25±2.0	13 (1.0)
+	−	0.5±0.1	38±2.1	73 (5.6)
−	+	8.7±1.3	189±17	22 (1.7)
+	+	4.5±0.6	203±21	45 (3.5)
$FIXa_{Y225P}$				
−	−	2.1±0.5[a]	0.3±0.1[a]	0.1 (1.0)
+	−	3.1±0.5	3.5±0.4	1.1 (11)
−	+	7.9±0.4	53.2±5.5	6.8 (68)
+	+	7.3±0.2	47.0±5.2	6.4 (64)

Increasing amounts of CBS 31.39 were added to reaction mixtures containing 125–375 nM FIXa or $FIXa_{Y225P}$ in 50 mM Tris (pH 7.4) containing 0.1% PEG 8000. To keep the ionic strength constant, the concentration of Na^+ was 200 mM in the absence of Ca^{2+} and 185 mM in the presence of 5 mM Ca^{2+}. The fold change in catalytic efficiency is given in parentheses. The results presented are the average of three experiments±SE.
[a] The rate for $FIXa_{Y225P}$ in the absence of Na^+ and Ca^{2+} was very slow even at high concentrations of substrate and an accurate determination of K_m and k_{cat} could not be carried out. The numbers given reflect the best estimates.

increased the affinity for the synthetic substrate, whereas Ca^{2+} binding lowered this affinity. The S1 site in serine proteases is known to play a dominant role in binding of synthetic substrates. Therefore, we examined whether the reactivity of the S1 site in FIXa is altered upon binding of Na^+ and/or Ca^{2+}. We used *p*AB as an S1 site probe for these studies. The interactions of *p*AB with FIXa, determined under each of the four salt conditions, are summarized in Table 3. In the presence of Na^+, the affinity of *p*AB for FIXa was increased fourfold; whereas Ca^{2+} had essentially no effect. Further, addition of Na^+ in the presence of Ca^{2+} had minimal effect on the affinity of FIXa for *p*AB. These data indicate that Na^+ may be primarily affecting the S1 site with smaller effects on the S2/S3/S4 sites; whereas, Ca^{2+} may be primarily affecting the S2/S3/S4 sites with additional effects on the S1 site in FIXa (Tables 1–3).

The S1 site of $FIXa_{Y225P}$ was probed using pAB under various conditions as described above for normal FIXa. These data are provided also in Table 3. K_{ipAB} in the presence of Na^+ was 813 μM for $FIXa_{Y225P}$ as compared to 25 μM for normal FIXa. However, in the presence of Ca^{2+} or both Ca^{2+} and Na^+, K_{ipAB} decreased to ~180 μM, a value that does not differ significantly from that of normal FIXa (119–166 μM) under comparable conditions. Since $FIXa_{Y225P}$ is impaired in binding to Na^+, the lowering of K_{ipAB} is due to binding of Ca^{2+} in the protease domain. Thus, in the mutant FIXa, Ca^{2+} binding can partially overcome the impairment caused by disruption of Na^+ binding. These results further indicate that the Ca^{2+} site is also linked to the S1 site in FIXa.

Carbamylation of Val16 using NaNCO

Since the S1 site in serine proteases is linked allosterically to salt-bridge formation between the free amino group of Val16 and the carboxyl group of Asp194,[11,12] we studied the rate of carbamylation of the free amino group of Val16 [181] in FIXa and $FIXa_{Y225P}$. Since NaNCO is the reagent used for carbamylation, studies could be performed only in the presence or in the absence of Ca^{2+}. These data are presented in Figure 4 and are summarized in Table 4. Ca^{2+} decreased the carbamylation rate of FIXa by 2.9-fold. Similarly, *p*AB reduced the rate of carbamylation by 3.5-fold. Addition of both Ca^{2+} and *p*AB reduced the carbamylation rate by 13.5-fold, indicating that Ca^{2+} and *p*AB both stabilize the H-bond between Val16 [181] and Asp194 [364], and shift the zymogen–enzyme equilibrium towards a more enzyme-like form in FIXa.

The effect of Na^+ binding to FIXa on the carbamylation of Val16 [181] was examined using

Table 3. Effect of Na^+ and Ca^{2+} on the inhibition constants for Interaction of *p*AB with FIXa and $FIXa_{Y225P}$

Protein	Conditions		
	Na^+	Ca^{2+}	K_{ipAB} (μM)
FIXa	−	−	99±10
	+	−	25±3
	−	+	166±14
	+	+	119±8
$FIXa_{Y225P}$	−	−	ND[a]
	+	−	813±22
	−	+	184±26
	+	+	179±21

To keep the ionic strength constant, the concentration of Na^+ was 200 mM in the absence and 185 mM in the presence of 5 mM Ca^{2+}. The results presented are the average of two K_i values obtained using 100 μM and 200 μM *p*AB at various concentrations of the synthetic substrate as described in Materials and Methods.

[a] ND, not determined.

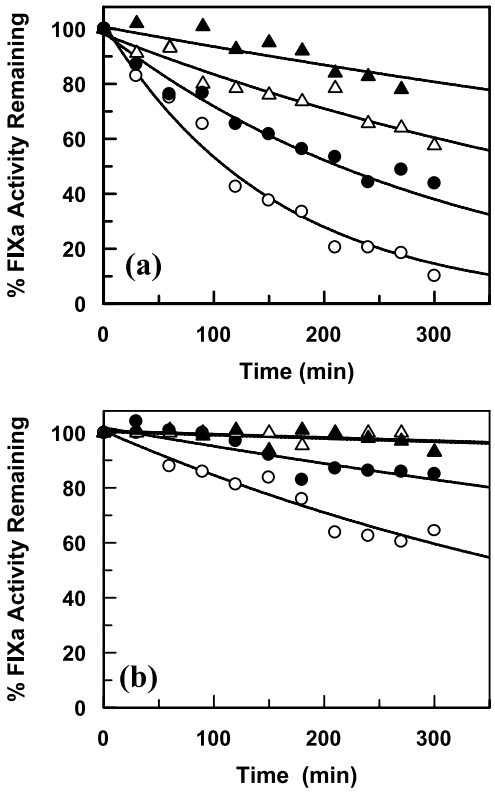


Figure 4. Carbamylation of NH_2-Val16 of FIXa and $FIXa_{Y225P}$ using NaNCO. (a) The effect of Ca^{2+} on carbamylation of FIXa and the Na^+ binding mutant $FIXa_{Y225P}$. (b) The effect of *p*AB and Ca^{2+}/*p*AB on carbamylation of FIXa and $FIXa_{Y225P}$. All reaction mixtures contained 2 μM FIXa or $FIXa_{Y225P}$ in HBSP. When *p*AB was used, its concentration was tenfold higher than K_{ip}AB under the particular Na^+/Ca^{2+} conditions. The reactions were started by the addition of 0.2 M NaNCO. Every 30 minutes, a 20 μl aliquot was removed, added to 140 μl of HBSP containing 1 mM CBS 31.39, and the residual activity determined immediately. The residual activities were then plotted as a percentage of initial activity *versus* time to obtain k_{obs} (pseudo first-order rate constant) for carbamylation. k_{obs} were then converted to second-order rate constants and are given in Table 4. (a) Open circles, $FIXa_{Y225P}$+EDTA; filled circles, FIXa+EDTA; open triangles, $FIXa_{Y225P}+Ca^{2+}$; filled triangles, $FIXa+Ca^{2+}$. (b) Open circles, $FIXa_{Y225P}$+*p*AB; filled circles, FIXa+*p*AB; open triangles, $FIXa_{Y225P}+Ca^{2+}$+*p*AB; filled triangles, $FIXa_{Y225P}+Ca^{2+}$+*p*AB.

Table 4. Rate constants for carbamylation of Val16 [181] in FIXa and $FIXa_{Y225P}$

Protein	Ca^{2+}	*p*AB	Rate (10^3 M^{-1} min^{-1})	Fold decrease in carbamylation
FIXa	−	−	14.85±1.6	1.0[a]
	+	−	5.14±1.0	2.9
	−	+	4.28±0.8	3.5
	+	+	1.10±0.3	13.5
$FIXa_{Y225P}$	−	−	34.80±3.0	1.0[a]
	+	−	8.05±1.0	4.3
	−	+	9.90±1.5	3.5
	+	+	1.25±0.3	27.8

The rates of carbamylation of the amino group of Val16 [181] were measured in the presence and in the absence of 5 mM Ca^{2+} over a period of five hours. Aliquots were removed every 30 minutes and assayed for residual FIXa activity. Saturating concentrations of *p*AB were used to examine the effect of S1 site occupancy on the carbamylation rate. The results presented are the averages of two experiments.

[a] Fold change is based upon the carbamylation rate in the absence of Ca^{2+} or *p*AB for normal FIXa or $FIXa_{Y225P}$.

the Na^+ binding mutant, $FIXa_{Y225P}$. The carbamylation of Val16 [181] in $FIXa_{Y225P}$ was 2.3-fold faster than normal FIXa, indicating that Na^+ binding acts to stabilize the salt-bridge formation. Addition of Ca^{2+} or saturating *p*AB corrected this impairment only partially. However, Ca^{2+} and saturating *p*AB together appear to nearly correct the defect in $FIXa_{Y225P}$ caused by impairment of the Na^+ site. This is expected, since S1 site occupancy is linked to the stability of the salt-bridge, and Ca^{2+} and *p*AB both affect the S1 site. Importantly, in the absence of any modulating ligands, the conformation of the protease domain of $FIXa_{Y225P}$ is shifted more towards the zymogen form as compared to normal FIXa.

Binding of FVIIIa to FIXa and $FIXa_{Y225P}$

Studies in the previous sections indicate that the zymogen–enzyme equilibrium in $FIXa_{Y225P}$ is shifted more towards the zymogen form. Here, we investigated whether active site-blocked $FIXa_{Y225P}$, which is locked in the enzyme conformation, is impaired in binding to FVIIIa. These data are presented in Figure 5. In this competition-based assay, dEGR-FIXa and dEGR-$FIXa_{Y225P}$ proteins were used to compete for FIXa binding to FVIIIa and inhibit FXa generation. K_{dapp} obtained under these conditions for dEGR-FIXa was 0.1 nM and for $FIXa_{Y225P}$ it was 0.4 nM. Thus, dEGR-$FIXa_{Y225P}$ is fourfold defective in binding to FVIIIa.

Kinetics of FX activation by FIXa and $FIXa_{Y225P}$

In the presence of Ca^{2+} and Na^+, $FIXa_{Y225P}$ is fourfold defective in hydrolyzing the synthetic substrate. Here, we investigated whether the Na^+ site mutant is defective in activating FX. These data are presented in Figure 6. In the presence of PL and Ca^{2+},with or without FVIIIa, $FIXa_{Y225P}$ activated FX with similar K_m values but threefold reduced k_{cat}.

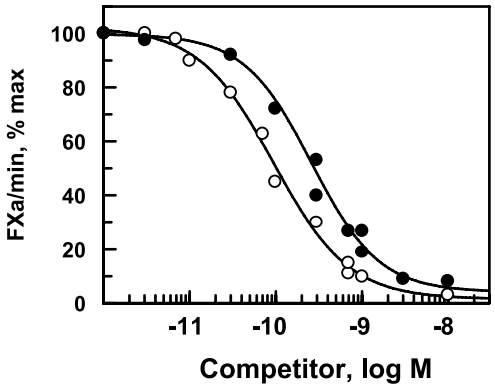


Figure 5. Inhibition of FXa generation by dEGR-FIXa and dEGR-$FIXa_{Y225P}$. Each reaction mixture contained 0.2 nM FIXa, 0.07 nM FVIIIa, 480 nM FX, 10 μM PL, 5 mM $CaCl_2$, and various concentrations of dEGR-FIXa or dEGR-$FIXa_{Y225P}$ in TBS with 1 mg/ml of BSA. The percentage of FXa generated at various concentrations of dEGR-FIXa (open circles) or dEGR-$FIXa_{Y225P}$ (filled circles) is depicted. The curves represent best fit to the IC_{50} four-parameter logistic equation (equation (3)).

Thus, $FIXa_{Y225P}$ is impaired in hydrolyzing the synthetic substrate as well as the macromolecular physiologic substrate, FX.

Discussion

The purpose of the present study was to further characterize the FIXa protease domain Ca^{2+} site as well as the Na^+ site and their roles in regulating the catalytic activity and binding to FVIIIa. The spatial relationship of the Ca^{2+} site, Na^+ site, autolysis loop, Asp189 [359] S1 site, and the catalytic triad is provided in Figure 1. Di Cera and colleagues reported that several serine proteases, including those involved in coagulation, possess a functional Na^+ site.[17] X-ray crystal structures of thrombin,[11,12] FXa,[13,38] APC,[14,43] and FVIIa[15,16] have been reported where the Na^+ site in these molecules is defined. The Na^+ site in thrombin uses a single loop involving the carbonyl oxygen atoms of residues 221A and 224 as well as four water molecules, whereas the Na^+ site in FXa, APC, and FVIIa uses two loops involving the carbonyl oxygen atoms of residues 184A, 185, 221A, and 224 as well as two water molecules. The nature of residue 225 plays an important role in orienting the carbonyl oxygen atom of residue 224 towards the Na^+-coordination shell.[17] As shown in Figure 7, the Na^+ site in FIXa resembles that of FXa, APC, and FVIIa but not thrombin. This may be due to the insertion of three residues in the 183 loop of thrombin. As a result of insertion, the carbonyl oxygen atoms of this loop in thrombin are spatially distant and unable to coordinate with Na^+. Instead, the cavity in thrombin is filled with water molecules, two of which are situated optimally to coordinate with Na^+.

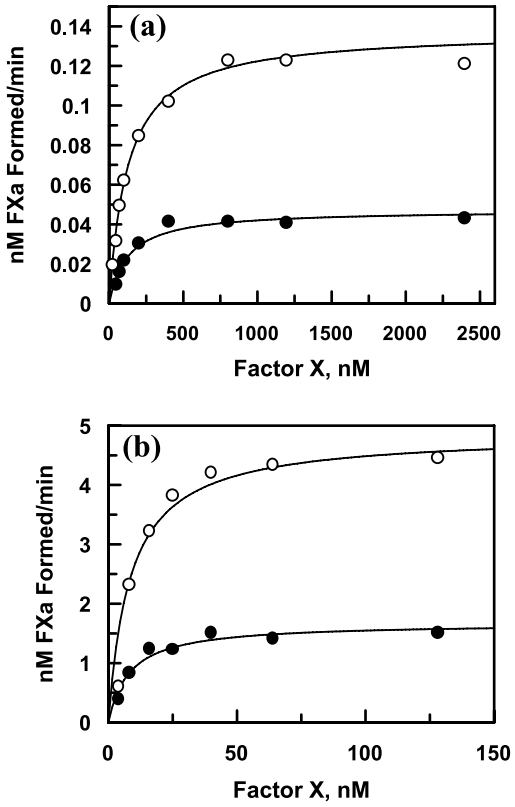


Figure 6. Activation of FX by FIXa and $FIXa_{Y225P}$. (a) FX activation in the absence of FVIIIa. Each reaction contained 20 nM FIXa or $FIXa_{Y225P}$, 25 μM PL, 5 mM $CaCl_2$, and increasing amounts of FX (25 nM to 3.6 μM). The reactions were carried out at 37 °C for 2.5–20 minutes and stopped by the addition of EDTA. The amount of FXa generated was measured as described in Materials and Methods. Open circles represent FIXa and filled circles represent $FIXa_{Y225P}$. K_m for FIXa was 124(±19) nM and for $FIXa_{Y225P}$ it was 126(±23) nM, and k_{cat} for FIXa was $7.0(\pm 0.6)\times 10^{-3}$ min^{-1} and for $FIXa_{Y225P}$ it was $2.3(\pm 0.3)\times 10^{-3}$ min^{-1}. (b) FX activation in the presence of FVIIIa. Each reaction contained 0.05 nM FIXa or $FIXa_{Y225P}$, 10 μM PL, 5 mM $CaCl_2$ and 14 nM FVIIIa, for each concentration of FX (3.5–512 nM). The activations were carried out for 15–150 seconds, at which time the reaction was stopped by addition of EDTA and the amount of FXa generated was measured. Open circles represent FIXa and filled circles represent $FIXa_{Y225P}$. K_m for FIXa was 8.5(±1.9) nM and for $FIXa_{Y225P}$ it was 8.2(±2.0) nM, and k_{cat} for FIXa was 105(±6.4) min^{-1} and for $FIXa_{Y225P}$ it was 33.4(±1.9) min^{-1}. K_m and k_{cat} values both in the absence and in the presence of FVIIIa represent the average of two experiments.

Previously, fluorescence binding studies yielded equilibrium dissociation constants for the binding *p*AB to FIXa of ~80 μM in the presence of both Ca^{2+} and Na^+, and 230 μM in the presence of Na^+ alone.[41] K_d in the presence of both cations (Na^+ and Ca^{2+}) is similar to that obtained in the present study and in other studies.[23,44] However, the value in the presence of Na^+ alone was considerably higher than the 25 μM value obtained in the present study using a competition-based assay. We repeated the fluorescence studies with FIXa and were again unable to observe significantly enhanced *p*AB fluorescence equivalent to K_{ipAB} of 25 μM (data not shown). The enhancement of fluorescence by binding of *p*AB is due primarily to the hydrophobic environment surrounding the S1 site,[45] and is dependent upon the serine protease under investigation. For example, it is ~230-fold for thrombin and ~50-fold for trypsin,[45] and it is insignificant for FIXa in the presence of Na^+ alone, corresponding to K_{ipAB} of 25 μM. Accordingly, the previous apparent K_{ipAB} of 230 μM for FIXa in the presence of Na^+ alone is in error. The lack of fluorescence increase in the presence of only Na^+ and the observable fluorescence increase in the presence of Ca^{2+} indicate that the environment of the S1 site is different under these two conditions. Whether it is related to the side-chain conformations of Tyr99 [266] and Lys98 [265], which are in the vicinity of the S1 site,[46] cannot be ascertained at this time.

In the absence of Ca^{2+}, Na^+ increased the affinity of CBS 31.39 to FIXa (Table 1) by ~6.5-fold and binding of *p*AB (Table 3) by fourfold. This suggests that the Na^+ site is linked allosterically to the S1 site and possibly to the S2/S3/S4 sites in FIXa. This conclusion is supported also by the kinetic data obtained with $FIXa_{Y225P}$. K_m obtained for $FIXa_{Y225P}$ is essentially equivalent to that observed for normal FIXa in the absence of Na^+ and the binding of *p*AB to $FIXa_{Y225P}$ is impaired severely. The linkage of the Na^+ site to the S1 site in FIXa is similar to that observed previously for APC and FXa.[14,38,43]

It is interesting to note that $FIXa_{Y225P}$ has substantially reduced activity as compared to FIXa in the absence of Na^+ and Ca^{2+}. One plausible explanation for this observation could be that in FIXa, the 220 loop may not be displaced substantially in the absence of Na^+; whereas in the mutant, the conformation of the 220 loop may be altered sufficiently to have a profound effect on catalysis. The rate of synthetic substrate hydrolysis for $FIXa_{E80V}$ was also much lower than observed for FIXa in the absence of Na^+ and Ca^{2+}. This could be attributed to a drastically different conformation of the Ca^{2+}-loop in which Glu80 [245] is replaced by a hydrophobic Val residue. In contrast, in normal FIXa, a water molecule could occupy the Ca^{2+} site and partially maintain the conformation of the Ca^{2+}-loop, resulting in higher activity than $FIXa_{E80V}$.

The S1 site in serine proteases is linked to the stability of the salt-bridge between residues 16 and 194.[2,42] The stability of this salt-bridge was impaired in $FIXa_{Y225P}$, supporting the kinetic data suggesting that the Na^+ site is linked to the S1 site. Further, Ca^{2+} increased the stability of this salt-bridge for both FIXa and $FIXa_{Y225P}$ (Table 3), indicating strongly that the Ca^{2+} site is also linked to the S1 site. The stability of this salt-bridge was increased by *p*AB in both FIXa and $FIXa_{Y225P}$, establishing a linkage between the S1 site and salt-bridge formation as has been reported for other

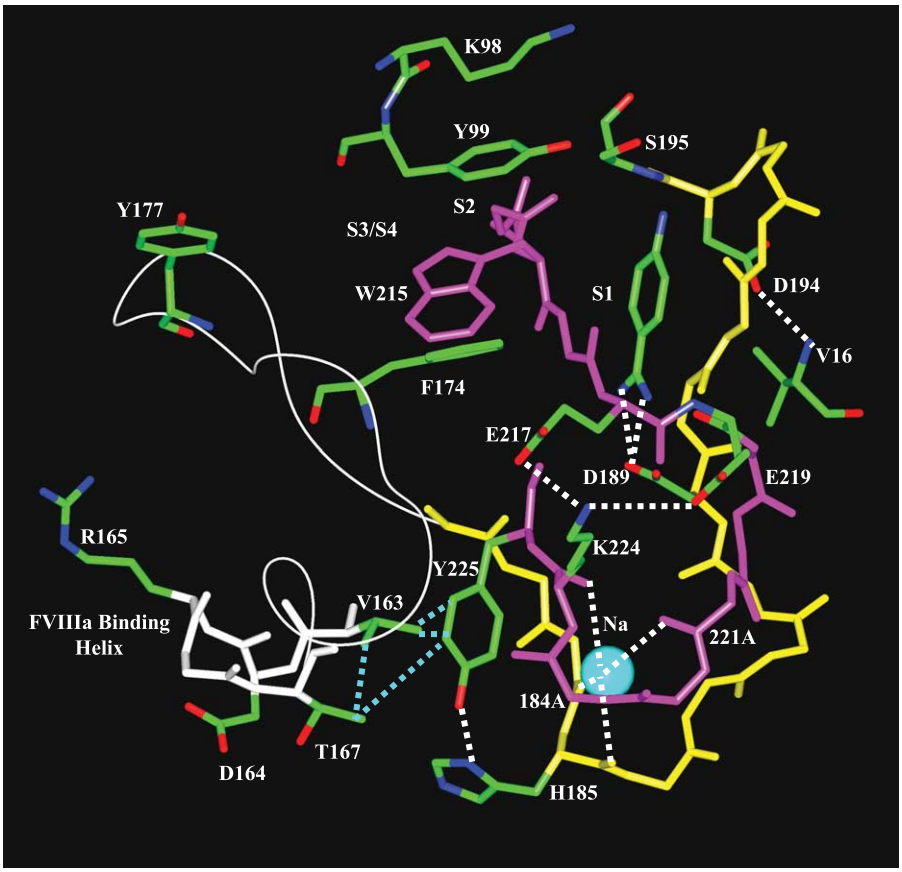


Figure 7. Relationship of the Na^+-binding site to the S1 site and FVIIIa binding helix in FIXa. The two Na^+ loops, 183–189 [353–359] colored *yellow*, and 221–225 [391–395] colored magenta, and the FVIIIa 162-helix [330-helix] colored white are shown. Na^+ is shown as a cyan sphere and is coordinated to the carbonyl O atoms of 184A [353], 185 [354], 221A [391], and 224 [394]. The benzene ring of Tyr225 [395] makes van der Waals contacts with Val163 [331] and Thr167 [335]. The hydroxyl group of Tyr225 [395] makes a H-bond with His185 [354] and Lys224 [394] makes H-bonds with Glu217 [387] and Glu219 [388]. Note that there is a one residue deletion in FIX as compared to chymotrypsin. The S1 site is occupied by *p*AB and its benzamidine moiety makes H-bonds with Asp189 [359], and the amino group of Val16 [181] makes a H-bond with the carboxylate group of Asp194 [364]. The H-bonds are shown with broken white lines and hydrophobic interactions are shown in broken cyan lines. Asp164 [332] and Arg165 [333] are two residues that are important in binding to FVIIIa. Residues Lys98 [265], Tyr99 [266], Phe174 [342], Tyr177 [345], and Trp215 [385] that play important roles in occupancy of the substrate at the S2/S3/S4 sites are depicted. All residues are colored by atom type, except Trp215 [385], which is colored magenta. Red represents oxygen, blue represents nitrogen, and green represents carbon. The locations of the S2 and S3/S4 sites are shown. The Figure is based upon the published atomic coordinates (PDB code 1rfn).[23]

serine proteases.[2,42] Ca^{2+} and saturating *p*AB together stabilized this salt-bridge in FIXa and $FIXa_{Y225P}$ to the same extent, which indicates that these two ligands can correct the impairment caused by the Y225P mutation. Thus, both Na^+ and Ca^{2+} affect the S1 site allosterically and Val16-Asp194 salt-bridge formation in FIXa, and shift the zymogen–enzyme equilibrium towards the enzyme conformer.

During conversion of the zymogen to enzyme, the four regions of the protease domain that are most affected are the N-terminal region, the autolysis loop (residues 140–152), the 183–194 loop, and the 216–225 loop.[2,47,48] In FIXa, Na^+ binding involves the 183 loop and the 216 loop. Further, the Lys224 [394] side-chain makes a H-bond with the carboxylate groups of Glu217 [387] and Glu219 [388], and His185 [354] makes a H-bond with the hydroxyl group of Tyr225 [395] (Figure 7). Thus, proper conformation of these two loops mediated by Na^+ binding may promote proper development of the S1 site.

In the case of Ca^{2+}, the autolysis loop is situated between the Ca^{2+}-binding loop (residues 70–80 [235–245]) and the N-terminal region (Figure 1), and binding of Ca^{2+} is known to protect the autolysis loop from proteolysis.[41,49] Thus, Ca^{2+}-binding could affect the conformation of the autolysis loop, which is one of the regions altered during zymogen activation. Hence, Ca^{2+} binding may affect the S1 site by maintaining the adjacent autolysis loop in a proper conformation.

In the absence of Na^+, Ca^{2+} increased K_m of FIXa for CBS 31.39 hydrolysis 4.6-fold (Table 2) but had essentially no effect on the binding of *p*AB (Table 3). However, addition of Ca^{2+} decreased K_{ipAB} for

$FIXa_{Y225P}$ from 813 μM to 179 μM. Moreover, addition of Na^+ in the presence of Ca^{2+} was largely ineffective in lowering K_{ipAB} of normal FIXa. Conceivably, Na^+ and Ca^{2+} effects overlap and are related to alteration of both the S1 and S2/S3/S4 sites. As shown (Table 1), Ca^{2+} binding is linked negatively to synthetic substrate binding. This may be attributable to the effects of protease domain Ca^{2+} binding on the side-chain conformation of Tyr99 [266] in FIXa (Figure 7). In the structure of human FIXa in the presence of Ca^{2+} (PDB code 1rfn), Tyr99 [266] occupies the S2 site[23] and will have a steric conflict with the C^{α}, amide N, and the side-chain of the P2 residue of the substrate. Since the P2 residue in CBS 31.39 is Gly, only a steric conflict between the hydroxyl of Tyr99 [266] and the amide N and C^{α} atoms of Gly is anticipated. Thus, the side-chain of Tyr99 [266] will have to move toward the S3/S4 site (Figure 7), as has been observed with the porcine FIXa inhibited with D-Phe-Pro-Arg-chloromethyl ketone,[50] which could then possibly interact with the D-Leu of CBS 31.39. Such influence of Ca^{2+} on synthetic substrate binding was not observed for APC or FXa.[14,38]

Previously, the Na^+ sites in FXa and APC were found to be linked allosterically to the Ca^{2+} site.[14,38] Our data would indicate that such is the case for FIXa, although the linkage appears to be weak. Scheme 1 and Table 1 illustrate the allosteric linkage between the protease domain Ca^{2+} site and the Na^+ site. Ca^{2+} binding to the protease domain increases the affinity of free FIXa for Na^+ and *vice versa*. This is important, because in the absence of Ca^{2+} at physiologic concentrations of Na^+ (~150 mM) only two-third of the free FIXa ($K_{E,N}$ = 89.9 mM) would be bound to Na^+; however, upon binding of Ca^{2+} ($K_{E,C}$ = 69.4 μM) the binding to Na^+ would be greatly favored ($K_{EC,N}$ = 54.8 mM) and the binding of Na^+ would then further favor binding to Ca^{2+} ($K_{EN,C}$ = 42.3 μM). Thus, free FIXa under physiologic conditions would exist in a Na^+ and Ca^{2+}-bound form, which would favor FVIIIa binding and protection against proteolysis in the autolysis loop, which leads to loss of FIXa clotting activity.[41]

The Na^+ site mutant, $FIXa_{Y225P}$ is impaired in binding to FVIIIa. This impairment in FVIIIa binding to $FIXa_{Y225P}$ could be due to the loss of hydrophobic interactions between Tyr225 [395] with Val163 [331] and Thr167 [335] of the FVIIIa binding helix[37] as well as to the loss of H-bond between the hydroxyl of Tyr225 [395] and His185 [354] (Figure 7). All of these residues, except His185 [354], are identical in FIX from different species. His185 [354] is conserved in FIX for many species; however, it is Arg in mouse and rat, and it is Asp in rabbit.[8] These two side-chains have the potential to form similar H-bonds with Tyr225 [395]. Therefore, it is possible that this H-bond plays a significant role in positioning the Tyr225 [395] for hydrophobic interactions with Val and Thr of the FVIIIa binding helix. Thus, disruption of these interactions in $FIXa_{Y225P}$ may lead to increased flexibility of the 162 helix [330 helix] and impaired binding to FVIIIa. Such an argument will apply also for change of Tyr225 [395] to His, a naturally occurring hemophilia B mutation.[39] Change of Tyr225 [395] to a larger Trp residue, another hemophilia B mutation, could seriously modify the local structure and disrupt both the FVIIIa-binding helix as well as the Na^+-binding loops. Thus, it appears that the role of Tyr225 [395] is twofold: to allow Na^+ binding; and to maintain the proper conformation of the FVIIIa-binding helix. We are in the process of expressing several mutants of FIX to test this assumption.

FIXa and $FIXa_{Y225P}$ activated FX with similar K_m values both in the presence and in the absence of FVIIIa (Figure 6). This is expected, since exosites distant from the active site determine the affinity of binding of the macromolecular substrate for the intrinsic tenase complex ($FIXa/FVIIIa/PL/Ca^{2+}$).[51] However, saturating amounts of FVIIIa did not correct the impairment in k_{cat} for $FIXa_{Y225P}$ activation of FX (Figure 6). Binding of FVIIIa to the 162 helix [330 helix] is thought to induce changes in the loop containing Phe174 [342] and Tyr177 [345] (Figure 7), which have been implicated in reorienting the conformation of Tyr99 [266] such that FX can restructure the substrate-binding region.[46] Since FVIIIa is unable to fully correct the impairment in $FIXa_{Y225P}$, it implies that Na^+ contributes to restructuring the active site for macromolecular substrate catalysis. Support for this concept comes from studies by Camire, in which binding of FVa to FXa_{Y225P} did not completely restore the catalytic activity of FXa_{Y225P}.[42]

In conclusion, Na^+ and Ca^{2+} binding to the protease domain contribute significantly in shifting the equilibrium to a more enzyme-like state of FIXa. This concept is supported by the carbamylation data, where Na^+ stabilizes the Val16-Asp194 salt-bridge by 2.3-fold and Ca^{2+} stabilizes it by 4.3-fold (Figure 4 and Table 4). By altering the zymogen-enzyme equilibrium towards a more enzyme-like form of FIXa, Na^+ and Ca^{2+} induce conformational rearrangements within the protease domain necessary for FVIIIa binding. Previous studies have shown that binding of Ca^{2+} to the protease domain of FIXa increases its affinity for FVIIIa ~15-fold *via* an allosteric mechanism.[41] Here, we show that in addition to the effects of Ca^{2+}, impairment of the Na^+-binding site in FIXa results in a fourfold reduction in affinity for FVIIIa as well as threefold reduction in k_{cat}. These data are in agreement with the tenfold reduced activity of FIX_{Y225P} measured in a plasma-based clotting assay.[52] It has been proposed that impairment of the Na^+ site in thrombin results in the protein existing in multiple conformations including the slow and fast forms.[18,53–55] Thus, it is possible that $FIXa_{Y225P}$ may represent a conformation in which the Na^+ binding is partially impaired. The possibility exists that the reduced catalytic activity in the $FIXa_{Y225P}$ mutant is due to conformational alterations in FIXa, independent of Na^+ binding. Nonetheless, studies with this mutant

illustrate a functional role of Tyr225 [395] and of Na^+ binding in physiologic clotting.

Materials and Methods

Reagents

CH_3SO_2-D-Leu-Gly-Arg-*p*NA (CBS 31.39) was purchased from Diagnostica Stago (France) and H-D-Ile-Pro-Arg-*p*NA (S-2288) and benzoyl-Ile-Glu-Gly-Arg-*p*NA (S-2222) were purchased from DiaPharma (West Chester, OH). Diisopropylfluorophosphate (DFP) and dansyl-Glu-Gly-Arg chloromethyl ketone (dEGRck) were purchased from Calbiochem. Fatty acid-free bovine serum albumin (BSA), *p*-aminobenzamidine (*p*AB), PEG 8000, phosphatidylcholine, phosphatidylserine, recombinant hirudin, and all other chemicals of the highest grade available were obtained from Sigma. Phospholipid vesicles containing 75% (w/v) phosphatidylcholine and 25% (w/v) phosphatidylserine were prepared as described.[56]

Proteins

Human FIX, human α-thrombin, human FX, human FXa, and human FXIa were purchased from Enzyme Research Laboratories (South Bend, IN). Purified human FVIII was obtained from the American Red Cross (Rockwell, MD). FVIII was activated to FVIIIa using 1 nM human α-thrombin in the presence of 0.1% (w/v) BSA and 5 mM $CaCl_2$ in TBS (50 mM Tris–HCl (pH 7.5), 150 mM NaCl) at 37 °C for two minutes as described,[37] followed by the addition of recombinant hirudin to inactivate thrombin.

SDS-PAGE

SDS-PAGE was performed using the Laemmli buffer system.[57] The acrylamide concentration used was 12% (w/v) and the gels were stained with Coomassie brilliant blue dye. All proteins used were ~98% pure.

Expression and purification of recombinant FIX proteins

Recombinant FIX_{WT}, FIX_{E80V} [245], and FIX_{Y225P} [395] were expressed in HEK 293 cells and purified using a FIX A-7 mAb column followed by a Mono-Q column as described.[41,52] Each FIX protein had ~12 Gla residues/mol and appeared homogeneous on both reduced and non-reduced SDS-PAGE with Mr ~57,000.[41,52]

Activation of FIX proteins

FIX_{NP}, FIX_{WT}, FIX_{E80V}, and FIX_{Y225P} were activated at 200 μg/ml with FXIa (2 μg/ml) for 30–90 minutes at 37 °C. The buffer used was TBS with 5 mM $CaCl_2$. SDS-PAGE analysis showed full activation to FIXa without significant degradation. To inactivate FXIa, DFP (final concentration 1 mM) was added to each reaction mixture followed by incubation at room temperature for 15 minutes and then on ice for one hour. In control experiments, FXIa was fully inactivated using this protocol and S-2288 as a substrate, while FIXa was not affected. Each FIXa protein sample was freed of Ca^{2+}, Na^+, and DFP by dialysis and/or a desalting column exactly as described.[58] The final concentration of Na^+ after these steps was <1 mM as measured by a conductivity meter.[58] The FIXa proteins were frozen at −80 °C in 20 μl aliquots, thawed, and used immediately. This freezing and thawing did not result in a measurable loss in activity. All proteins were used within two weeks of freezing.

Preparation of dEGRck inhibited FIXa

FIX was activated to FIXa as described above and dEGR-IXa was prepared as described.[37,41] The excess dEGRck was removed using dialysis and/or a Centricon 30 concentrator.

Measurement of CBS 31.39 amidolytic activity of FIXa derivatives

The concentration of $FIXa_{NP}$, $FIXa_{WT}$, $FIXa_{E80V}$ or $FIXa_{Y225P}$ used was between 125 and 375 nM. The CBS 31.39 concentration ranged from 50 μM to 15 mM. The buffer used was 50 mM Tris–HCl (pH 7.4) containing 0.1% (w/v) PEG 8000 and various salt combinations given in the legends to the appropriate Figures. Rubidium, a larger monovalent cation was used to keep the ionic strength constant at 0.2 M. The *p*-nitroaniline (*p*NA) release was measured continuously (ΔA_{405nm} min^{-1}) for up to 30 minutes using a SpectraMAX 190 plate reader from Molecular Devices. An extinction coefficient of 9.9 $mM^{-1}cm^{-1}$ at 405 nM was used in calculating the amount of *p*NA released.[59]

Determination of steady-state kinetic constants

The data were analyzed using the indicated equations by non-linear, least-squares regression analysis with the Marquardt algorithm,[60] and the quality of fit was evaluated as described.[61] The fitted parameters are given ±95% confidence limits. Initial velocity measurements of CBS 31.39 hydrolysis were analyzed using the Henri–Michaelis–Menten equation to yield K_m and V_{max}.

Global analysis of initial velocity data

Initial velocities of peptidyl substrate cleavage determined at increasing concentrations of Na^+ in the absence or in the presence of Ca^{2+} or determined with increasing concentrations of Ca^{2+} in the presence or in the absence of Na^+ were analyzed globally using the ordinary differential equations resulting from Scheme 1. Global fitting was performed by applying the rapid equilibrium assumption and the program Dynafit,[62] to yield fitted values for the equilibrium dissociation constants and rate constants listed in Scheme 1. Errors in the fitted terms reflect linear approximations of the 95% confidence limits. When equilibrium constants were calculated from fitted terms, uncertainty was estimated by propagating the errors of the fitted parameters. In Scheme 1, E C, N and P denote FIXa, Ca^{2+}, Na^+, peptidyl substrate and product respectively and equilibrium constants are subscripted to denote the interacting species. For example, $K_{ENS,C}$ denotes the dissociation constant for interaction Ca^{2+} with FIXa that is bound to Na^+ and substrate.

Determination of K_{ipAB} of binding of *p*AB to FIXa and $FIXa_{Y225P}$

K_i of binding of pAB to FIXa and FIXaY225P was determined by its ability to competitively inhibit CBS 31.39 hydrolysis in the absence and in the presence of Na^+, with or without Ca^{2+}. Each reaction mixture contained 125–375 nM FIXa, a given amount of pAB

(50 μM, 100 μM, 200 μM, 500 μM, or 1000 μM) and increasing amounts of CBS 31.39 in 50 mM Tris–HCl (pH 7.4), 0.1% PEG 8000 in four different Na^+/Ca^{2+} conditions: (1) 200 mM RbCl, 1 mM EDTA; (2) 200 mM NaCl, 1 mM EDTA; (3) 185 mM RbCl, 5 mM $CaCl_2$; and (4) 185 mM NaCl, 5 mM$CaCl_2$. The data were fit to equation (1) using non-linear, least-squares analysis:

$$v = V_{max}[S]/(K_m(1 + [I]/K_i) + [S]) \quad (1)$$

where v is the reaction velocity, V_{max} is the maximum velocity at the given concentration of FIXa, $[S]$ is the concentration of substrate, $[I]$ is the concentration of *p*AB, and K_m is the value under the given Na^+ and Ca^{2+} conditions.

Carbamylation of Val16 [181] in FIXa and FIXa$_{Y225P}$ by reaction with NaNCO

These experiments were performed as described.[42] Briefly, each reaction mixture contained 1 μM FIXa mutant or normal protein in HBSP (20 mM Hepes (pH 7.0), 0.15 M NaCl, 0.1% (w/v) PEG 8000). Experiments were performed in the presence of either 1 mM EDTA or 2 mM Ca^{2+}. When *p*AB was included, its concentration was tenfold the K_{ip}AB under the specified conditions. The reactions were started by the addition of 1 M NaNCO for a final concentration of 0.2 M. The final pH of the reaction after the addition of NaNCO was 7.5. Every 30 minutes, a 20 μl aliquot was removed and added to 130 μl of TBS/BSA containing 5 mM Ca^{2+} and 1 mM CBS 31.39. The residual activity was determined from the initial linear rates of hydrolysis using a Beckman DU 800 spectrophotometer. The residual activity was plotted as a percentage of initial activity and k_{obs} for carbamylation were determined using equation (2):

$$A_t = A_0 \exp(-k_{obs} t) \quad (2)$$

where A_t and A_0 are the percentage FIXa activity at time t and time zero, respectively.

Measurement of factor X activation

FX activation by FIXa and FIXa$_{Y225P}$ was measured under two sets of conditions:[37] (1) in the presence of Ca^{2+} and PL; and (2) in the presence of Ca^{2+}, PL, and FVIIIa. In the absence of FVIIIa, each reaction contained 20 nM FIXa, 25 μM PL, 5 mM $CaCl_2$, and various concentrations of FX ranging from 25–2400 nM. The activations were carried out for 2.5–20 minutes. In the presence of FVIIIa, each reaction contained 0.05 nM FIXa, 10 μM PL, 5 mM $CaCl_2$, 14 nM (saturating) FVIIIa and various concentrations of FX from 3.5–480 nM. The activations were carried out for 15–150 seconds. The buffer used in all experiments was TBS/BSA (pH 7.5), and the reaction volume was 50 μl. The reactions were carried out at 37 °C for different lengths of time, after which 2 μl of 0.5 M EDTA was added to stop the reaction. Then 100 μl of TBS/BSA (pH 7.5) and 10 μl of S-2222 (final concentration 100 μM) were added. From this mixture, 150 μl was removed and placed in a well on an Immulon 4 HBX flat-bottom 96-well microtiter plate (Dynex Technologies) and the *p*-nitroaniline release was measured (ΔA_{405}/minute) for up to 30 minutes. The FXa generated was calculated from a standard curve constructed using FXa prepared by insolubilized Russell's viper venom. K_m and k_{cat} were obtained using the program GraFit from Erithacus Software.

Binding of FIXa$_{WT}$ and FIXa$_{Y225P}$ to FVIIIa

K_{dapp} of binding of FIXa$_{WT}$ or FIXa$_{Y225P}$ to FVIIIa was determined by the ability of dEGR-IXa and dEGR-IXa$_{Y225P}$ to inhibit the FVIIIa potentiation of FX activation by FIXa.[37] Reaction mixtures contained 0.2 nM FIXa, 0.07 nM FVIIIa, 480 nM FX, 10 μM PL, and various concentrations of dEGR-IXa derivatives in TBS/BSA (pH 7.5) containing 5 mM $CaCl_2$. The IC_{50} (concentration of inhibitor required for 50% inhibition) was determined by fitting the data to IC_{50} four-parameter logistic equation of Halfman:[63]

$$y = \frac{a}{1 + (x/IC_{50})^s} \quad (3)$$

where y is the rate of FXa formation in the presence of a given concentration of dEGR-IXa or dEGR-IXa$_{Y225P}$ represented by x, a is the maximum rate of FXa formation in the absence of dEGR-IXa, and s is the slope factor. Each point was weighted equally and the data were fit to equation (3) using the non-linear regression analysis program, GraFit from Erithcus Software. To obtain apparent K_d values for the interaction of dEGR-IXa proteins with FVIIIa, we used the following equation as described by Cheng and Prusoff[64] and further elaborated by Craig:[65]

$$K_d = \frac{IC_{50}}{1 + ([S]K_m)} \quad (4)$$

where S is the concentration of FIXa$_{WT}$, IC_{50} is the concentration of dEGR-FIXa or dEGR-IXa$_{Y225P}$ that gives 50% inhibition, and K_m is the concentration of FVIIIa that represents the midpoint of the maximum potentiation of FX activation by FIXa under these conditions.[37]

Modeling of the Na^+ site in FIXa

X-ray crystal structures of FXa,[13] FVIIa,[16] APC,[43] thrombin,[11,12] and FIXa[23,50] were superimposed using the program O.[66] The sodium ion was placed in the FIXa structure based upon the position of Na^+ in homologous proteins, namely, Xa, APC, and FVIIa.

Acknowledgements

This work was supported by National Institutes of Health grants HL-36365 and HL-70369 (to S.P.B.) and HL-62523 and HL-74124 (to S.K.). A.E.S. is supported, in part, by an American fellowship from the American Association of University Women Educational Foundation. The costs of publication of this article were defrayed, in part, by the payment of page charges. This article must be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 to indicate this fact.

References

1. Neaurath, H. & Walsh, K. A. (1976). Role of proteolytic enzymes in biological regulation. *Proc. Natl Acad. Sci. USA*, **73**, 3825–3832.
2. Huber, R. & Bode, W. (1978). Structural basis of the activation and action of trypsin. *Accts. Chem. Res.* **11**, 114–122.
3. Daci, E., Everts, V., Torrekens, S., Van Herck, E., Tigchelaar-Gutterr, W., Bouillon, R. & Carmeliet, G. (2003). The role of the plasminogen system in bone resorption in vitro. *J. Bone Miner. Res.* **18**, 1167–1176.

4. Barrett, A. J. & Rawlings, N. D. (1995). Families and clans of serine peptidases. *Arch. Biochem. Biophys.* **318**, 247–250.
5. Blow, D. (1990). Enzymology. More of the catalytic triad. *Nature*, **343**, 694–695.
6. Schechter, I. & Berger, A. (1967). On the size of the active site in proteases I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157–162.
7. Halfon, S. & Craik, C. S. (1998). Trypsin. In *Handbook of Proteolytic Enzymes* (Barrett, A. J., Rawlings, N. D. & Woessner, J. F., eds), pp. 12–21, Academic Press, San Diego, CA.
8. Bajaj, S. P. & Birktoft, J. J. (1993). Human factor IX and factor XIa. *Methods Enzymol.* **222**, 96–128.
9. Bode, W. & Schwager, P. (1975). The refined crystal structure of bovine beta-trypsin at 1.8 Å resolution II. Crystallographic refinement, calcium binding site, benzamidine binding site and active site at pH 7.0.. *J. Mol. Biol.* **98**, 693–717.
10. Mattos, C., Rasmussen, B., Ding, X., Petsko, G. A. & Ringe, D. (1994). Analogous inhibitors of elastase do not always bind analogously. *Nature Struct. Biol.* **1**, 55–58.
11. Di Cera, E., Guinto, E. R., Vindigni, A., Dang, Q. D., Ayala, Y. M., Wuyi, M. & Tulinsky, A. (1995). The Na^+ binding site of thrombin. *J. Biol. Chem.* **270**, 22089–22092.
12. Zhang, E. & Tulinsky, A. (1997). The molecular environment of the Na^+ ion binding site of thrombin. *Biophys. Chem.* **63**, 185–200.
13. Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R. *et al*. (1993). Structure of human des(1–45) factor Xa at 2.2 Å resolution. *J. Mol. Biol.* **232**, 947–966.
14. Schmidt, A. E., Padmanabhan, K., Underwood, M. C., Bode, W., Mather, T. & Bajaj, S. P. (2002). Thermodynamic linkage between the S1 site, the Na^+ site and the Ca^{2+} site in the protease domain of human activated protein C (APC). Sodium ion in the APC crystal structure is coordinated to four carbonyl groups from two separate loops. *J. Biol. Chem.* **277**, 28987–28995.
15. Banner, D. W., D'Arcy, A., Chene, C., Winkler, F. K., Guha, A., Koningberg, W. H. & Nemerson, Y. (1996). The crystal structure of the complex of blood coagulation in factor VIIa with soluble tissue factor. *Nature*, **380**, 41–46.
16. Bajaj, S. P., Padmanabhan, K., Bajaj, M. S., Liesum, A., Dumas, J., Prevost, D. & Schreuder, H. (2003). Crystal structure of benzamidine-VIIa/tissue factor at 1.87 Å resolution: mapping of Ca^{2+}, Mg^{2+}, Na^+, and Zn^{2+} sites in the VIIa strucure. *J. Thromb. Haem*. Supplement, OC449.
17. Dang, Q. D. & Di Cera, E. (1996). Residue 225 determines the Na^+-induced allosteric regulation of catalytic activity in serine proteases. *Proc. Natl Acad. Sci. USA*, **93**, 10563–10656.
18. Guinto, E. R., Caccia, S., Rose, T., Futterer, K., Waksman, G. & Di Cera, E. (1999). Unexpected crucial role of residue 225 in serine proteases. *Proc. Natl Acad. Sci. USA*, **96**, 1852–1857.
19. Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W. & Kurachi, K. (1985). Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry*, **24**, 3736–3750.
20. Di Scipio, R. G., Kurachi, K. & Davie, E. W. (1978). Activation of human factor IX (Christmas factor). *J. Clin. Invest.* **61**, 1528–1538.
21. Osterud, B. & Rapaport, S. I. (1977). Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation. *Proc. Natl Acad. Sci. USA*, **74**, 5260–5264.
22. Bajaj, S. P., Rapaport, S. I. & Russell, W. A. (1983). Redetermination of the rate-limiting step in the activation of factor IX by factor XIa and by factor VIIa/tissue factor. Explanation for different electrophoretic radioactivity profiles obtained on activation of 3H- and 125I-labeled factor IX. *Biochemistry*, **22**, 4047–4053.
23. Hopfner, K. P., Lang, A., Karcher, A., Sichler, K., Kopetzki, E., Brandstetter, H. *et al*. (1999). Coagulation factor IXa: the relaxed conformation of Tyr99 blocks substrate binding. *Struct. Fold. Des.* **7**, 989–996.
24. Mann, K. G. (1999). Biochemistry and physiology of blood coagulation. *Thromb. Haemost.* **82**, 165–174.
25. Shikamoto, Y., Morita, T., Fujimoto, Z. & Mizuno, H. (2003). Crystal structure of Mg^{2+}- and Ca^{2+}-bound Gla domain of factor IX complexed with binding protein. *J. Biol. Chem.* **278**, 24090–24094.
26. Huang, M., Furie, B. C. & Furie, B. (2004). Crystal structure of the calcium-stabilized human factor IX Gla domain bound to a conformation-specific anti-factor IX antibody. *J. Biol. Chem.* **279**, 14338–14346.
27. Rao, Z., Handford, P., Mayhew, M., Knott, V., Brownlee, G. G. & Stuart, D. (1995). The structure of a Ca^{2+}-binding epidermal growth factor-like domain: its role in protein-protein interactions. *Cell*, **82**, 131–141.
28. Bajaj, S. P., Sabharwal, A. K., Gorka, J. & Birktoft, J. J. (1992). Antibody-probed conformational transitions in the protease domain of human factor IX upon calcium binding and zymogen activation: putative high-affinity Ca^{2+}-binding site in the protease domain. *Proc. Natl Acad. Sci. USA*, **89**, 152–156.
29. Freedman, S. J., Blostein, M. D., Beleja, J. D., Jacobs, M., Furie, B. C. & Furie, B. (1996). Identification of the phospholipid binding site in vitamin K dependent blood coagulation protein factor IX. *J. Biol. Chem.* **271**, 16227–16236.
30. Ahmad, S. S., London, F. S. & Walsh, P. N. (2003). Binding studies of the enzyme (factor IXa) with the cofactor (factor VIIIa) in the assembly of factor-X activating complex on the activated platelet surface. *J. Thromb. Haemost.* **1**, 2348–2355.
31. Zhong, D., Smith, K. J., Birktoft, J. J. & Bajaj, S. P. (1994). First epidermal growth factor-like domain of human blood coagulation factor IX is required for its activation by factor VIIa/tissue factor but not by factor XIa. *Proc. Natl Acad. Sci. USA*, **91**, 3574–3578.
32. Zhong, D., Bajaj, M. S., Schmidt, A. E. & Bajaj, S. P. (2002). The N-terminal EGF-like domain in factor IX and factor X represents an important recognition motif for binding to tissue factor. *J. Biol. Chem.* **277**, 3622–3631.
33. Blostein, M. D., Furie, B. C., Rajotte, I. & Furie, B. (2003). The Gla domain of factor IXa binds to factor VIIIa in the tenase complex. *J. Biol. Chem.* **278**, 31297–31302.
34. Mertens, K., Celie, P. H., Kolkman, J. A. & Lenting, P. J. (1999). Factor VIII-factor IX interactions: molecular sites involved in enzyme-cofactor complex assembly. *Thromb. Haemost.* **82**, 209–217.
35. Nishimura, H., Takeya, H., Miyata, T., Suehiro, K., Okamura, T., Niho, Y. & Iwanaga, S. (1993). Factor IX Fukuoka.Substitution of Asn92 by His in the second epidermal growth factor-like domain results in defective interaction with factors VIIIa/X. *J. Biol. Chem.* **268**, 24041–24046.
36. Chang, Y. J., Wu, H. L., Hamaguchi, N., Hsu, Y. C. &

Lin, S. W. (2002). Identification of functionally important residues of the epidermal growth factor-2 domain of factor IX by alanine-scanning mutagenesis. Residues Asn(89)-Gly(93) are critical for binding factor VIIIa. *J. Biol. Chem.* **277**, 25393–25399.
37. Mathur, A. & Bajaj, S. P. (1999). Protease and EGF1 domains of factor IXa play distinct roles in binding to factor VIIIa. Importance of helix 330 (helix 162 in chymotrypsin) of protease domain of factor IXa in its interaction with factor VIIIa. *J. Biol. Chem.* **274**, 18477–18486.
38. Underwood, M. C., Zhong, D., Mathur, A., Heyduk, T. & Bajaj, S. P. (2000). Thermodynamic linkage between the S1 site, the Na^+ site, and the Ca^{2+} site in the protease domain of human coagulation factor Xa. Studies in catalytic efficiency and inhibitor binding. *J. Biol. Chem.* **275**, 36876–36884.
39. Giannelli, F., Green, P. M., Sommer, S. S., Poon, M., Ludwig, M. & Schwaab, R. (1998). Haemophilia B: database of point mutations and short additions and deletions, 8th edition. *Nucl. Acids Res.* **26**, 267–270.
40. Schmidt, A. E., Stewart, J. E. & Bajaj, S. P. (2003). Functional linkage between the Na^+ site, substrate site, and the Ca^{2+} site in the protease domain of human factor IXa (FIXa). *Blood*, 1074.
41. Mathur, A., Zhong, D., Sabharwal, A. K., Smith, K. J. & Bajaj, S. P. (1997). Interaction of factor IXa with factor VIIIa: Effects of protease domain Ca^{2+}-binding site, proteolysis in the autolysis loop, phospholipid and factor X. *J. Biol. Chem.* **272**, 23418–23426.
42. Camire, R. M. (2002). Prothrombinase assembly and S1 site occupation restore the catalytic activity of FXa impaired by mutation at the sodium-binding site. *J. Biol. Chem.* **277**, 37863–37870.
43. Mather, T., Oganessyan, V., Hof, P., Huber, R., Foundling, S., Esmon, C. & Bode, W. (1996). The 2.8 Å crystal structure of Gla-domainless activated protein C. *EMBO J.* **15**, 6822–6831.
44. Kolkman, J. A. & Mertens, K. (2000). Surface-loop residue Lys316 in blood coagulation factor IX is a major determinant for factor X but not antithrombin recognition. *Biochem. J.* **350**, 701–707.
45. Evans, S. A., Olson, S. T. & Shore, J. D. (1982). *p*-Aminobenzamidine as a fluorescent probe for the active site of serine proteases. *J. Biol. Chem.* **257**, 3014–3017.
46. Sichler, K., Kopetzki, E., Huber, R., Bode, W., Hopfner, K. P. & Brandstetter, H. (2003). Physiological FIXa activation involves a cooperative conformational rearrangement of the 99-loop. *J. Biol. Chem.* **278**, 4121–4126.
47. Wang, D., Bode, W. & Huber, R. (1985). Bovine chymotrypsinogen A X-ray crystal structure analysis and refinement of a new crystal form at 1.8 Å resolution. *J. Mol. Biol.* **185**, 595–624.
48. Perera, L., Darden, T. & Pedersen, L. G. (2001). Modeling human zymogen factor IX. *Thromb. Haemost.* **85**, 596–603.
49. Kisiel, W., Smith, K. J. & McMullen, B. A. (1985). Proteolytic inactivation of blood coagulation factor IX by thrombin. *Blood*, **66**, 1302–1308.
50. Brandstetter, H., Bauer, M., Huber, R., Lollar, P. & Bode, W. (1995). X-ray structure of clotting factor IXa: active site and module structure related to Xase activity and hemophilia B. *Proc. Natl Acad. Sci. USA*, **92**, 9796–9800.
51. Krishnaswamy, S. (2005). Exosite-driven substrate specificity and function in coagulation. *J. Thromb. Haemost.* **3**, 54–67.
52. Mathur, A. (1999). Role of the protease domain of factor IXa in its interaction with factor VIIIa. PhD thesis, Saint Louis University, St Louis, MO.
53. Huntington, J. A. & Esmon, C. T. (2003). The molecular basis of thrombin allostery revealed by a 1.8 Å structure of the "slow" form. *Structure (Camb)*, **11**, 469–479.
54. Carter, W. J., Myles, T., Gibbs, C. S., Leung, L. L. & Huntington, J. A. (2004). Crystal structure of anticoagulant thrombin variant E217K provides insights into thrombin allostery. *J. Biol. Chem.* **279**, 26387–26394.
55. Pineda, A. O., Carrell, C. J., Bush, L. A., Prasad, S., Caccia, S., Chen, Z. W. *et al*. (2004). Molecular dissection of Na^+ binding to thrombin. *J. Biol. Chem.* **279**, 31842–31853.
56. Husten, E. J., Esmon, C. T. & Johnson, A. E. (1987). The active site of blood coagulation factor Xa. Its distance from the phospholipid surface and its conformational sensitivity to components of the prothrombinase complex. *J. Biol. Chem.* **262**, 12953–12961.
57. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriaphage T4. *Nature*, **227**, 680–685.
58. Wells, C. M. & Di Cera, E. (1992). Thrombin is a Na^+-activated enzyme. *Biochemistry*, **31**, 11721–11730.
59. Lottenberg, R. & Jackson, C. M. (1983). Solution composition dependent variation in extinction coefficients for *p*-nitroaniline. *Biochim. Biophys. Acta*, **742**, 558–564.
60. Bevington, P. R. & Robinson, K. D. (1992). *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.
61. Straume, M. & Johnson, M. L. (1992). Analysis of residuals: criteria for determining goodness-of-fit. *Methods Enzymol.* **210**, 87–105.
62. Kuzmic, P. (1996). Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV proteinase. *Anal. Biochem.* **237**, 260–273.
63. Halfman, C. J. (1981). Concentrations of binding protein and labeled analyte that are appropriate for measuring at any analyte concentration range in radioimmunoassays. *Methods Enzymol.* **74**, 481–508.
64. Cheng, Y-C. & Prusoff, W. H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–3108.
65. Craig, D. A. (1993). The Cheng-Prusoff relationship: something lost in the translation. *Trends Pharmacol. Sci.* **14**, 89–91.
66. Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crysallog. sect. A*, **47**, 110–119.

Edited by R. Huber

(Received 23 February 2005; received in revised form 20 April 2005; accepted 25 April 2005)