Mapping of the Catalytic Groove Preferences of Factor Xa Reveals an Inadequate Selectivity for Its Macromolecule Substrates*

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Factor Xa (FXa) hydrolyzes two peptide bonds in prothrombin having (Glu/Asp)-Gly-Arg-(Thr/Ile) for P3′-P2′-P1′-P2-P1-P1′ residues, but the exact preferences of its catalytic groove remain largely unknown. To investigate the specificity of FXa, we synthesized full sets of fluorescence-quenched substrates carrying all natural amino acids (except Cys) in P3, P2, P1, P2′, and P3′, and determined the $k_{cat}/K_m$ values of cleavage. Contrary to expectation, glycine was not the “best” P2 residue; peptide with phenylalanine was cleaved slightly faster. In fact, FXa had surprisingly limited preferences, barely more pronounced than trypsin; in P2, the ratio of the $k_{cat}/K_m$ values for the most favorable side chain over the least was 289 (12 with trypsin), but in P1′, this ratio was only 30 (versus 80 with trypsin). This unexpected selectivity undoubtedly distinguished FXa from thrombin, which exhibited ratios higher than 19,000 in P2 and P1′. Thus, with respect to the catalytic groove, FXa resembles a low-efficiency trypsin rather than the highly selective thrombin. The rates of cleavage of the peptidyl substrates were virtually identical whether or not FXa was in complex with factor Va, suggesting that the cofactor did not exert a direct allosteric control on the catalytic groove. We conclude that the remarkable efficacy of FXa within prothrombinase originates from exosite interaction(s) with factor Va and/or prothrombin rather than from the selectivity of its catalytic groove.

At the confluence of the formerly named intrinsic and extrinsic pathways, factor Xa (FXa) is the midway protease of the blood clotting waterfall (1). FXa belongs to clan SA of the S1 family of serine peptidases along with thrombin and trypsin (2–5). Without cofactors, activation of prothrombin by FXa is slow; it becomes efficient only when FXa complexes factor Va to form prothrombinase (6). Rapid inhibition of FXa by antithrombin and heparin cofactor II. In contrast to FXa, however, thrombin alone rapidly catalyzes a number of critical reactions in the cascade: cleavage of fibrinogen, activation of factors V and VIII, and activation of protease-activated receptor 1 (6, 10, 15). Trypsin, the archetypal endopeptidase of the digestive tract, does not require cofactors to rapidly hydrolyze (in appropriate conditions) most peptide bonds that follow an arginine or a lysine. The notable specificity of the blood coagulation peptidases result from at least four molecular mechanisms: (i) constraints built up by subunits 3 to 3′ (S3 to S3′)2 of the catalytic groove, which accommodate the P3-P3′ residues of the substrate or inhibitor; (ii) strict restrictions caused by surface loops surrounding the catalytic groove; (iii) exosites remote from the catalytic groove, which may anchor complementary motifs of the overall substrate protein; and (iv) cofactors that may overturn the specificity of the protease (4, 16–21).

A comparison of the P3′-P2′ sequences of the known substrates and inhibitors of FXa suggests that glycine in P2 and serine in P1′ could favor catalysis. In this paper, we report a comprehensive study of FXa substrates preferences using fluorescence-quenched substrates. We also compared FXa preferences with those of thrombin and trypsin. Some of the substrate preferences of FXa were unexpected, but the main surprise came from the overall limited selectivity of its catalytic groove. Addition of factor Va, phospholipid, and calcium had no detectable influence on FXa preferences or on its catalytic efficiency. Thus, we conclude that the remarkable efficacy of FXa within prothrombinase must rely on exosite(s) interaction(s) rather than on a purely allosteric mechanism involving its catalytic groove.

**EXPERIMENTAL PROCEDURES**

Proteins and Reagents—Prothrombin was purified from human plasma and converted to thrombin as described previously (22). Bovine factor V/Va was from Kordia (Leiden, The Netherlands), and phospholipids vesicles were prepared by sonication of a 1 mg/ml mixture of phosphatidylcholine (80% w/w) with phosphatidyserine (20% w/w) (Sigma) as described previously (23).

The residues of the substrates are numbered from P1 to P2′, where P2 and P2′ refer to six residues remote from the cleavage site on the N- and C-terminal side, respectively. The corresponding subsites on the enzyme are numbered from S1 to S3′.

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Factor Xa Specificity

The five series of fluorescence-quenched substrates had ABz-VQFRSLGDQ-EDDnp for a common framework. The values reported were obtained for the hydrolysis of the substrates by recombinant FXa and represent the weighted mean of at least three determinations (standard error was 18% or less). Cleavage always occurred between the arginine and the serine, even when a second arginine (or a lysine) was in the sequence. The fluorescence-quenched substrates were performed in parallel under the conditions, and the $k_{cat}/K_m$ values were deduced from the progress curve, assuming a simple Michaelis-Menton mechanism with the encounter of enzyme and substrate limiting. The amino acid in P3(P2, P1), (resistant to hydrolysis) was set to ordifor conditions, and the $k_{cat}/K_m$ values that varies in each series is listed together with the estimated value of $k_{cat}/K_m$ (in $\text{M}^{-1}\text{s}^{-1}$) within each series, amino acids are classified from the most favorable to the least favorable. 

| $P_3$ | $k_{cat}/K_m$ | $P_2$ | $k_{cat}/K_m$ | $P_1'$ | $k_{cat}/K_m$ | $P_1$ | $k_{cat}/K_m$ | $P_0$ | $k_{cat}/K_m$ |
|-------|--------------|-------|--------------|-------|--------------|-------|--------------|-------|--------------|
| Q     | 2.6 x 10^4  | F     | 2.6 x 10^4  | S     | 2.6 x 10^4  | L     | 2.6 x 10^4  | S     | 3.0 x 10^4  |
| G     | 2.6 x 10^4  | G     | 2.1 x 10^4  | A     | 1.9 x 10^4  | G     | 1.9 x 10^4  | H     | 2.9 x 10^4  |
| H     | 2.5 x 10^4  | W     | 1.2 x 10^4  | T     | 1.5 x 10^4  | F     | 1.8 x 10^4  | T     | 2.6 x 10^4  |
| V     | 2.0 x 10^4  | Y     | 7.4 x 10^3  | G     | 9.1 x 10^3  | I     | 1.8 x 10^4  | N     | 2.6 x 10^4  |
| R     | 1.9 x 10^4  | P     | 5.1 x 10^3  | V     | 7.8 x 10^3  | Y     | 1.8 x 10^4  | G     | 2.6 x 10^4  |
| W     | 1.9 x 10^4  | L     | 4.0 x 10^3  | A     | 5.8 x 10^3  | A     | 1.6 x 10^4  | K     | 2.5 x 10^4  |
| A     | 1.7 x 10^4  | L     | 2.6 x 10^3  | H     | 5.8 x 10^3  | V     | 1.5 x 10^4  | K     | 2.0 x 10^4  |
| F     | 1.7 x 10^4  | S     | 1.9 x 10^3  | Y     | 3.7 x 10^4  | S     | 1.4 x 10^4  | M     | 1.8 x 10^4  |
| N     | 1.6 x 10^4  | R     | 1.7 x 10^3  | I     | 3.3 x 10^3  | W     | 1.2 x 10^4  | R     | 1.8 x 10^4  |
| L     | 1.6 x 10^4  | V     | 1.7 x 10^3  | F     | 3.2 x 10^3  | T     | 1.2 x 10^4  | A     | 1.8 x 10^4  |
| M     | 1.4 x 10^4  | Q     | 1.5 x 10^3  | L     | 3.1 x 10^3  | N     | 1.1 x 10^4  | E     | 1.7 x 10^4  |
| T     | 1.4 x 10^4  | E     | 1.2 x 10^3  | Q     | 2.6 x 10^3  | R     | 8.6 x 10^3  | Q     | 1.6 x 10^4  |
| S     | 1.3 x 10^4  | H     | 1.2 x 10^3  | R     | 2.6 x 10^3  | H     | 8.5 x 10^3  | P     | 1.4 x 10^4  |
| K     | 1.3 x 10^4  | T     | 1.1 x 10^3  | M     | 2.4 x 10^3  | E     | 8.1 x 10^3  | I     | 1.0 x 10^4  |
| P     | 1.3 x 10^4  | N     | 1.1 x 10^3  | D     | 2.4 x 10^3  | D     | 8.1 x 10^3  | I     | 9.0 x 10^3  |
| E     | 1.2 x 10^4  | I     | 8.2 x 10^3  | Q     | 1.9 x 10^3  | E     | 9.1 x 10^3  | Q     | 9.0 x 10^3  |
| E     | 1.1 x 10^4  | M     | 7.4 x 10^2  | W     | 1.0 x 10^3  | K     | 6.1 x 10^3  | V     | 7.5 x 10^3  |
| Y     | 6.0 x 10^3  | K     | 3.9 x 10^2  | E     | 8.6 x 10^3  | M     | 4.1 x 10^3  | L     | 7.2 x 10^3  |
| D     | 4.6 x 10^3  | D     | 9.0 x 10^1  | P     | ND           | P     | 1.4 x 10^3  | W     | 5.3 x 10^3  |

Tritiation of Trypsin, FXa, and Thrombin—The active site concentration of a stock trypsin solution (bovine, tosylphenylalanyl chloromethyl ketone-treated; Worthington, Lorne Laboratories, Twyford, UK) was determined by titration with p-nitrophenyl-p'-guanidinobenzoate. This tritated trypsin was used to determine the precise concentration of aliquots in 1 mM HCI of d-Phe-Phe-Arg-CH2Cl and d-Phe-Pro-Arg-CH2Cl (Calbiochem, Meudon, France). Briefly, 200 nM trypsin in kinetic buffer was incubated for 3 h at room temperature with various amount of each chloromethyl ketone (0.03–3 M). The reaction mixture was diluted 1:10 in kinetic buffer containing 100 µM S-2222, and the remaining free enzyme concentration was estimated from the rate of A222 increase. The initial concentrations of chloromethyl ketone aliquots were deduced from the intercept to the x axis of a linear plot of the remaining activity versus the amount of inhibitor added. The active site concentrations of FXa (recombinant or human) and of thrombin were determined in the same buffer system and experimental conditions using the calibrated aliquots of chloromethyl ketone. For FXa titration, the remaining free enzyme concentration (extinction coefficient 25 M⁻¹ cm⁻¹) was incubated with 0.1–5 µM d-Phe-Phe-Arg-CH2Cl, and the remaining free enzyme concentration was measured with 100 µM S-2222 as substrate. For thrombin titration, 20 nM enzyme according to the procedure described in Alves et al. (28). Lyophilized substrates were resuspended in a minimum volume N,N-dimethylformamide, such that concentration in the stock solutions was about 5 mM, to ensure that during all kinetic experiments, the final amount of N,N-dimethylformamide never exceeded 0.5% (v/v). The concentration of the fluorescence-quenched substrates was estimated from their A222 assuming an absorption coefficient of 10^5 M⁻¹ cm⁻¹. Determination of the $k_{cat}/K_m$ values of hydrolysis was performed essentially as described previously (16, 29). Assuming that the reaction...
obey a simple Michaelis-Menten mechanism with the encounter of enzyme and substrate limiting, when the reaction is performed at a substrate concentration below \( K_{\text{m}} \), the progress curve kinetic allows estimation of the \( k_{\text{cat}}/K_{\text{m}} \) of the reaction. Briefly, hydrolysis at 37 °C was monitored by measuring the fluorescence at \( \lambda_{\text{em}} = 414 \text{ nm} \) (slit, 4 nm) and \( \lambda_{\text{ex}} = 325 \text{ nm} \) (slit, 10 nm) in a LS50B spectrofluorimeter (PerkinElmer Life Sciences) equipped with a thermostatted microplate reader. A microparticle of 180 µl of substrate (1.8-µM in a solution), containing a 0.2% (v/v) Tween 20 was left in the thermostatted compartment until the temperature equilibrium was attained (10–15 min), and the reaction was started by adding 20 µl of enzyme (0.1–200 nm). Care was taken to keep the ionic strength constant, in particular the NaCl concentration (30–32). The increase in fluorescence with time was monitored for up to 4 h (until at least 75% completion of the reaction). The pseudo-first-order rate constant \( k \), the initial fluorescence \( (F_0) \), and the maximum fluorescence intensity \( (I_{\text{max}}) \) were estimated by nonlinear curve fitting analysis of the fluorescence intensity \( (I) \) dependence on time \( (t) \) using the following equation:

\[
INT = I_0 + I_{\text{max}}(1 - \exp(-kt))
\]

where \( k \) is the enzyme concentration. The values of \( I_0 \) and \( I_{\text{max}} \) estimated through the nonlinear curve fitting analysis were always consistent with the fluorescence intensities measured before and after 1 h of incubation of the substrate with 0.1 µM trypsin (corresponding to the intact and fully cleaved peptide, respectively). The apparent rate constant will be pseudo-first order and will equal \( k_{\text{cat}}/K_{\text{m}} \), provided that the initial substrate concentration is lower than its \( K_{\text{m}} \) value for the enzyme. To assess whether this condition was met, progress curve kinetics were systematically performed at two initial concentrations of the substrate. When comparing the results obtained at both substrate concentrations, the enzyme concentration was variable. With all of the substrates assayed \( k \), was, within the experimental error, identical at the two substrate concentrations. Thus, in this concentration range, the rate of hydrolysis was not dependent upon the amount of substrate, suggesting that \( k \) could be equated to the \( k_{\text{cat}}/K_{\text{m}} \) value.

When substrates carried several potential cleavage sites, the preferred bond of hydrolysis was determined after purification of the products by reverse phase chromatography. Kinetics were performed in conditions identical to those used for the fluorescence studies except that the reactions were quenched at timed intervals by adding H3PO4.
Factor Xa Specificity

A general comparison of the selectivity indexes of FXa, thrombin, and trypsin. The selectivity indexes (ratio of the \( k_{cat}/K_m \) value for the most favorable amino acid over the least) with FXa (closed circles), thrombin (closed squares), and trypsin (open circles) are plotted against S3, S2, S1, S1', S2', and S3'. Thrombin is by far the most selective protease, whereas the selectivity of FXa is only better than that of trypsin for the P2 residue.

\[ \text{Selectivity index} = \frac{k_{cat}}{K_m} \]

RESULTS

Preferred P2 Amino Acid for FXa Catalysis Was Phenylalanine—To investigate the catalytic groove preferences of FXa, we synthesized five series of fluorescence-quenched substrates, having a common 10-amino acid-long framework (ABz-VQ-RRSLGDDQ-EDDnp). The peptides carried a strongly fluorescent ABz group at the N-terminal end, but a C-terminal EDDnp quenched this fluorescence by resonance energy transfer. The \( k_{cat}/K_m \) value for the cleavage of each peptide by FXa was estimated by analysis of the increase of the fluorescence intensity upon hydrolysis. In each series of peptides, either the P3, P2, P1', P2', or P3' amino acid was varied, such that complete sets of peptides were prepared covering all natural amino acids (except cysteine). Proline in P1' was also avoided because it is known to prohibit trypsin cleavage.

Study of the P2 preferences of FXa revealed that, contrary to expectation, glycine was not the most favorable amino acid; the peptide having a phenylalanine residue in P2 was cleaved slightly faster than the one with a glycine (Table I). Overall, substrates with aromatic side chains in P2 were cleaved efficiently, whereas the least favorable amino acid was asparagine. The FXa cleavage sites in prothrombin and factor VII have glycine in P2. In fact, a number of studies have established that FXa prefers Gly over Phe in P2 position with chromogenic or fluorogenic substrates (30, 33–37). Furthermore, x-ray analysis suggests that Tryp99 (in the chymotrypsinogen numbering system) normally blocks S2 of FXa (2, 3). Thus, it was quite surprising that bulky side chains (phenylalanine and tryptophan) could be as efficient as glycine in our study. The discrepancy could originate from the influence of the P3/P4 apolar \( \beta \)-amino acid present in the substrates used in most previous studies on FXa specificity. Binding of a P3/P4 \( \beta \)-amino acid is highly favorable and clearly improves the catalytic efficiency of FXa (30). It is conceivable that binding of a \( \beta \)-side chain in S3/S4 distorts the neighboring S2', hence prohibiting the binding of a bulky P2 side chain. On the other hand, as opposed to small P2-P3 peptideyl substrates, our fluorescence-quenched substrates bind to substrates on both sides of the scissile bond. Thus, the observed specificity could also result from cooperative effects that would influence the P2 preferences of FXa. To investigate such possible cooperative effects, we synthesized a series of chromogenic substrates (acetyl-VQXR-pNA, where X was F, T, G, or P).

Analysis of the progress curves of cleavage confirmed that, when constituted exclusively by L-amino acids, a chromogenic substrate having phenylalanine in P2 is hydrolyzed slightly faster than its counterpart with glycine (4.1 ± 0.2 versus 3.4 ± 0.2 \( \times 10^3 \) m⁻¹ s⁻¹). Another possibility is that binding provides the necessary energy to move the aromatic ring system of FXa (Phe⁷⁴, Tyr⁹⁹, and Tryp¹⁵), perhaps by a simple rotation of Tyr⁹⁹ toward S3/S4, as is observed in kalirin and factor IXa (2, 3, 38, 39). Such movement would be prohibited when a \( \beta \)-amino acid side chain occupies S3/S4. In support of this hypothesis, FXa selects sequences with phenylalanine or tyrosine in P2 (in addition to those having glycine) when offered a library of fusion proteins displayed on phages as substrates (40) or a library of combinatorial fluorogenic substrates (41). In addition, binding of TFPI evidently requires that Tryp⁹⁹ of FXa swing away from the “normal” S4 (42). Finally, in the study of Castillo et al. (43) that also used fluorescence-quenched substrates, Gly as the “best” P2 amino acid for FXa was only measured in comparison with substrates with Val, Ser, or Thr at this position.

From the P2 series of fluorescence-quenched substrates, a value of 289 was calculated as the S2 selectivity index of FXa. This selectivity index simply represented the ratio of the \( k_{cat}/K_m \) values between the best and the worst side chains in P2. Study of the P1' preferences of FXa revealed a surprisingly low specificity. The selectivity index was only 30 (i.e. 10 times less than for the P2 residue). In general, FXa preferred small side chains in P1' (serine, threonine, and alanine), whereas amino acids with bulky or charged side chains were detrimental to catalysis (Table I). Preferences of FXa in P2' were even less pronounced with a selectivity index of 19. Nevertheless, FXa exhibited a slight preference for apolar or aromatic P2' residues. Selectivity indexes in S1 and S1' were less than 6, meaning that it was difficult to discern preferences, although P3' hydrophilic side chains possibly favored catalysis. Overall, only the P2 side chain of the fluorescence-quenched substrates allowed a marked selectivity to FXa.

The Catalytic Groove of FXa Behaves More as a Low Efficient Trypsin Than a Highly Selective Thrombin—In a previous study (29), we reported selectivity indexes of 636 and 20 in S2' for thrombin and trypsin, respectively. Accordingly, S2' of FXa would have a selectivity comparable with that of trypsin. To resolve this paradox, we completed the subsite mapping of thrombin and trypsin (Table II). As expected, trypsin was the most efficient and least selective protease. Within the P2 series for instance, thrombin cleaved its best substrate with a \( k_{cat}/K_m \) value comparable with that of trypsin, whereas its worst substrate was hydrolyzed with a \( k_{cat}/K_m \) value 5500-fold lower. Thus, thrombin emerged as a very efficient and selective enzyme. The selectivity index of FXa was 24-fold higher than that.
but use of FXa commercialized by Kordia gave identical results. Tested. The confirmed this observation with all fluorescence-quenched peptides prothrombinase complex, FXa hydrolysis was indistinguishable. We higher than the corresponding indexes of FXa and trypsin. S 2 most discriminating subsites were S2 and S 1 except for cleavage of the preferred site. FXa and thrombin cleaved all of the fluorescence-quenched substrates used in this study exclusively after the generic arginine.

FIG. 2. Analysis of the products of ABz-VQFRLGDQ-EDDnp hydrolysis by trypsin. The P3-P' 3' residues within this substrate are ambiguous because two potential cleavage sites coexist; in fact, substrate was cleaved at both positions by trypsin. Aliquots of the reaction mixture (100 μl in 1% H3PO4) were loaded on a C18 reverse phase column developed with a 10–80% linear gradient acetonitrile in 0.5% H3PO4. The curves represent the chromatograms of aliquots taken after (from front to back) 0, 1, 2, 4, 6, 8, 10, 15, 20, 25, 30, and 40 min of incubation of 10 μM ABz-VQFRLGDQ-EDDnp with 5 nM trypsin. The peaks were detected by monitoring the A280, such that only peptides containing the EDDnp group are visible. The peptides were quantified by peak surface integration analysis and identified by N-terminal sequencing. The insets represent progress curves of the amount of each product and of the remaining intact substrate (expressed in percentages of the maximum). Considering that two cleavages were performed simultaneously by trypsin, the kcat/Km value reported in Table II constitutes a lower limit because the alternate site must have competed for cleavage of the preferred site. FXa and thrombin cleaved all of the fluorescence-quenched substrates used in this study exclusively after the generic arginine.

Factor Xa Specificity

FIG. 3. Factor Va does not influence FXa hydrolysis of the fluorescence-quenched substrates. Progress curve kinetics (monitored by fluorescence) of the hydrolysis of 5 μM substrate by 50 nM FXa in the absence (black curve) or in the presence (gray curve) of 250 nM factor Va, 35 μM phospholipid, and 5 mM calcium. The top curves represent hydrolysis of ABz-VQFRSLGDQ-EDDnp, the middle curves represent hydrolysis of the same substrate with asparagine instead of serine, and the bottom curves represent hydrolysis of the same substrate with an isoleucine for the P3 residue. Whether or not in the prothrombinase complex, FXa hydrolysis was indistinguishable. We confirmed this observation with all fluorescence-quenched peptides tested. The curves on this graph were obtained with recombinant FXa, but use of FXa commercialized by Kordia gave identical results.

of trypsin in P3, but for all other subsites, indexes of FXa were either comparable or even lower than for trypsin. Conversely, selectivity indexes of FXa were far lower than for thrombin, including in P3 (65-fold less). Fig. 1 represents a general comparison of the selectivity indexes for the three proteases. The most discriminating subsites were S2 and S' 3 for thrombin, with selectivity indexes of 19,000 and 24,706, respectively, far higher than the corresponding indexes of FXa and trypsin. S2 was the most selective subsite of FXa, whereas for trypsin it was S' 3. Overall, the selectivity indexes suggested that the specificity of the catalytic groove of FXa resembled that of trypsin more than that of thrombin. The highest kcat/Km value obtained with FXa remained 177-fold lower than the kcat/Km value obtained with trypsin for the same substrate (3.0 104 versus 5.3 × 106 m−1 s−1). Moreover, the peptide ABz-VQFRRSLGDQ-EDDnp had the most favorable side chains from P3 to P' 3' for FXa, whereas this sequence was quite distant from the optimum for trypsin (see below). Thus, in contrast to thrombin, FXa emerged not only as enzyme with low selectivity but also as a protease with relatively poor efficiency (30).

Assuming no cooperative effects, the optimal P3-P' 3' sequence for FXa hydrolysis would be QRFR-SL, that for thrombin would be MPR-SFR, and the theoretical optimal sequence for trypsin would be MRR-RVG. The latter sequence is ambiguous, because it contains three potential cleavage sites. To resolve this uncertainty, we isolated and identified the products resulting from trypsin hydrolysis of the equivocal sequences. Trypsin cleavage of ABz-VQFRRSLGDQ-EDDnp (Fig. 2) released ABz-VQFRR and ABz-VQFR with similar rate constants (9.5 and 9.2 × 105 m−1 s−1). Cleavage of ABz-VQRRSLGDQ-EDDnp occurred with a slight preference after the second arginine (2.8 versus 2.3 × 105 m−1 s−1). Moreover, the peptide ABz-VQFRRSLGDQ-EDDnp released ABz-VQFR with a rate constant twice that of ABz-VQFRK (8.2 versus 4.5 × 105 m−1 s−1), and ABz-VQKRRSLGDQ-EDDnp was cleaved exclusively after the arginine in fifth position. From these data, it can be concluded that (i) trypsin slightly prefers Arg over Phe in P3 and/or Arg over Leu in P' 3', (ii) trypsin slightly prefers Arg over Gln for the same substrate (3.0 104 versus 5.3 × 106 m−1 s−1). Cleavage of ABz-VQFRKLGDQ-EDDnp released ABz-VQFR with a rate constant twice that of ABz-VQFRK (8.2 versus 4.5 × 105 m−1 s−1), and ABz-VQKRSLGDQ-EDDnp was cleaved exclusively after the arginine in fifth position. Therefore, the preferred P3 and P' 3' amino acids must both be arginine, and consequently, the hypothetical optimal sequence for trypsin would be MRR-RVG with preferential cleavage after the second arginine. Interestingly, the above preferences of bovine trypsin differ slightly from that of rat trypsin (44–47).
**Factor Xa Specificity**

*FIG. 4. Diagram of the theoretical $k_{\text{cat}}/K_m$ values for cleavage of the main sites involved in blood coagulation by FXa and thrombin.*

The values were calculated assuming complete independence of the subsites. The outer circle represents $k_{\text{cat}}/K_m$ value of $10^6 \text{ M}^{-1} \text{s}^{-1}$, the second circle represents $10^5 \text{ M}^{-1} \text{s}^{-1}$, the third circle represents $10^4 \text{ M}^{-1} \text{s}^{-1}$. All P$_1$-P$_3$ sequences (given in parentheses) are from the human protein. Selectivity of the catalytic groove of FXa (open squares) appears inadequate to select its natural substrates and inhibitors. On the contrary, the selectivity of thrombin allows rationalization in part for its role in blood clotting; the most favorable sequences correspond to reactions catalyzed, and the least favorable ones correspond to reactions resisting thrombin. The protein C sequence is restrictive to thrombin, but activation requires thrombomodulin as a cofactor; the fibrinogen sequence is also restrictive, but thrombin uses exosite interactions for its cleavage. F, factor; Prot. S, protein S; PAR-1 and PAR-2, protease-activated receptors 1 and 2; ATIII, antithrombin; FV, factor V inactivating site; tPA, tissue type plasminogen activator; Prot. C, protein C; Fg-A, A-chain of fibrinogen; Fg-B, B-chain of fibrinogen. The asterisks with protein S and TFPI indicate that Cys was taken as Ala to calculate the $k_{\text{cat}}/K_m$ value.

**Factor Va Influences Neither the Intrinsic Specificity nor the Efficiency of the Catalytic Groove of FXa**—In complex with factor Va within the prothrombinase complex, FXa activates prothrombin very rapidly and selectively. Obviously the cofactor converts FXa from a poor trypsin analogue to a highly specific and efficient prothrombin activator, raising the question as to whether factor Va improves the catalysis and/or the selectivity of the catalytic groove of FXa. We compared the $k_{\text{cat}}/K_m$ values for the hydrolysis by FXa of representative fluorescence-quenched substrates in the presence and absence of saturating amounts of factor Va. With each peptidyl substrate assayed, the $k_{\text{cat}}/K_m$ value obtained in presence of factor Va (250 nM), calcium (5 mM), and phospholipid (35 μM) was virtually identical to that obtained without cofactors (Fig. 3). Whether or not the peptidyl substrate presented a favorable sequence for FXa catalysis, factor Va had no detectable influence. Thus, factor Va did not improve the catalytic machinery of FXa for fluorescence-quenched substrate cleavage. The fact that factor Va was unable to alter FXa catalysis also implied that factor Va had no detectable influence on the selectivity of the catalytic groove. Consequently, rather than inducing a conformational change in the catalytic groove of FXa, factor Va likely provides a secondary binding site to the substrate (prothrombin) and/or modifies the conformation of prothrombin, allowing a better alignment of the scissile bond.

**DISCUSSION**

By using a collection of fluorescence-quenched substrates, our study provides a precise and complete mapping of FXa subsites. The most striking inference is that FXa emerges as a protease with loose selectivity and relatively low catalytic efficiency. The addition of factor Va influenced neither the selectivity nor the efficacy of FXa toward small, unconstrained peptides. Therefore, the high selectivity and efficiency of FXa within prothrombinase likely originates from secondary binding site interactions rather than from a remodeling of its catalytic groove by the cofactor.

A number of cleavage reactions have been attributed to FXa (prothrombin, factors V, VII, and VIII, and PAR-2 activation, as well as protein S inactivation), although the physiological relevance of all of these reactions has not been fully demonstrated. Nevertheless, the vast majority of the potential cleavage sequences found in proteins associated with blood coagulation resist FXa hydrolysis. It is therefore surprising that the overall specificity of FXa suggests that it could cleave (at least slowly) most, if not all, of the corresponding peptidyl substrates. Our results allow us to compute a theoretical $k_{\text{cat}}/K_m$ for cleavage by FXa of the P$_1$-P$_3$ sequences of the main blood clotting reactions (Fig. 4). The most favorable sequence would be the reactive site loop of antithrombin. The calculated $k_{\text{cat}}/K_m$ value of cleavage is $1.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, remarkably close to the association rate constant of the macromolecules. Interestingly, the least favorable reaction would be the release of fibrinopeptide A from fibrinogen. Several of the cleavages that are known to be performed by FXa are among the relatively favorable sequences. In addition to that of antithrombin, sequences that would be cleaved with $k_{\text{cat}}/K_m$ values higher than $10^4 \text{ M}^{-1} \text{s}^{-1}$ include PAR-2, the (nonactivating) cleavage of prothrombin, the activating site of factors VII, and several of the activating cleavages in factor V and VIII. Conversely, besides the release of fibrinopeptide A, sequences that would be cleaved with $k_{\text{cat}}/K_m$ values less than $10^2 \text{ M}^{-1} \text{s}^{-1}$ include a number of reactions that FXa is unable to fulfill: its own activation, that of
protein C, and the inactivating sites in factor V and VIII. Inconsistent with this picture, however, activation of plasminogen would be favorable, whereas the activation site of prothrombin and the reactive site loop of TFPI would not. In this regard, the requirement for a cofactor is amply documented for prothrombin activation only. Overall, the difference between the calculated $k_{cat}/K_{m}$ values is rather limited (636-fold at the most). Furthermore, the same evaluation with thrombin renders the above reasoning relatively pointless. The most favorable sequence for thrombin within the clotting cascade (one of the activating sites in factor VIII) would be cleaved with a $k_{cat}/K_{m}$ value 10$^4$ times higher than the least favorable sequence (activation of plasminogen). It is remarkable that reactions catalyzed by thrombin exhibit $k_{cat}/K_{m}$ values higher than 10$^4$ M$^{-1}$ s$^{-1}$, whereas reactions that thrombin is unable to catalyze mostly exhibit lower values. Exceptions include protein C and factor XI activation (both reactions require a cofactor) and fibropeptide A cleavage, which involves secondary sites of thrombin remote from the catalytic groove (4, 16, 17, 48, 49). TFPI also has a quite favorable sequence, but it is likely that, as for the basic pancreatic trypsin inhibitor (25), a sterical hindrance prohibits binding. In agreement with the above rationale, chimeras of antithrombin carrying one of the prothrombin cleavage sites in place of the normal reactive site loop are slightly less effective inhibitors of FXa but are unable to inhibit thrombin (50), and mutating the P$_{3}$, P$_{2}$, P$_{1}$, or P$_{0}$' residues in the reactive site loop of antithrombin has little influence on its reactivity toward FXa (51). Clearly, the subsites of thrombin control in part its specificity, whereas FXa cannot rely on its catalytic groove to select substrates.

X-ray data and NMR studies are consistent with the concept that FXa is a “weak trypsin” rather than an enzyme efficient and selective as thrombin. First, the catalytic groove of FXa is not hindered, quite similarly to the widely opened groove of trypsin, whereas that of thrombin is much narrower (2, 4, 52). Clearly, part of the specificity of thrombin originates from an exclusion by sterical hindrance of numerous P$_{0}$-P$_{4}$’ sequences (14, 25, 53). Lack of insertions leaves the catalytic triad exposed in FXa and trypsin, certainly explaining in part their low selectivity toward peptidyl substrates. FXa was not only poorly selective, it was also a low efficiency enzyme. Indeed, thrombin exhibited $k_{cat}/K_{m}$ values in the 10$^7$ M$^{-1}$ s$^{-1}$ range for its best fluorescence-quenched substrates, which compared favorably with the values obtained with trypsin. In contrast, none of the peptidyl substrates were cleaved by FXa with $k_{cat}/K_{m}$ values higher than 3 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$, even when the P$_{0}$-$P_{3}$’ sequence was nearly optimal. Thus, the selectivity of FXa was barely higher than that of trypsin, but its catalytic efficiency was a thousand times lower on average.

Within prothrombinase, the dramatic increase in the catalytic efficiency of prothrombin activation by FXa results from a 100-fold decrease of $K_{m}$ and a 3000-fold increase of $k_{cat}$ (54). Furthermore, factor Va exclusively enhances prothrombin activation, rendering FXa extremely selective as well as efficient. Cofactors may act by several mechanisms that are not mutually exclusive. The simplest mechanism would be a conformational change in the active site of the enzyme induced by cofactor binding. Structural alterations would improve catalysis by rearranging the geometry of the charge stabilizing system and/or that of the S$_1$ pocket. In this model, all reaction rates improve upon binding of the cofactor, simply because of the more efficient catalytic machinery. Such a mechanism does not apply to the FXa/factor Va interaction; cleavage rates of the fluorescence-quenched substrates were virtually identical whether or not factor Va was bound to FXa. Alternatively, still in a purely allosteric mechanism, the cofactors could ensure remodeling of one or several subsites, allowing a better binding and/or alignment of the scissile bond. Such a mechanism also does not apply to the FXa/factor Va interaction, at least with unconstrained peptidyl substrates; irrespective of the sequences of the fluorescence-quenched substrates, cleavage rates with or without factor Va were virtually identical. The same is true with peptidyl chloromethyl ketone inhibitors as reported by Walker and Krishnaswamy (54). Whether the probably constrained activation site in prothrombin requires a particular geometry to be cleaved by FXa remains to be explored.

Besides possible remodeling of the catalytic groove, cofactors could induce formation of a secondary binding site for the substrate (55–57). The subsequent site could be either on the enzyme (resulting from a remodeling induced by the cofactor) and/or carried by the cofactor itself (20, 21). In either case influence of cofactor binding on the hydrolysis of unconstrained peptidyl substrates would not be detectable. Providing a supplementary site for the substrate suggests at first that the $K_{m}$ value rather than the $k_{cat}$ value of the reaction would improve. However, it is conceivable that anchoring also drives the scissile bond of the substrate into a conformation allowing a better alignment with respect to the catalytic groove, thus improving both the $K_{m}$ and $k_{cat}$ values of the reaction. Our results establish that factor Va has no detectable influence on the catalytic efficiency and selectivity of the active site cleft of FXa toward small and unconstrained peptides. The inference is that factor Va improves the binding of prothrombin and/or allows a better alignment of the scissile bonds through the creation of secondary binding sites rather than by a purely allosteric mechanism exclusively involving the catalytic groove of FXa.

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