Physiological fIXa Activation Involves a Cooperative Conformational Rearrangement of the 99-Loop*

Coagulation factor IXa (fIXa) plays a central role in the coagulation cascade. Enzymatically, fIXa is characterized by its very low amidolytic activity that is not improved in the presence of cofactor, factor VIIIa (fVIIIa), distinguishing fIXa from all other coagulation factors. Activation of the fIXa-fVIIIa complex requires its macromolecular substrate, factor X (fX). The 99-loop positioned near the active site partly accounts for the poor activity of fIXa because it adopts a conformation that interferes with canonical substrate binding in S2-S4. Here we show that residues Lys-98 and Tyr-99 are critically linked to the amidolytic properties of fIXa. Exchange of Tyr-99 with smaller residues resulted not only in an overall decreased activity but also in impaired binding in S1. Replacement of Lys-98 with smaller and uncharged residues increased activity. Simultaneous mutagenesis of Lys-98, Tyr-177, and Tyr-94 produced an enzyme with 7000-fold increased activity and altered specificity. This triple mutant probably mimics the conformational changes that are physiologically induced by cofactor and substrate binding. It therefore provides a cooperative two-step activation model for fIXa. Tyr-177 locks the 99-loop in an inactive conformation which, in the physiologic complex, is released by cofactor fVIIIa. fX is then able to rearrange the unlocked 99-loop and subsequently binds to the active site cleft.

The blood coagulation factor IXa is a trypsin-like vitamin K-dependent serine protease that circulates in the plasma as a single chain inactive zymogen (1, 2). Its activation requires cleavage of two peptide bonds by either the activated factor VII (fVIIa)-tissue factor complex or activated factor XI (fXIa) (3, 4) to remove a 35-residue activation peptide. The active enzyme, rf9a, lacking the N-terminal Gla and epidermal growth factor-1 domains. In detail, residues Lys-98 and Tyr-99 were deleted, and we constructed multiple mutants. In addition, two solvent-exposed alanine residues from the two crystal structures of fIXa reported to date (11, 13). In particular, Lys-98/ Arg-99 are critical for substrate binding in the S2-S4 sites. These observations suggest a critical role of Tyr-99 and spatially neighboring amino acids for substrate binding. rf9a results in a 22-fold increase in activity toward synthetic substrates. Kolkman and Mertens (16) further demonstrate that the 99-loop restricts enzymatic activity toward fX and synthetic substrates in the absence, but not in the presence, of the cofactor fVIIIa.

The side chain of Tyr-99 adopts different conformations in the two crystal structures of fIXa reported to date (11, 13). In both structures Tyr-99 is in steric conflict with canonical substrate binding in the S2-S4 sites. These observations suggest a critical role of Tyr-99 and spatially neighboring amino acids for the substrate dependent activity of fIXa. In particular, Lys-98 is likely to electrostatically interfere with the basic substrate preference of fIXa.

Important questions remain open, however, including (i) What are the distinguishing mechanisms of substrate-assisted activity enhancement of fIXa?, (ii) What are the mechanisms underlying the cofactor-assisted activity enhancement of fIXa?, and (iii) Are these mechanisms independent from each other or coupled on a molecular basis?

To address these questions, we introduced a series of point mutations in the 99-loop of a truncated version of fIXa, designated rf9a, lacking the N-terminal Gla and epidermal growth factor-1 domains. In detail, residues Lys-98 and Tyr-99 were exchanged with smaller and/or uncharged residues. In addition, two solvent-exposed alanine residues from the 99-loop were deleted, and we constructed multiple mutants, where several interactions stabilizing the 99-loop were simultaneously abolished. We investigated the influence of the primary specificity pocket on activity by mutating the

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§ The abbreviations used are: fIXa, activated factor IX; MS-D-Phe-Gly-Arg-pNA, N-methylsulfonyl-L-Phe-Gly-Arg-4-nitroanilide acetate; Nle, norleucine; Ac-CONH-c-Nle-Gly-Arg-pNA, methoxycarbonyl-Nle-norleucyl-Gly-Arg-4-nitroanilide acetate; PABA, para-aminobenzamidine; RVV, Russell's viper venom.

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Cofactor- and Substrate-assisted Activation of fIXa

EXPERIMENTAL PROCEDURES

Chemicals—N-Methylsulfonyl-D-Phenylalanyl-4-nitroanilide acetate (MS-D-Phenylalanyl-4-nitroanilide acetate (MS-D-Phenylalanyl-4-nitroanilide acetate (MS-D-Phe-Arg-pNA), methoxycarbonyl-D-Norleucyl-Gly-Arg-4-nitroanilide acetate (MOC-D-Nle-Gly-Arg-pNA), and human antithrombin III were purchased from Roche Diagnostics GmbH (Mannheim, Germany), and Q-Sepharose FF and Heparin-Sepharose CL 6B were from Amersham Biosciences. para-Aminobenzamidine (PABA), porcine heparin (grade I-A) and crude Russell’s viper snake venom (RVV) were obtained from Sigma Aldrich. The IX activator RVV-X was purified as described elsewhere (17). 4-Nitrophenyl-4-guanidiniumbenzoate and benzamidine were from Merck. Purified human α-thrombin was a generous gift from Dr. Wirths (Roche Diagnostics GmbH). Restriction enzymes and enzymes used for PCR and cloning were from Roche Diagnostics GmbH and New England Biolabs (Schwalbach, Germany). All other materials were of the highest grade commercially available.

Bacterial Strains and Plasmids—Cloning and expression of proteins was done in the Escherichia coli K12 strain UT5600 (18) carrying the lacIq repressor plasmid pUBS520 (19). The plasmid pSa (20) was used for expression of recombinant proteins.

DNA Manipulation Techniques—All techniques used for amplification, mutagenesis, and cloning of DNA followed published protocols (21). Commercial kits were used according to the manufacturer's instructions.

Construction of Recombinant Proteins—The rf9 variant encoded the second epidermal growth factor domain, the activation peptide, and the catalytic domain of human fIX (15). The mutant proteins were produced using the plasmids pf9 and pf9 — DNA Manipulation Techniques. Activation was achieved using RVV-X, and the concentration of active sites was determined using thrombin-titrated antithrombin III and heparin as active site reactants (15, 22).

Amidolytic Assays and Determination of Kinetic Parameters—Experiments were performed in 50 mM Tris, 150 mM NaCl, 5 mM CaCl2, 0.1% polyethylene glycol 8000, pH 8.0, at 25 °C in a temperature-controlled Kontron-Uvikon 933 spectrophotometer (Kontron Instruments, Milano, Italy). Substrate concentrations ranged from 30 μM to 5 mM. Enzyme concentrations were between 2 and 200 nM. Reactions were started by adding the enzyme to the pre-warmed reaction buffer containing different substrate concentrations. The change in absorption at 405 nm was monitored for typically 2 min, and Δε/min was calculated from the linear part of the curve. Kinetic parameters were calculated by nonlinear curve-fitting of the initial rates to the Michaelis-Menten equation using the SigmaPlot software (Jandel GmbH, Erkrath, Germany). Inhibition constants of PABA were measured by competition with MOC-D-Nle-Gly-Arg-pNA. A 7 × 6 matrix was created by varying the concentration of MOC-D-Nle-Gly-Arg-pNA (45 μM to 3 mM) and PABA (0–2 mM) by repeated dilution by a factor of 2 and 3, respectively. The highest concentration was usually 5–10-fold the K_i of MOC-D-Nle-Gly-Arg-pNA or K_i of PABA. Kinetic data were calculated by global nonlinear least square fitting of the reaction rate v to v = v_eK_i/[S] + 1/K_i + [I] + 1/K_i + [I]), where v_e, [S], and [I] denote the total concentration of enzyme, substrate, and the inhibitor PABA, respectively. The influence of ethylene glycol on the reactivity of the enzymes was examined by measuring their activity in the presence of different ethylene glycol concentrations and 1 mM substrate.

RESULTS

Conformational and Catalytic Properties of Mutant Enzymes—The location of the mutations is summarized in Fig. 1. We examined enzymatic activity and conformational aspects of the mutants. The amidolytic activity and changes in selectivity in S2/S4 were probed by using the peptidic substrates MOC-D-Nle-Gly-Arg-pNA (substrate A) and MS-D-Phe-Arg-pNA (substrate B). To measure the effects of the mutations on the conformation of the primary specificity pocket, we also determined the binding constant K_i of the reversible S1 site inhibitor PABA in competition with substrate B. The results for the point mutations of Tyr-99 and Lys-98 as well as the Ala95a/Ala95b deletion mutant are summarized in Table II.
activity increased, the more the preference was altered toward the selectivity for the two tested substrates. The stronger the activity and inhibitor binding, the mutations also influenced the original value (see Table II). In addition to the impact on the amidolytic activity. In contrast, rf9a-K98M resulted in a 6.7-fold increase in activity compared with the single mutant rf9a-K98T, increasing activity when compared with rf9a and a 1000-fold increase activity, in particular K98T, we constructed multiple mutants to mimic the cooperative mode of fIXa activation in the physiological context. The kinetic parameters (activity, selectivity, and charge of the residue at position 98. Replacement of Lys-98 with smaller residues. These substitutions always resulted in a decreased activity, and the reductions inversely correlated with the size of the introduced residue; the activity of rf9a-Y99L with substrate A was reduced to 41%, whereas rf9a-Y99A retained only 5% of its original activity. Likewise, the affinity for the inhibitor PABA was decreased depending on the reduction of the side chain size. Despite these effects on activity and inhibitor binding, only the mutation Y99T had an influence on the selectivity of the enzyme as measured with two synthetic substrates.

As summarized in Fig. 1, structure analysis indicated that Tyr-99 hinders the direct substrate access to the S4 binding site (11, 13). Therefore, we replaced Tyr-99 with several smaller residues. These substitutions always resulted in a decreased activity, and the reductions inversely correlated with the size of the introduced residue; the activity of rf9a-Y99L with substrate A was reduced to 41%, whereas rf9a-Y99A retained only 5% of its original activity. Likewise, the affinity for the inhibitor PABA was decreased depending on the reduction of the side chain size. Despite these effects on activity and inhibitor binding, only the mutation Y99T had an influence on the selectivity of the enzyme as measured with two synthetic substrates.

Fig. 1 also suggests that the positively charged side chain of Lys-98 might interfere with the access of basic residues to the S1 pocket (11). Therefore we set out to test the role of both size and charge of the residue at position 98. Replacement of Lys-98 with the larger but equally charged arginine had only a minor effect on the amidolytic activity. In contrast, rf9a-K98M resulted in a 2.8-fold, and rf9a-K98T resulted in a 6.7-fold increase in activity with substrate A. Although PABA binding was hardly affected in rf9a-K98R, it was improved in the other 98 mutants, leading to a K_i reduced to ~60% that of the original value (see Table II). In addition to the impact on activity and inhibitor binding, the mutations also influenced the selectivity for the two tested substrates. The stronger the activity increased, the more the preference was altered toward the substrate A, which was less preferred in the wild-type enzyme.

Ala95a-Ala95b is a factor IXa-specific two-residue insertion as compared with trypsin-like serine proteases. This distinct sequential motif prompted us to probe the role of this insertion on rf9a amidolytic activity. Deletion of Ala95a/Ala95b, thus, aimed to reconstitute the 99-loop in a conformation resembling that of related coagulation factors. Nevertheless, this deletion had only little influence on all parameters examined.

Multiple Mutants Probing the Conformation of the 99-Loop—The conformation of the 99-loop in fIXa relates to a series of more subtle structural characteristics in addition to a two-residue insertion (Ala95a-Ala95b). Its conformation is stabilized by a hydrogen bond between the carbonyl of Lys-98 and the hydroxyl of Tyr-94, which is specific to fIXa. Also, Tyr-177 and Lys-98 would collide if the fIXa-99-loop were to adopt an fXa-like conformation. Moreover, the structure analysis revealed that Tyr-177 stabilizes and locks the 99-loop in its inactive conformation by interactions with Asn-97 and Asn-100 (cf. Fig. 1). Given the impact of mutants in the 99-loop on the activity, in particular K98T, we constructed multiple mutants to mimic the cooperative mode of fIXa activation in the physiological context. The kinetic parameters (activity, selectivity, and inhibitor binding) for these enzymes together with the corresponding wild-type data are summarized in Table III.

The triple mutant rf9-Y94F/K98T/Y177T had a 7000-fold increased activity when compared with rf9a and a 1000-fold increased activity compared with the single mutant rf9a-K98T, resulting in an amidolytic activity comparable with that of rf10a. rf9–99loop(F10)–Y94F/Y177T had a 3-fold higher activity than rf9–99loop(F10) and a 51-fold increased activity compared with rf9a. The affinity for the S1-directed inhibitor PABA in the triple mutant was essentially the same as in rf9a, whereas in rf9–99loop(F10)–Y94F/Y177T it resembled that of rf9–99loop(F10) and increased ~2-fold compared with rf9a. The selectivity of the mutants for the two substrates was in between those of rf9a and rf10a.

Influence of S1 Site Mutations on the Activity of fIXa—Glu-219 is positioned at the entrance frame to the S1 site of fIXa. It is particularly interesting because Gly-219 is conserved in almost all trypsin-like serine proteases, including trypsin itself and the coagulation factors. Because Glu-219 has an apparently unchanged backbone conformation compared with trypsin (11), we exchanged Glu-219 against Gln, thereby removing the substrate A, which was less preferred in the wild-type enzyme.

Ala95a-Ala95b denotes the two-residue deletion mutant where the fIXa-specific insertions Ala95a and Ala95b are removed.

| Enzyme             | kcat/Km | Selectivity, A/B | K_i, PABA |
|--------------------|---------|------------------|-----------|
| rf9a-wild type     | 134     | 159              | 0.85      | 201 |
| rf9a-Y99L          | 55      | 74               | 0.74      | 230 |
| rf9a-Y99T          | 33      | 22               | 1.49      | 690 |
| rf9a-Y99A          | 7       | 9                | 0.72      | 561 |
| rf9a-K98R          | 165     | 120              | 1.37      | 230 |
| rf9a-K98M          | 374     | 236              | 1.58      | 119 |
| rf9a-K98T          | 902     | 433              | 2.08      | 123 |
| rf9a-99loop(F10)   | 145     | 181              | 0.80      | 345 |

As summarized in Fig. 1, structure analysis indicated that Tyr-99 hinders the direct substrate access to the S4 binding site (11, 13). Therefore, we replaced Tyr-99 with several smaller residues. These substitutions always resulted in a decreased activity, and the reductions inversely correlated with the size of the introduced residue; the activity of rf9a-Y99L with substrate A was reduced to 41%, whereas rf9a-Y99A retained only 5% of its original activity. Likewise, the affinity for the inhibitor PABA was decreased depending on the reduction of the side chain size. Despite these effects on activity and inhibitor binding, only the mutation Y99T had an influence on the selectivity of the enzyme as measured with two synthetic substrates.
the salt bridge with Lys-224 and Ser-190 against Ala. The kinetic parameters for these mutations and the wild type as well as our earlier data are shown in Table IV.

rf9-E219Q had a 30% decreased activity with substrate A, paralleled by a 20% decreased affinity for PABA. The selectivity for the two substrates was unchanged. rf9-S190A had a rather unaltered activity, whereas $K_i$ was increased by 82%. The selectivity was altered toward a preference for substrate $A$.

The Ethylene Glycol Effect Depends on the 99-Loop—The reactivity of some mutants in the presence of ethylene glycol was examined with two different substrates. The results are summarized in Fig. 2. All tested rf9a variants had a maximum stimulation at ~35% ethylene glycol. The extent of stimulation correlates inversely with the catalytic turnover, which depends on both the mutant enzyme and the used substrate. The maximum 20-fold activity enhancement was observed with the wild-type rf9a and the worse substrate A (see Fig. 2). Analogously, the more active mutants showed less stimulation. The fXa mutant rf10a-99loop(F9), containing the fXa-derived loop, was stimulated to a maximum of 1.5-fold at 15–20% ethylene glycol and was inhibited at concentrations above 27 and 37%, depending on the substrate. Wild type fXa is inhibited by ethylene glycol already at low concentrations.

Proteolytic Activation of fIX Occurs in Two Steps—Even at millimolar concentrations, all zymogens were unable to auto-activate. This behavior contrasts that of related enzymes such as factor VII where the zymogen autoactivates spontaneously (23). Instead, the wild-type and mutant fIX enzymes required an exogenous protease (RVV-X) to cleave the peptide bond between Ala-146, isopropyl, and Ser-190 against Ala. The active site mutant rf9a-S195A was cleaved only once in the presence of RVV-X, thereby converting single chain rf9-S195A into the active conformation. Two-chain rf9a-S195A was not further processed. This contrasts the situation in related serine proteases, which display residual activity when carrying the corresponding mutation of the catalytic residue Ser-195 (26–28).

Tyr-99 Stabilizes the 99-Loop—Structure analysis indicated that Tyr-99 hinders the direct substrate access to the S4 binding site. Therefore, we replaced Tyr-99 with several smaller residues. Counterintuitively, the activity of these mutants decreased. The reactivity was lowest with the smallest side chain substitution, Y99A. Moreover, binding of the S1 inhibitor PABA was also impaired. This indicates that the removal of Tyr-99 results in complex changes, affecting not only the S2–S4 but also the S1 site by either blocking access to the primary specificity site or by even destabilizing its conformation. The side chain of Tyr-99 is the part of the 99-loop closest to the S1 pocket. Removal of the bulky side chain of Tyr-99 probably causes the loop to slide further into the S2–S4 substrate binding cleft. As a consequence, the positively charged side chain of Lys-98 comes closer to the entrance to S1 and repels basic substrate residues from binding into the primary specificity pocket.

**DISCUSSION**

**fIX Lacks Any Zymogen Activity**—**In vitro** the activation of fIX requires two distinct enzymatic events. RVV-X performs the initial cleavage, at Arg-180–Val-181, of single chain fIX to produce fIXaa but is unable to complete activation to fIXab. Instead, the second cleavage, at Arg-145–Ala-146, is performed auto-catalytically by fIXa. This observation implies that proteolytic activation of fIX critically depends on the presence of an already active enzyme, contrasting the situation of, for example, fVII, which is able to auto-activate without a prior “jump start” (23, 24). Given the sequence similarity of both activation peptide cleavage sites, we expect active fIXa to be able to cleave at both sites. The need for an exogenous activator indicates that single-chain fIX is unable to undergo the conformational switch necessary for proteolytic activity (23, 25).

The active site mutant rf9a-S195A was cleaved only once in the presence of RVV-X, thereby converting single chain rf9-S195A into the active conformation. Two-chain rf9a-S195A was not further processed. This contrasts the situation in related serine proteases, which display residual activity when carrying the corresponding mutation of the catalytic residue Ser-195 (26–28).

**Charge and Size of Lys-98 Disturb Substrate Binding**—To elucidate the influence of charge and size of Lys-98 on substrate binding, the activity of mutants with uncharged and shorter side chains in position 98 was examined. rf9a-K98M has an increased activity, confirming the working hypothesis of an electrostatic repulsion of basic substrate residues by Lys-98. The activity of rf9a-K98T is even higher, in line with the assumption that in the wild-type enzyme both charge- and size-hinder substrate binding. The binding affinity of the S1...
inhibitor PABA is doubled by the charge removal but independent of the size of the side chain at position 98. This indicates that, whereas the binding of positively charged P1 residues is electrostatically hindered by Lys-98, the size of the side chain at position 98 presumably only affects the accessibility to the S2/S4 site but not to the S1 site.

The Ala95a/Ala95b Insertion in the 99-Loop Does Not Affect the Amidolytic Activity of fIXa—The Ala95a/Ala95b insertion is specific to fIXa and, based on sequence comparison, is the most prominent difference to the related enzymes. Therefore, the deletion of these residues represents a straightforward approach to examine whether the low catalytic activity originates from these amino acids. However, the deletion of Ala95a/Ala95b did not have a significant impact on either activity or selectivity of rf9a.

Tyr-177 Locks the 99-Loop in an Inactive Conformation, Which Is Released by Cofactor fVIIIa and Rearranged by fX—The unique conformation of the 99-loop in fIXa relates to several elements, including a two-residue insertion (Ala95a-Ala95b) and a series of more subtle structural characteristics. Its conformation is stabilized by a hydrogen bond between the carbonyl of Lys-98 and the hydroxyl of Tyr-94. Because IXa has a Phe-94, this hydrogen bond is not existent in IXa. Also, Tyr-177 and Lys-98, both of which are threonines (or serines) in fIXa, fVIIa, and protein C, would collide if the fIXa-99-loop were to adopt an IXa-like conformation. Moreover, the structure analysis revealed that Tyr-177 stabilizes and locks the 99-loop in its inactive conformation by interactions with Asn-97 and Asn-100.

This rationale was examined by constructing a triple mutant rf9a-Y94F/K98T/Y177T as well as a chimerical rf9a containing the 99-loop of IXa and the IXa-like environment of the loop (Y94F/Y177T). Both mutants had a largely increased amidolytic activity. rf9a-99loop(F10)-Y94F/Y177T did not achieve the same activity as the triple mutant. Although the access to the substrate binding cleft is facilitated, and the steric clash between Lys-98 and Tyr-177 has been removed, the heterologous loop can obviously not adopt the optimal conformation.

In the highly active triple mutant rf9a-Y94F/K98T/Y177T, the 99-loop and the 177-segment probably approach the physiologically active conformation and open the active site cleft for substrate binding. These data suggest a mechanism of the physiological fIXa activation by cofactor and substrate binding. In this model, cofactor binding close to the 177-segment (5, 10) will release the lock of the inactive 99-loop imposed by Tyr-177. Only the physiological substrate fX is able to rearrange the released 99-loop, paving itself its way into the active site cleft of fIXa. Both steps of the fIXa activation are combined in the triple mutant, explaining its high activity toward peptidic substrates.

Notably, the conformation of the S1 site remains mainly unaltered in both multiple mutants as shown by the binding affinity of the S1 site inhibitor PABA (Table III). Thus, the introduced multiple mutations mainly affect the S2-S4 recognition sites.

S1 Site Mutations Decrease the Activity of fIXa—In serine proteases, the S1 site usually contributes most to substrate recognition and catalysis. Therefore, we examined the impact of mutations localized at the S1 site on the fIXa activity. None of the S1-directed mutations presented in this work and in earlier work (15) resulted in an improved activity.

Glu-219 is particularly intriguing because Gly-219 is conserved in almost all trypsin-like serine proteases, including trypsin itself and the coagulation factors. Although energetically unfavorable, Glu-219 has an apparently unchanged backbone conformation compared with trypsin. We attempted to clarify the importance of the charge of Glu-219 and its salt bridge with Lys-224 for fIXa activity by isosterically replacing it with Gln. Both activity and inhibitor binding were slightly impaired, indicating a moderate disturbance of the S1 site geometry. In the S190A mutant, the absence of a hydroxyl in the S1 site explains the weakening of PABA binding.

If the S1 site is affected during physiological activation, these changes are very subtle and are difficult to mimic by mutagenesis. Alternatively, the primary specificity pocket may be only of minor relevance for cofactor activation. The latter hypothesis is supported by only small differences in PABA binding between fIXa and IXa (Table IV).

Ethylene Glycol Binding at the 60-Loop AllostERICALLY Stimulates the Activity of fIXa by Reorganizing the 99-Loop—Stürzebecher and co-workers (29) speculate that the stimulation of fIXa activity by ethylene glycol and other alcohols is mediated by interaction of these alcohols with the 99-loop. Kolkman and Mertens (16) report that a fIXa variant with mutations in the 99-loop and increased amidolytic activity was stimulated by ethylene glycol to a lesser extent than the wild type but resulted in the same maximum activity. We recently showed that fIXa and fVIIa have a common ethylene glycol binding site between residues 60 and 90 that is not accessible in IXa (23). Occupation of this site by ethylene glycol appears to mimic the effects of macromolecular substrate binding on the 99-loop. Thereby it stimulates catalysis of synthetic substrates in a way that is usually only observed for macromolecular substrates that are able to occupy both sites (60-loop region and active site cleft) simultaneously in the presence of fVIIa. The decreased stimulation of the more active 99-loop mutants by...
ethylene glycol underlines this interpretation; the 99-loop is already reorganized in these mutants, thus precluding a further activity enhancement.

FXa is not stimulated by ethylene glycol. An FXa variant with the 99-loop of FXa was stimulated but only to less than 10% compared with FXa. The inferior activity modulation of this mutant can be explained by the lack of a proper ethylene glycol binding site in FXa despite the presence of a 99-loop that is in principle susceptible to ethylene glycol stimulation in this mutant.

The Closed Conformation of the 99-Loop Is a Major Insurance Against Premature Onset of Coagulation—FXa has evolved as an enzyme with a strictly regulated activity. It is virtually inactive in the absence of cofactor and physiologic substrate. This can be attributed mainly to the 99-loop. The side chains of Tyr-99 and Lys-98 impair substrate binding and are only removed by major reorganizations of several parts of the enzyme after binding of both the cofactor and the substrate. This “closed conformation” of the active site cleft is guaranteed by several mechanisms. Lys-98, Tyr-94, and Tyr-177 prevent a closed conformation of the active site cleft is guaranteed by

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