Surface Loop 199–204 in Blood Coagulation Factor IX Is a Cofactor-dependent Site Involved in Macromolecular Substrate Interaction

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Joost A. Kolkman, Olivier D. Christophe‡, Peter J. Lenting, and Koen Mertens§

From the Department of Plasma Protein Technology, CLB, Amsterdam, The Netherlands

In factor IX residues 199–204 encompass one of six surface loops bordering its substrate-binding groove. To investigate the contribution of this loop to human factor IX function, a series of chimeric factor IX variants was constructed, in which residues 199–204 were replaced by the corresponding sequence of factor VII, factor X, or prothrombin. The immunopurified and activated chimeras were indistinguishable from normal factor IXa in hydrolyzing a small synthetic substrate, indicating that this region is not involved in the interaction with substrate residues. In contrast, replacement of loop 199–204 resulted in a 5–25-fold reduction in reactivity toward the macro-molecular substrate factor X. This reduction was due to a combination of increased $k_{\text{on}}$ and reduced $k_{\text{off}}$. In the presence of factor VIIIa the impaired reactivity toward factor X was largely restored for all factor IXa variants, resulting in a more pronounced stimulation by factor X was largely restored for all factor IXa variants, presence of factor VIIIa the impaired reactivity toward substrates. Collectively, our data demonstrate that loop 199–204 plays an important role in the interaction of factor IXa with macromolecular substrates.

Human factor IX (FIX) is a vitamin K-dependent zymogen which plays an important role in blood coagulation. A defect or a deficiency in FIX is associated with the X-linked bleeding disorder hemophilia B. During the coagulation process FIX is activated by factor XIa (FXIa) or factor VIIa (FVIIa)-tissue factor complex (1). Activated FIX (FIXa) then converts factor X (FX) into FXa in the presence of calcium ions, phospholipids, and activated factor VIII (FVIIIa) by limited proteolysis (2). FIXa is a two-chain, disulfide-linked molecule, composed of a light chain ($M_\text{r}$, 18,000) and a heavy chain ($M_\text{r}$, 28,000). The N-terminal light chain consists of a $\gamma$-carboxyglutamic acid (Gla)-containing domain, followed by a short hydrophobic stack and two epidermal growth factor-like domains (3–5). The C-terminal heavy chain comprises the catalytic domain of FIXa. This domain possesses a trypsin-like serine protease fold consisting of two interconnected $\beta$-barrel domains with the catalytic residues His$^{221}$ [c57], Asp$^{509}$ [c102], and Ser$^{565}$ [c195] (chymotrypsin numbering in brackets) found at the interface of the two domains (6). This architecture is well conserved among the numerous members of the serine protease family. In addition to this common framework, each member contains a number of surface loops connecting the secondary structure elements. The size and amino acid sequence of these loops are characteristic for each individual serine protease. Serine proteases recognize specific cleavage sites within their natural substrates and are also targets of protease inhibitors, which play an important role in the inactivation of these enzymes. The variable surface loops bordering the substrate binding groove are believed to contribute to the specific interaction with these inhibitors and substrates. Six separate loops are involved in the construction of this large groove; loop 199–204 [c34–40], loop 223–229 [c59–64], loop 235–245 [c70–80], loop 256–268 [c91–101], loop 312–322 [c143–154], and loop 340–347 [c172–179] (for review, see Refs. 7 and 8).

In comparison with other coagulation enzymes, FIXa has remarkably low activity toward natural and synthetic substrates. The nature of the surface loops surrounding FIXa’s active site cleft may underlie this low catalytic activity. Binding of FVIIIa overcomes this limitation and results in a dramatic increase of FIXa-dependent conversion of FX (9, 10). This increase is mediated, at least in part, by a conformational change in FIXa’s active site region (11). In contrast, the catalytic activity of FIXa toward small synthetic substrates is not enhanced in the presence of FVIIIa (12, 13). These observations may indicate that the stimulating effect of FVIIIa on the activity of FIXa is due to a rearrangement of substrate-binding region(s) beyond the active site. Studies for other serine proteases revealed that one of the regions involved in macromolecular substrate and inhibitor binding comprises residues c34–40 (14–16). This surface loop, which has previously been referred to as Variable Region 1 by Furie et al. (17), has also been implicated in the interaction with cofactors and activators. For example, in $\alpha$-thrombin and FXa residues in this region have been reported to contribute to the interaction with cofactors thrombomodulin and factor Va, respectively (18, 19). In protein C a cluster of three basic residues located in Variable Region 1 is essential for efficient activation by the thrombin-thrombomodulin complex (20).

In the present study we investigated the contribution of loop
The Role of Loop 199–204 in Factor IX Function

Oligonucleotides used for the construction of cDNAs encoding chimeric FIX variants

| FIX variant | Oligonucleotide (5' → 3') |
|------------|--------------------------|
| FIX199–204/FX | ATC AAT GAG GAA AAC GAG GGT TTC TGT GGA GGC TCT ATC |
| FIX199–204/FVII | ACC CTC GTT CTC CTC ATT GAT CAA AAC AAC CTG CCA AGG |
| FIX199–204/FII | CGG AAG ACT CCC CAG GCT TTC TGT GGA GCC GTC ATC |
| FIX199–204/FXI | CTC CCG GGC ACT CAC AAG ACC ATC CCA AGG |
| FIX199–204/FX | GTT GTG TTC CCG AAG GTT GAT GCA TTC TGT GGA |
| FIX199–204/FVII | TGC ATC AAC CTT CCG GAA CAA AAC ACG CTG CCA AGG |
| FIX202–204/FII | AAT GGT AAA AGT CCC GTT GAT GCA TTC TGT GGA GGC TCT ATC |
| FIX199–204–359 | ATC AAT GAG GAA AAC GAG GGT TTC TGT GGA GGC TCT ATC |

199–204 to human FIX function with particular reference to inhibitor and substrate recognition and its role in the FVIII-dependent stimulation of FX activation. For this purpose, chimeric FIX variants were constructed in which loop 199–204 or parts thereof were replaced by the corresponding sequence of other serine proteases of the blood coagulation cascade. The chimeric proteins were expressed in mammalian cells and after purification functionally characterized for the interaction with substrates and inhibitors in the presence and absence of cofactors.

EXPERIMENTAL PROCEDURES

Materials—Protein A-Sepharose CL-4B, CNBr-Sepharose CL-4B, and Q-Sepharose FF were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). t-α-Phosphatidylserine, t-α-phosphatidylycholine, heparin (grade 1-A), and vitamin K were obtained from Sigma. Cell factories (6000 cm²) were from Nunc A/S (Roskilde, Denmark). Mikrotiter plates (Immulon) were from Dynatech (Plochingen, Germany). Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, and fetal calf serum were purchased from Life Technologies, Inc. (Breda, The Netherlands). FPL polymerase was obtained from Stratagene (Cambridge, United Kingdom). Oligonucleotide primers were from Perkin-Elmer (Gouda, The Netherlands). CH₃SO₂-LGR-Arg-Ile-Ala-Glu-Cys-Thr-Val-Glu-Cys-Glu-Gln-Ile-Leu-Glu-Cys-Tyr-Glu, product name CBS 31.39, was purchased from Diagnostica Stago (Asnières, France).

Proteins—The monoclonal anti-FIX antibody CLB-FIX 14 has been described previously (21). Polyclonal antibodies against FIX were obtained as described (22). Antibodies were purified employing Protein A-Sepharose as recommended by the manufacturer. Antibodies were conjugated with horseradish peroxidase as described (23). Normal plasma-derived FIX (pd-FIX) was purified from the activation mixture employing anion exchange chromatography as outlined previously (24).

Protein Concentrations—FIX antigen was measured by enzyme-linked immunosorbent assay employing a previously described method (25). FIX antigen was assayed in units, where 1 unit represents the amount of FIX antigen in 1 ml of normal human plasma. Protein was quantified by the method of Bradford (29), using HSA as a standard.

Hydrolysis of CH₃SO₂-LGR-Arg-Ile-Ala-Glu-Cys-Thr-Val-Glu-Cys-Glu-Gln-Ile-Leu-Glu-Cys-Tyr-Glu was initiated by addition of FIXa. FXa formation was quantified by addition of FIXa. FXa formation was stopped by addition of EDTA (0.01 m final concentration) and subsequently quantified employing the chromogenic substrate S2222 or S2276 (Chromogenix AB, Mölndal, Sweden). The amount of FVIII in 1 ml of human plasma (1 unit/ml) was assumed to correspond to 0.35 nM. The concentration of FIXa was determined by active-site titration with antithrombin (24). In preliminary experiments it was observed that in the presence of heparin, mutant and normal FIXa were completely inhibited by antithrombin within 30 min. To ensure that residual activities represent true end points, 1-h incubation periods were maintained in the active-site titration experiments.

Data were analyzed by using linear regression with minitab or by addition of FIXa. FXa formation was stopped by addition of EDTA (0.01 m final concentration) and subsequently quantified employing the chromogenic substrate S2222 or S2276 (Chromogenix AB, Mölndal, Sweden). The relationship between substrate hydrolysis and FXa concentration was determined using a active-site titrated FXa reference preparation. During the activation period less than 5% FXa was converted and FXa formation was linear in time. In experiments using various FVIIa concentrations (0–1.75 nM), unactivated FVIIa was added to the reaction mixture containing phospholipid (0.1 m), FIXa (0.1 m), and thrombin (5 nM). After 1 min of incubation, FXa activation was monitored at 0.2 m/s.

Inhibition Kinetics—Inhibition of mutant and normal FIXa by antithrombin was measured using the slow-binding kinetic approach (31). A series of inhibition progress curves was generated at 37 °C under pseudo first-order conditions with at least a 10-fold excess of antithrombin over FIXa. Each FIXa variant was added in a final concentration of 28 (28). Purification of recombinant FIX from concentrated medium was performed using the same immunopurification step as outlined previously (28). The specific antigen of the purified recombinant proteins was at least 150 units/mg. Analysis by SDS-PAGE and Coomassie Brilliant Blue staining revealed that all recombinant proteins migrated with a similar Mr, as pd-FIX, demonstrating that no propeptide-containing unprocessed FIX was present. As established previously, recombinant FIX produced by this expression system displays normal calcium-dependent properties and substrate specificity for recombinant wt-FIX and pd-FIX (26, 28). Recombinant and pd-FIX were converted into FIXa by FIXa inactivation and pd-FIX was purified from the activation mixture employing anion exchange chromatography as outlined previously (24).
Amino acid sequences of surface loop 199–204 and adjacent regions in FIX, FIX199–204/FX, FIX199–204/FVII, FIX199–204/FII, FIX199–203/FII, FIX199–202–204/FII, and FIX199–204–205 are shown. Mutated residues are indicated in boldface type.

| 198 | Loop 199–204 | 205 |
|-----|--------------|-----|
| FIX | PWQVVL | NGK–VDA | FCGLS |
| FIX199–204/FX | PWQVVL | INER–NEG | FCGLS |
| FIX199–204/FVII | PWQVVL | LVN–GAQ | FCGLS |
| FIX199–204/FII | PWQVVL | FRKSPQEL | FCGLS |
| FIX199–201/FII | PWQVVL | FRK–VDA | FCGLS |
| FIX199–202–204/FII | PWQVVL | NGK–QEL | FCGLS |
| FIX199–204–205 | PWQVVL | NGKSPVDA | FCGLS |

10 nM to a prewarmed solution containing various concentrations of antithrombin (0–3.5 μM) and CH₃SO₂-LGR-pNA (3 mM) as a competing substrate. Data were collected for up to 10 h. However, data analysis was restricted to the reaction phase in which substrate consumption was less than 10%. The progress curves were fitted to the integrated rate equation for slow-binding (31).

\[
A = v_0 t + (v_0 - v) (1 - e^{-k'} t) / k' + A_0
\]

(Eq. 1)

where \( A \) is absorbance at 405 nm at time \( t \), \( v_0 \) and \( v \) are initial and steady-state velocity, \( k' \) is the apparent first-order rate constant, and \( A_0 \) is the initial absorbance at 405 nm. Fitting generates values for \( k' \), \( v_0 \), and \( v \) for each progress curve. If the association between enzyme and inhibitor proceeds in a single step mechanism, a plot of \( k' \) versus the inhibitor concentration \( [I] \) yields a straight line, the slope of which represents the association rate constant \( (k_{a}) \) according to Equation 2.

The dissociation rate constant \( (k_{d}) \) is equal to the intercept with the y-axis.

\[
k' = k_{a} + \frac{k}{1 + [S]/K_{m}}
\]

(Eq. 2)

Analysis of FIXa-Antithrombin Complexes by SDS-PAGE—Complex formation of normal and mutant FIXas with antithrombin was analyzed by SDS-PAGE as described previously (24). The reactions were carried out in 5 mM CaCl₂, 0.1 M NaCl, 0.05 mM Tris (pH 7.4) with 1 μM FIXas and 2 μg antithrombin for 1 h in the presence of 2 mM g/ml heparin or overnight in the absence of heparin. After incubation, the samples were boiled for 5 min in 0.01% (w/v) bromphenol blue, 10% glycerol (v/v), 2% (w/v) SDS, 0.05 M Tris (pH 6.8). Complexes were applied to SDS-PAGE and detected by Coomassie Brilliant Blue staining.

RESULTS

Chimeric FIX Variants—The role of surface loop 199–204 in human FIX function was investigated employing chimeric FIX variants in which this loop was replaced by the corresponding sequence of coagulation factors FX, FVII, or prothrombin (Table II). These chimeras were expressed under transcription of Madin-Darby canine kidney cells and purified by immunoadfinity chromatography as described under “Experimental Procedures.” The chimeric FIX proteins could be converted completely to the FIXaβ form (M, 48,000) using FIXa and the conversion rate was similar to pd-FIX. The final preparations of activated normal and mutant FIX were more than 90% active as assessed by active site titration with antithrombin.

Amidolytic Activity—The contribution of loop 199–204 to the catalytic activity of FIXa was examined first by measuring the activity of normal and mutant FIXa toward the synthetic substrate CH₃SO₂-LGR-pNA. Hydrolysis rates at varying substrate concentrations were used to calculate the catalytic efficiency (\( k_{cat}/K_{m} \)). The apparent catalytic efficiency (\( k_{cat}/K_{m} \)) for FIXa was 1.9 (±0.2) × 10⁷ M⁻¹ s⁻¹ for FIXa, and 2.0 (±0.1) × 10⁷ M⁻¹ s⁻¹ for FIXa199–204/FII, FIXa199–204/FVII, and normal FIXa, respectively. Thus, replacement of loop 199–204 by the FX, FVII, or prothrombin counterpart leaves FIXa’s ability to hydrolyze a small tripeptide substrate lacking residues on the C-terminal side of the scissile bond (P* residues) unaffected.

FX Activation in the Absence of FVIIa—To assess the influence of the loop 199–204 replacements on large substrate interaction, the proteolytic activity of FIXa toward its physiological substrate FX was evaluated. The Michaelis-Menten parameters for FX activation in solution are presented in Table III. In comparison with normal FIXa, FIXa199–204/FX, FIXa199–204/FVII, and FIXa199–203/FII displayed significantly higher \( K_{m} \) values (8.4 μM versus 21.9, 21.3, and 23.5 μM, respectively) for normal FIXa, FIXa199–204/FX, and FIXa199–204/FVII, respectively, suggesting that FX is a less suitable substrate for the three chimeric FIXa variants. The increase in \( K_{m} \) values was accompanied by a decrease in \( k_{cat} \) for all three enzymes, resulting in a 24-, 27-, and 6-fold reduction in catalytic efficiency (\( k_{cat}/K_{m} \)) for FIXa199–204/FX, FIXa199–204/FVII, and FIXa199–204/FII, respectively.

FX activation was also studied in the presence of phospholipids as the catalytic surface. As shown in Fig. 1A, the activation of FIXa by FIXa199–204/FX, FIXa199–204/FVII, and FIXa199–203/FII is strongly reduced in comparison with normal FIXa, with 25-, 20-, and 5-fold lower apparent \( k_{cat}/K_{m} \) values, respectively. This reduction in catalytic efficiency was similar to that observed in the absence of phospholipids for all three FIX mutants, indicating that the defect in FX activation is phospholipid-independent. Overall, these results demonstrate
that loop 199–204 contributes to the proteolytic activity of FIXa toward FX both in the presence and absence of phospholipids. Interestingly, replacement of loop 199–204 by the FVII and FX sequence results in a 20–27-fold reduction in FX activity toward FX by a factor of 28, 47, and 26 \( \times 10^3 \), respectively, while normal FIXa was stimulated only 5 \( \times 10^2 \)-fold. Together, these data clearly demonstrate that in the presence of FVIIIa the impaired enzymatic activity is partially restored for all FIXa variants. The kinetic data shown in Fig. 1B were used to calculate the apparent dissociation constant (apparent \( K_a \)) for FVIIIa binding. As summarized in Table IV, the apparent \( K_a \) for both mutant and normal FIXa was approximately 0.7 nm, indicating that replacement of loop 199–204 in FIX has no effect on the affinity for FVIIIa.

**Inhibition by Antithrombin in the Presence and Absence of Heparin**—As the chimeric FIX variants displayed decreased activity toward FX, it was of interest to assess the influence of the loop 199–204 replacements on the inhibition by the macromolecular inhibitor antithrombin. Therefore, inhibition experiments under slow-binding conditions (see “Experimental Procedures”) were performed, in which the activated FIX chimeras and normal FIXa were incubated with various concentrations of antithrombin and a competing amide substrate. The inhibition curves obtained in the absence of heparin are shown in Fig. 2. As shown in panels C and D, the inhibition curves obtained for FIXa \( 199–204 \) were similar to those for normal FIXa. Apparently, replacement of loop 199–204 by the prothrombin sequence has a limited effect on the inhibition of FIXa by antithrombin. In contrast, FIXa \( 199–204 \) was almost resistant to antithrombin inhibition (Fig. 2, A and B). Inhibition of mutant and normal FIXa by

### Table IV

|                | \(-\text{FVIIIa} \times 10^5\) | Stimulation factor \times 10^3 | Apparent \( K_a \) |
|----------------|-------------------------------|-------------------------------|-------------------|
| Normal FIXa    | 40.9                          | 5                             | n/a               |
| FIXa\(^{199–204}\)/FX | 1.7                       | 28                            | 0.7               |
| FIXa\(^{199–204}\)/FVII | 2.1                       | 47                            | 0.8               |
| FIXa\(^{199–204}\)/FII | 8.0                       | 26                            | 0.7               |
| FIXa\(^{199–204}\)/FVII | 2.6                       | 52                            | 0.8               |
| FIXa\(^{199–204}\)/FII | 1.9                       | 49                            | 0.6               |
| FIXa\(^{199–204-100}\) | 6.6                       | 24                            | 0.5               |

*The catalytic activity (\( k_{cat}/K_m \)) of FX inactivation in the absence or presence of FVIIIa (1 unit/ml) was determined in the presence of phospholipids as described under “Experimental Procedures.” Stimulation factor represents the ratio of \( k_{cat}/K_m \) in the presence and absence of FVIIIa. Apparent \( K_a \) values for FVIIIa binding of normal and mutant FIXa were derived from the kinetics of FX activation as presented in Fig. 1B. Data represent mean values of multiple independent experiments. S.D. values were <10% of the mean given.*

**Fig. 2. Slow-binding kinetics for the inhibition of mutant and normal FIXa by antithrombin in the absence of heparin.** Inhibition curves of FIXa \( 199–204\)/FVII (A), FIXa \( 199–204\)/FX (B), FIXa \( 199–204\)/FII (C), and normal FIXa (D) are shown for representative experiments. FIXa (10 nM) and CH\(_3\)SO\(_2\)-LGR-pNA (3 mM) were incubated in 5 mM CaCl\(_2\), 0.1 mM NaCl, 0.2 mg/ml HSA, 0.05 mM Tris (pH 7.4) in the absence of antithrombin (open circles) or in the presence of 0.35 (closed circles), 0.7 (open squares), 1.0 (closed squares), 1.7 (open triangles), or 2.5 \( \mu \)M (closed triangles) antithrombin. Chromogenic substrate hydrolysis, expressed as absorbance, is plotted on the y axis versus the reaction time (min) on the x axis.
antithrombin was also examined in the presence of heparin. Addition of heparin resulted in irreversible inhibition of enzymatic activity for all four enzymes. The activity of FIXα<sup>199–204</sup>/FI and normal FIXa toward CH<sub>3</sub>SO<sub>2</sub>-LGR-pNA was inhibited instantaneously under the experimental conditions, whereas the inhibition rate for FIXα<sup>199–204</sup>/FVII and FIXα<sup>199–204</sup>/FX was only slightly reduced compared with normal FIXa and FIXα<sup>199–204</sup>/FII (data not shown).

Complex formation between antithrombin and FIXa was monitored directly by SDS-PAGE analysis. To this end, FIXα<sup>199–204</sup>/FVII, FIXα<sup>199–204</sup>/FI, and normal FIXa were incubated with a 2-fold excess of antithrombin in the presence and absence of heparin. As shown in Fig. 3, normal FIXa and FIXα<sup>199–204</sup>/FI formed high molecular weight complexes with antithrombin in the absence of heparin. In contrast, no complex was formed between FIXα<sup>199–204</sup>/FVII and antithrombin under the same conditions (lane 5). In the presence of heparin, complex formation with antithrombin occurred for normal FIXa and the two activated chimeras (lanes 1–3). Collectively, these findings indicate that the defect in antithrombin inhibition observed for FIXα<sup>199–204</sup>/FVII and FIXα<sup>199–204</sup>/FX in the absence of heparin is partially restored in the presence of this cofactor. In concordance with the FX activation experiments, the interaction between FIXα<sup>199–204</sup>/FI and antithrombin was only slightly affected.

**Partial Replacements in FIX Loop 199–204 by Prothrombin Counterparts**—To investigate the background of the relatively mild reduction in FX activation and antithrombin inhibition observed for FIXα<sup>199–204</sup>/FI, a second set of FIX variants was constructed. In these FIX variants the C- or N-terminal part of loop 199–204 was replaced by the corresponding prothrombin sequence (Table II). In addition, FIXα<sup>199–204</sup>/ST<sup>C</sup> was constructed in which prothrombin residues Ser-Pro were inserted into loop 199–204 (Table II). After purification and activation by FIXa (see “Experimental Procedures”), all three FIXa variants were found to be indistinguishable from normal FIXa with regard to amidolytic activity (data not shown). In contrast, the activity toward FX in the absence of FVIIIa was 16–22, and 6-fold reduced for FIXα<sup>199–204</sup>/FVII, FIXα<sup>202–204</sup>/FVII, and FIXα<sup>199–204</sup>/ST<sup>C</sup>, respectively (Table IV). Interestingly, the rate of FX activation observed for FIXα<sup>199–204</sup>/ST<sup>C</sup> was almost identical to that of FIXα<sup>199–204</sup>/FI. Similar to our findings in the FX activation experiments with the first set of FIX chimeras, the impaired proteolytic activity was partially restored in the presence of FVIIIa, resulting in a considerable increase in FVIIIa stimulation compared with normal FIXa (Table IV). To study the inhibition by antithrombin in the absence of heparin, the FIX chimeras were subjected to slow-binding inhibition experiments. In Fig. 4 the apparent first-order rate constant (k’) obtained at each antithrombin concentration is plotted versus the inhibitor concentration. For comparison FIXα<sup>199–204</sup>/FI and normal FIXa were included. It is obvious from these data that FIXα<sup>199–204</sup>/FI and FIXα<sup>202–204</sup>/FI were virtually insensitive to antithrombin inhibition, whereas the inhibition rate for FIXα<sup>199–204</sup>/ST<sup>C</sup> was decreased to the same extent as for FIXα<sup>199–204</sup>/FI. These data suggest that the prothrombin-specific insertion (Ser-Pro) compensates for the adverse effect of the other substitutions in FIXα<sup>199–204</sup>/FI.

**DISCUSSION**

In serine proteases the substrate binding groove is surrounded by a number of highly variable loops (Fig. 5). One of these surface loops comprises FIX residues 199–204. Mutagenesis studies and crystal structures of serine proteases in complex with inhibitors demonstrated that this loop plays a major role in the recognition of macromolecular substrates and inhibitors (14–16, 33). In order to investigate the contribution of loop 199–204 to human FIX activity, chimeric FIX variants were constructed, in which loop 199–204 was replaced by the corresponding sequence of FVII, FX, or prothrombin. Despite the similarity in their structures, FVIIa, FIXa, FXa, and thrombin display a large variation in activity and specificity. For example, FIXa shares its natural substrate FX with FVIIa, but the catalytic efficiency of FX activation in the absence of cofactors is considerably higher for FIXa (30, 34). Although FXa and thrombin differ substantially from FIXa with regard to substrate specificity, all three enzymes are sensitive to antithrombin inhibition. In contrast, FIXa is only susceptible to antithrombin inhibition in the presence of heparin (35).

In this study, we demonstrated that both FX activation and antithrombin inhibition are strongly reduced for chimeras FIXα<sup>199–204</sup>/FVII and FIXα<sup>199–204</sup>/FX (Figs. 1A and 2). Determination of the kinetic parameters for FX activation in the absence of FVIIIa and phospholipids revealed a 2.5-fold increase in K<sub>m</sub> and a 10-fold reduction in k<sub>cat</sub> (Table III). It seems reasonable to assume that in the absence of these cofactors the catalytic turnover of FX is slow relative to the dissociation of FX from the Michaelis complex and therefore the increase in K<sub>m</sub> values observed for the two FIXa variants implies that replacement of loop 199–204 reduces the binding affinity for FX. The reduction in k<sub>cat</sub> may indicate that replacement of loop 199–204 by the corresponding sequence of FVII or FX interferes with a correct orientation of the FX cleavage site with respect to FIXa’s active site. It is of interest to note that complex formation between FIXα<sup>199–204</sup>/FX and antithrombin was strongly affected, whereas antithrombin is a potent inhibitor of FXa (36). In addition, no detectable inhibition by tissue factor pathway inhibitor was observed for this FIXa variant (not shown), although electrostatic interactions between the FX counterpart of loop 199–204 and tissue factor pathway inhibitor play a major role in the interaction between FXa and tissue factor pathway inhibitor (14). Apparently, replacement of loop 199–204 is not sufficient to obtain FIXa variants with FXa-like inhibitor reactivity. This observation is in line with the study of Kurth et al. (37), in which replacement of the corresponding trypsin loop by the sequence of chymotrypsin did not reconstitute chymotrypsin-like specificity in the S<sub>1</sub>-binding site, unless a second trypsin loop (loop 223–229 in FIX) was replaced by its chymotrypsin counterpart.

In contrast to FIXα<sup>199–204</sup>/FVII and FIXα<sup>199–204</sup>/FX, FIXα<sup>199–204</sup>/FI displayed a limited reduction in antithrombin inhibition and FX activation. This finding was investigated in more detail by functional characterization of FIXa variants.
FIXa\textsuperscript{199–201}/FII, FIXa\textsuperscript{202–204}/FII, and FIXa\textsuperscript{199–204+SIP} (Table II). Substitution of the N- or C-terminal part of loop 199–204 by the corresponding prothrombin sequence was associated with a much more dramatic functional defect than substitution of the entire loop. The FIXa variant containing the prothrombin specific insertion Ser-Pro, however, was affected to the same extent as FIXa\textsuperscript{199–204}/FII, suggesting that these two residues are responsible for the unexpectedly mild functional defect observed for FIXa\textsuperscript{199–204}/FII. Apparently, the Ser-Pro insertion neutralizes the adverse effect of the other substitutions in this FIXa variant.

Several studies established that inhibition of FIXa by antithrombin and FIXa-dependent activation of FX is stimulated by several orders of magnitude in the presence of heparin and FVIIIa, respectively (9, 10, 36). The mechanisms of stimulation are dissimilar, however. In the antithrombin-dependent inhibition of FIXa, heparin most likely serves as a template, bringing enzyme and inhibitor in close proximity and thereby accelerating complex formation (38). In this study we observed that in the presence of heparin all FIXa variants form stable complexes with antithrombin leading to irreversible inhibition of their activities. This suggests that the addition of heparin as a template provides sufficient binding energy to counterbalance the unfavorable interaction between antithrombin and loop 199–204 in the FIXa chimeras.

FVIIIa enhances FIXa-dependent conversion of FX mainly by increasing the \( k_{\text{cat}} \) (9). This increase is associated with a conformational change in FIXa's active site environment (11). In the present study we demonstrated that the adverse effect of mutations in loop 199–204 [c34–40] on FX conversion is alleviated in the presence of FVIIIa (Fig. 1 and Table IV). A similar cofactor dependence has previously been reported for two other serine proteases. First, introduction of multiple mutations in loop c34–40 of tissue-type plasminogen activator resulted in an approximately 25-fold reduction in catalytic efficiency toward plasminogen in the absence of fibrin as a cofactor, whereas \( k_{\text{cat}}/K_m \) remained unchanged in the presence of fibrin (39).

Second, Le Bonniec et al. (16) suggested that binding of thrombomodulin to thrombin alleviates the disadvantageous interaction between Asp present in P9 of protein C and Glu\textsuperscript{39} located in loop c34–40 of thrombin. It should be noted that thrombomodulin interacts directly with this thrombin loop (18). However, our data led us to conclude that in FIXa loop 199–204 is not involved in cofactor binding, since replacement of this loop appeared to have no detrimental effect on FVIIIa interaction (Table IV). Several mechanisms may underlie the partial recovery of enzymatic activity in the presence of FVIIIa observed for all chimeric FIXa variants. One possible explanation is that the cofactor-dependent rearrangement of FIXa's active site region involves surface loop 199–204. Displacement of this loop upon FVIIIa binding may increase the ability of FIXa to orient FX productively in its active site. In this respect it is of interest to note that comparison of free thrombin and a thrombin variant in complex with basic pancreatic trypsin inhibitor revealed that a major reorganization of surface loops bordering the active site cleft is necessary for formation of this enzyme-inhibitor complex (40). Because there seems to be no direct interaction between FVIIIa and loop 199–204, we propose that the conformation of this region is altered by an allosteric mechanism. A similar mechanism has been proposed for the FVIIa-tissue factor interaction (41). Formation of this enzyme-cofactor complex is accompanied by a conformational change in a region that comprises one of the other surface loops bordering the active site cleft (equivalent to loop 235–245 in FIXa, see Fig. 5. Representation of the protease domain of porcine FIXa (6) showing the positions of the six surface loops bordering the substrate binding groove. The catalytic residues Ser\textsuperscript{265}, His\textsuperscript{221}, and Asp\textsuperscript{269} are depicted in the center of the molecule. The covalently bound inhibitor \( \text{d-Phe-Pro-Arg-chloromethyl ketone} \) is also shown with Phe, Pro, and Arg labeled P3, P2, and P1, respectively.
Fig. 5), whereas this region and tissue factor are not in direct contact (42). In addition to the role of loop 199–204 as a cofactor-dependent site, at least two alternative explanations exist that cannot be excluded at this stage. First, participation of FVIIIa in the FX-activating complex may alter the orientation of the substrate FX, thereby alleviating inhibitory contacts between FX and loop 199–204 and FX. This hypothesis would be supported by the notion that in the analogous prothrombinase complex the cofactor factor Va induces a conformational change in the protease domain of the substrate prothrombin (43). Second, it seems conceivable that in the presence of FVIIIa additional participation of FVIIIa (42, 44–46), make it tempting to speculate that a protease domains of FIXa and FVIIa are involved in cofactor binding (42, 44–46), make it tempting to speculate that a common mechanism is responsible for stimulation of the enzymatic activity of FIXa and FVIIa, and possibly other coagulation enzymes.

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