Surface-loop residue Lys\textsuperscript{316} in blood coagulation Factor IX is a major determinant for Factor X but not antithrombin recognition

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The active site of activated Factor IX (FIXa) and related blood-coagulation enzymes is surrounded by a number of highly variable surface loops, which contribute to the characteristic substrate specificity of each individual enzyme. FIX residue Lys\textsuperscript{316} is located in one of these loops and mutation of this residue to Glu is associated with haemophilia B. In the present study we investigated the functional role of Lys\textsuperscript{316} in human FIXa by analysing the purified and activated FIX mutants FIXa-K316E and FIXa-K316A. FIXa-K316E was indistinguishable from normal FIXa in binding the competitive active-site inhibitor \textit{p}-aminobenzamidine. In addition, substitution of Glu for Lys\textsuperscript{316} had no significant effect on the reactivity towards various synthetic tripeptide substrates. Inhibition by the macromolecular inhibitor antithrombin was only slightly reduced for both FIXa mutants (less than 2-fold). In contrast, proteolytic activity of FIXa-K316E towards the natural substrate Factor X (FX) was virtually lacking, while the Lys\textsuperscript{316} to Ala mutation resulted in a more than 10-fold reduction in FX activation. Thus residue Lys\textsuperscript{316} plays a key role in FIXa activity towards FX. The requirement for Lys at position 316 for FX activation was also evident in the presence of the cofactor activated Factor VIII, although to a lesser extent than in its absence. These data demonstrate that Lys\textsuperscript{316} specifically determines the reactivity of FIXa towards its natural substrate FX, but not to synthetic peptide substrates or antithrombin.

Key words: haemophilia B, Factor IX, Factor VIII, serine protease, site-directed mutagenesis.

INTRODUCTION

Factor IX (FIX) is the precursor of a serine protease required for blood coagulation. The structural organization of FIX is similar to that of other vitamin-K-dependent plasma proteins, such as Factor VII (FVII) and Factor X (FX) [1]. These proteins consist of an N-terminal \(\gamma\)-carboxyglutamic acid (Gla) domain, which is necessary for phospholipid binding, two epidermal growth factor (EGF)-like domains, an activation peptide region and the C-terminal serine protease domain with the catalytic centre [1,2]. FIX circulates in plasma as a single-chain zymogen and can be activated by either activated-FVII–tissue-factor complex or activated Factor XI (FXIa) [3,4]. Upon activation the peptide bonds Arg\textsuperscript{143}–Ala\textsuperscript{146} and Arg\textsuperscript{194}–Val\textsuperscript{198} are cleaved to yield a two-chain, disulphide-linked molecule, activated FIX (FIXa), and an activation peptide of 35 amino acids. The primary role of FIXa in coagulation is to convert FX into activated FX (FXa) in a process that requires calcium ions, a membrane surface and a protein cofactor, activated Factor VIIIa (FVIIIa); for review see [5]. FIXa alone displays extremely low proteolytic activity towards FX, but when assembled into the FX-activating complex it becomes a potent activator of FX [6,7].

The majority of enzymes involved in blood coagulation, including FIXa, belong to the family of trypsin-like serine proteases. A prominent characteristic of these proteases is the presence of a number of surface loops that display high variability in size and amino acid sequence. These loops border the substrate-binding groove and therefore are likely to control the substrate specificity and enzyme activity of each individual enzyme (for review see [8,9]). This notion is exemplified by a recent study from our laboratory in which mutations in one of these surface loops of FIXa, i.e. loop 256–268 [c91–101] (with the chymotrypsin numbering in brackets), enhanced the catalytic activity towards synthetic and natural substrates [10].

Furthermore, we have demonstrated that the reactivity of FIXa towards FX and antithrombin is impaired when loop 199–204 [c34–40] is exchanged for the corresponding sequence of related coagulation enzymes [11]. These mutational effects on FIXa activity were abolished in the presence of FVIIIa, suggesting that the surface loops are reoriented upon FVIIIa binding. This cofactor effect may provide an explanation for the fact that the haemophilia B database contains no mutations in loop region 199–204 [12]. Examination of the three-dimensional structure of human FIXa [13] reveals that the opposite edge of the substrate-binding groove is constituted by another surface loop comprising residues 315–322 [c146–154] (Figure 1). It seems conceivable, therefore, that this loop, which has previously been referred to as autolysis loop [14] or Variable Region 4 [15], serves a role similar to that of loop 199–204. One argument against this view, however, is the notion that in loop 315–322 several mutations have been identified that are associated with severe haemophilia B. These mutations occur at positions 316 (Lys \(\rightarrow\) Glu), 317 (Gly \(\rightarrow\) Arg, Trp or Glu) and 320 (Ala \(\rightarrow\) Asp or Val) [12]. In the present study we investigated this distinctive feature of loop 315–322 in more detail by functional analysis of FIXa variants in which Lys\textsuperscript{316} [c148] was replaced by Glu or Ala. Lys\textsuperscript{316} was selected for mutagenesis because this amino acid has, in contrast to Gly\textsuperscript{317} and Ala\textsuperscript{308}, a large basic side chain that is fully exposed to solvent and as such is a candidate for being in direct contact with substrates. The FIX mutants were expressed in mammalian cells, purified by immunoaffinity chromatography and subsequently activated by FXIa. Functional characterization of the activated mutants revealed that Lys\textsuperscript{316} is indispensable for efficient activation of FX in both the absence and presence of FVIIIa.

Abbreviations used: FIX, Factor IX; FIXa, activated FIX; FVIII, Factor VIII; FVIIIa, activated FVIII; FXa, activated Factor X; FX, Factor X; FXa, activated FX; FVII, Factor VII; HSA, human serum albumin; CH\textsubscript{3}SO\textsubscript{2}LGR-pNA, CH\textsubscript{3}SO\textsubscript{2}-\textit{l}-leucyl-\textit{l}-glycyl-\textit{l}-arginyl-p-nitroanilide; EGF, epidermal growth factor.

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Materials and Methods

Materials

t-α-Phosphatidyl-t-serine, t-α-phosphatidylcholine and heparin (grade 1-A) were purchased from Sigma (St. Louis, MO, U.S.A.). Q-Sepharose FF and the Thermo Sequenase cycle sequencing kit were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). p-Aminobenzamidine was obtained from Merck. Cell factories (6000 cm²) were from Nunc A/S (Roskilde, Denmark). Microtitre plates (Immulon) were from Dynatech (Plockingen, Germany). Oligonucleotide primers, restriction enzymes, DNA-modifying enzymes, Dulbecco’s modified Eagle’s medium, fetal calf serum, Geneticin 418 sulphate, penicillin} streptomycin and Fungizone were obtained from Life Technologies (Breda, The Netherlands).

Proteins

FXIa was obtained from Enzyme Research Laboratories (South Bend, IN, U.S.A.). Plasma-derived FIX was purified as described elsewhere [16]. Factor VIII (FVIII) was purified as outlined previously [17]. FX was purified as described in [18]. Antithrombin and human serum albumin (HSA) were obtained from the Division of Products of CLB (Amsterdam, The Netherlands). The monoclonal anti-FIX antibody CLB-FIX 14 has been described in [19]. Polyclonal antibodies against FIX were obtained as described in [17].

Protein concentrations

Protein concentrations were determined by the method of Bradford [20], using HSA as standard. FIX antigen was measured by ELISA employing a previously described method [16]. FVIII cofactor activity was measured using Coatest FVIII (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. FIXa concentrations were determined by antithrombin active-site titration in the presence of heparin [16].

Construction of mutant FIX cDNA

Plasmid pKG5 containing human FIX cDNA [21] was used as a template to construct cDNAs encoding FIX-K316A and FIX-K316E. Site-directed mutagenesis was performed by a PCR-based method, using oligonucleotide primers 5'-AAA GGG AGA TCT GCT TTA GTT CTT CAG TAC-3' (forward), 5'-TAA AGC A G AT C TC C CT T CG T GG A AG A CT C TT C CCA-3' (reverse, FIX-K316E) and 5'-TAA AGC AGA TCT CCC TGC GTG GAA GAC TCT TCC CCA-3' (reverse, FIX-K316A). A BglII restriction site (underlined) was introduced by silent mutagenesis to facilitate further cloning. The mutated FIX cDNA fragments were digested and subsequently ligated into the BamHI and HindIII restriction sites of pKG5. The final FIX constructs were verified by DNA sequence analysis.

Recombinant FIX

Recombinant FIX was expressed in Madin–Darby canine kidney cells, which were grown in Dulbecco’s modified Eagle’s medium
supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin. The expression plasmid pKGG containing mutant FIX cDNA was transfected into the cells using the calcium phosphate co-precipitation method. Geneticin-resistant clones were picked and propagated in selective medium to obtain stable cell lines. Secretion of FIX antigen into the medium was measured by ELISA. Cell lines with appropriate expression levels of FIX antigen were transferred to 1-litre cell factories as described in [22]. As reported previously, this expression system yields recombinant FIX molecules with normal calcium-dependent properties and similar activities for recombinant wild-type and plasma-derived FIXa [22]. Recombinant FIX was purified from concentrated medium by immunoaffinity chromatography using monoclonal antibody CLB-FIX 14 as outlined previously [22]. Mutant and normal FIX were converted to FIXa by incubation with FXIa at an enzyme/substrate ratio of 1:200 for 15 min. FIXa was purified from the activation mixture employing anion-exchange chromatography as outlined previously [16].

**Amidolytic activity**

Cleavage of amide substrates was assayed in 30% (v/v) ethylene glycol/5 mM CaCl₂/0.2 mg/ml HSA/0.1 M NaCl/0.05 M Tris, pH 7.4, at 37 °C. Initial rates of substrate hydrolysis were determined by monitoring the absorbance at 405 nm over time. Absorbance values were converted into molar concentrations using a molar absorption coefficient of 9650 M⁻¹·cm⁻¹ for p-nitroanilide and a pathlength of 0.35 cm for a 100 μl volume. The catalytic efficiency of CH₃SO₂-LGR-pNA hydrolysis was determined in the absence of ethylene glycol as described previously [11].

**FX activation**

FX activation in the absence of FVIIIa was assayed as outlined previously [23]. Briefly, phospholipid vesicles (phosphatidylserine/phosphatidylcholine, 1:1, mol/mol) and calcium ions were preincubated for 10 min at 37 °C, subsequently FX was added and after 1 min the reaction was started by the addition of FIXa. FXa formation was stopped by addition of EDTA (10 mM final concentration) and subsequently quantified employing the chromogenic substrate S2222 (Chromogenix AB). In experiments using various FVIIIa concentrations (0–1.75 nM), unactivated FXIIIa (40 nM), thrombin (5 nM) and calcium ions (0.1 mM), FIXa (0.1 nM) and thrombin (5 nM). After 1 min of incubation FX activation was initiated by addition of FX (0.2 μM). Initial rates of FXa formation were determined as described above.

**Analysis of FIXa–antithrombin complexes by SDS/PAGE**

Complex formation of FIXa with antithrombin was analysed by SDS/PAGE under non-reducing conditions. Protein was visualized by Coomassie Brilliant Blue staining.

**Slow-binding kinetics**

Inhibition of FIXa by antithrombin was examined using the slow-binding kinetic approach [11,24]. In these experiments FIXa was added at a final concentration of 10 nM (normal FIXa) or 20 nM (mutant FIXa) to a prewarmed solution containing various concentrations of antithrombin (0–1 μM) and CH₃SO₂-LGR-pNA (2.5 mM) as a competing substrate. The apparent first-order rate constant (k') was obtained for each antithrombin concentration by fitting the data from the progress curves to the integrated rate equation for slow binding [24]. A plot of k' versus the inhibitor concentration yields the association and dissociation rate constants (kₐ and kᵯᵤ), according to eqn. (2) in [11].

**Inhibition by p-aminobenzamidine**

The interaction of p-aminobenzamidine with FIXa was assessed in 5 mM CaCl₂/0.1 M NaCl/0.05 M Tris, pH 7.4, by competition with CH₃SO₂-LGR-pNA. In these experiments three concentrations of CH₃SO₂-LGR-pNA (0.5, 1 and 2 mM) were combined with a range of p-aminobenzamidine concentrations (50 μM–2 mM) in a 96-well microtitre plate. The reaction was initiated by addition of FXa (150 nM final concentration). Initial rates of substrate hydrolysis (v) were determined by measuring the absorbance at 405 nm over time. Inhibition constants (Kᵯ) were derived from a single-reciprocal (Dixon) plot of vᵱ versus the inhibitor concentration.

**RESULTS**

**FIX mutants**

To investigate the functional role of Lys³¹⁶ in surface loop 315–322 of FIX, we constructed two variants with mutations at this position. In FIX-K316E this residue was replaced by Glu, which corresponds to the reported haemophilia B mutation in this position [12]. In the FIX-K316A variant, Lys³¹⁶ was exchanged for a small and neutral Ala residue. These mutants were stably expressed in Madin–Darby canine kidney cells and purified using FXIa and the activation rate was similar to normal FIX.

**Inhibition by p-aminobenzamidine**

The contribution of Lys³¹⁶ to FIXa function was examined first, by evaluating the affinity of normal FIXa and FIXa-K316E for p-aminobenzamidine. This competitive inhibitor binds reversibly to the S1 binding site of a large number of serine proteases, including FIXa. The inhibition constant (Kᵯ) for p-aminobenzamidine was determined by competition with the chromogenic substrate CH₃SO₂-LGR-pNA. As listed in Table 1, FIXa-K316E and normal FIXa display similar values for Kᵯ, indicating that Lys³¹⁶ is not involved in the interaction of FIXa with p-aminobenzamidine.

**Amidolytic activity**

To investigate the role of Lys³¹⁶ in small substrate interaction, we determined the catalytic efficiency (kₙᵱ/kᵯᵱ) of normal FIXa and

**Table 1** Catalytic efficiency of CH₃SO₂-LGR-pNA hydrolysis and inhibition constants for p-aminobenzamidine

<table>
<thead>
<tr>
<th></th>
<th>CH₃SO₂-LGR-pNA</th>
<th>p-Aminobenzamidine</th>
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<tbody>
<tr>
<td></td>
<td>[10⁻² × kᵱ/kᵯ (M⁻¹·s⁻¹)]</td>
<td>[K (μM)]</td>
</tr>
<tr>
<td>Normal FIXa</td>
<td>1.9 ± 0.1</td>
<td>212 ± 12</td>
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<tr>
<td>FIXa-K316E</td>
<td>1.2 ± 0.1</td>
<td>203 ± 9</td>
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</tbody>
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Table 2  Hydrolysis of various amide substrates by normal FIXa and FIXa-K316E

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Normal FIXa (µM·min⁻¹)</th>
<th>FIXa-K316E (µM·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COO-CHG-NVal-Arg-pNA</td>
<td>3.7 ± 0.2</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>CH₃COO-CHG-Gly-Arg-pNA</td>
<td>15.4 ± 0.5</td>
<td>11.1 ± 0.2</td>
</tr>
<tr>
<td>CH₃COO-CHT-NVal-Arg-pNA</td>
<td>2.0 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>H-CHG-NVal-Arg-pNA</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.1</td>
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FX activation in the absence of FVIIa

To investigate the effect of the Lys³¹⁶ mutation on macro-molecular substrate interaction, we examined the proteolytic activity towards the natural substrate FX. To this end, FXa formation by mutant and normal FIXa was measured in the presence of phospholipids, calcium ions and various FX concentrations. As shown in Figure 2, substitution of Glu for Lys³¹⁶ virtually abolished the proteolytic activity towards FX (< 1% relative to normal FIXa). In order to investigate if the charge reversal was responsible for the severity of this FX activation defect, we also measured the proteolytic activity of FIXa-K316A towards FX. As shown in Figure 2, replacement of Lys³¹⁶ by the small neutral amino acid Ala resulted in a severe, but less dramatic, reduction in FX activation. Comparison of the kinetic parameters for FIXa-K316A and normal FIXa revealed that the Ala substitution did not affect the apparent $K_{cat}$ (0.18 ± 0.03 µM and 0.17 ± 0.02 µM, respectively; means ± S.D. are shown), while the apparent $k_{cat}$ was decreased by approx. 10-fold [(4.4 ± 0.2) × 10⁻⁵ min⁻¹ versus (45.3 ± 2.3) × 10⁻⁵ min⁻¹]. The extremely low rates of FX activation observed for FIXa-K316E did not allow for reliable estimation of the kinetic parameters. Similar experiments using bovine FX as a substrate revealed that the catalytic efficiency of FIXa-K316E for bovine FX was impaired to the same extent as for human FX (results not shown). Collectively, these data illustrate that Lys³¹⁶ plays a crucial role in the FIXa-dependent activation of FX.

Interaction with antithrombin

The role of Lys³¹⁶ in macromolecular substrate interaction was explored further by evaluating complex formation with the physiological inhibitor antithrombin. For this purpose, FIXa-K316E and normal FIXa were incubated with antithrombin in the presence or absence of heparin. Formation of FIXa–antithrombin complexes was examined by SDS/PAGE. As shown in Figure 3(A), normal FIXa formed high-molecular-mass complexes with antithrombin in the presence and absence of heparin (Figure 3A, lanes 3 and 5). Under the same conditions, FIXa-K316E also formed high-molecular-mass complexes with antithrombin, although the efficiency was lower in the absence of heparin (Figure 3A, lanes 4 and 6). This observation was addressed in more detail by measuring complex formation between FIXa and antithrombin over time. To this end, we performed inhibition experiments under slow-binding conditions in which FIXa-K316E, FIXa-K316A and normal FIXa were incubated with various concentrations of antithrombin in the presence of CH₃SO₄-LGR-pNA. In Figure 3(B), the apparent first-order rate constant (k') obtained at each antithrombin concentration was plotted versus the inhibitor concentration. The chromatographic substrate concentration used in these experiments was much lower than the $K_{cat}$ for all three enzymes, i.e. [S] < $K_{cat}$ in eqn. (2) of [11]. Thus the slope of each line directly represents the association rate constant ($k_{ass}$). The $k_{ass}$ values were (5.5 ± 0.3) × 10⁶ M⁻¹ min⁻¹ for normal FIXa, (3.0 ± 0.4) × 10⁶ M⁻¹ min⁻¹ for FIXa-K316E and (2.8 ± 0.2) × 10⁶ M⁻¹ min⁻¹ for FIXa-K316A. The $k_{dis}$ values were all within the range of (2.5–3.0) × 10⁻⁸ min⁻¹. Thus mutation of loop residue Lys³¹⁶ has only a minor effect on antithrombin interaction in the absence of heparin.

FX activation in the presence of FVIIa

Activation of FX was also assessed in the presence of the physiological cofactor FVIIa. In these experiments, the activity of mutant and normal FIXa towards FX was measured in the
presence of various concentrations of FVIIIa. As shown in Figure 4, FVIIIa enhanced the FIXa-dependent activation of FX in a dose-dependent and saturable manner. Similar to our observations in the absence of FVIIIa, the cofactor-dependent activity of FIXa-K316E and FIXa-K316A was considerably reduced relative to that of normal FIXa. However, it is obvious from these data that the defect in FX activation was less severe in the presence of FVIIIa for both FIXa variants. The kinetic data were used to calculate the apparent dissociation constant ($K_d$) for FVIIIa binding. The apparent $K_d$ values for normal and mutant FIXa were all between 0.7 and 1 nM, illustrating that mutation of Lys$^{316}$ has no significant effect on the affinity of FIXa for FVIIIa.

DISCUSSION

The blood-coagulation cascade comprises a series of sequential steps in which serine protease precursors are converted into active enzymes by limited proteolysis [25]. These serine proteases show considerable similarity to each other in amino acid sequence, domain organization and three-dimensional structure [1,8]. In spite of these common features, each enzyme displays high specificity for a limited number of plasma-protein substrates. It seems conceivable that specific interactions between the variable surface loops bordering the substrate-binding groove and the natural substrates play a significant role in the characteristic substrate specificity of each individual enzyme. In the present study we investigated the contribution of residue Lys$^{316}$, which is located in one of these surface loops, to the substrate specificity of human FIXa. To this end, we functionally characterized the FIX variants FIXa-K316E and FIXa-K316A.

The recently published crystal structure of a recombinant two-domain construct of human FIXa comprising EGF2 and the protease domain shows that Lys$^{316}$ extends into solvent [13]. The structure of full-length porcine FIXa [26] suggests, however, that residue 316 forms an intramolecular salt bridge with Glu$^{146}$ [219], which is located in one of the three $\beta$-strands that constitute the wall of the S1 binding pocket. Similarly, in the related coagulation protease thrombin, residue Glu$^{146}$ forms a salt bridge with Arg$^{221}$ [27]. Functional characterization of a thrombin variant in which Glu$^{146}$ was replaced by Ala revealed that disruption of this salt bridge alters the conformation of the S1 binding pocket [28]. In the present study we used $p$-aminobenzamidine to probe the conformation of this pocket in normal FIXa and FIXa-K316E. Our observation that both FIXa molecules display similar $K_d$ values for this inhibitor (Table 1) is in agreement with the view that, in the structure of human FIXa, Lys$^{316}$ is not in direct contact with the S1 binding site [13]. In addition, determination of the reactivity towards various amide substrates revealed that replacement of residue Lys$^{316}$ has no significant influence on the interactions in the S2 and S3 binding sites (Tables 1 and 2).

With regard to macromolecular substrate interaction, we observed that mutation of Lys$^{316}$ has only a slight effect on the inhibition by antithrombin (Figure 3), with a less than 2-fold reduction in the association rate constant. In this respect, it is of interest to note that replacements in the opposite surface loop, 199–204, strongly decreased the susceptibility to antithrombin inhibition [11], indicating that Lys$^{316}$ plays a secondary role in...
antithrombin interaction when compared with residues in loop, 199–204. In contrast to antithrombin inhibition, the enzymic activity of FIXa towards the natural substrate FX was dramatically reduced upon substitution of Lys316 (Figure 2). Comparison of the kinetic parameters for normal FIXa and FIXa-K316A revealed that mutation of Lys316 only influenced the \( k_{cat} \) of FX activation. This observation is in line with the study of Ruf [29], in which an Ala replacement for the analogous FVII residue (Arg290) reduced the \( k_{cat} \) of FX activation by approx. 5-fold, whereas the \( k_{m} \) remained unaffected. It may seem surprising that mutations in the active-site cleft of these two enzymes affect the \( k_{cat} \), rather than the affinity (\( K_{m} \)) for the FX substrate. This opens the possibility that interactions distant from the active-site region contribute to macromolecular substrate recognition. Indeed, for FIXa it has been reported that fragments consisting of either the two EGF-like domains linked together or the N-terminal EGF-like domain alone inhibit FX activation in the absence of cofactors [30,31]. Based on these observations the authors concluded that the EGF-like domains of FIX contribute directly to FX interaction. Moreover, in a recent study from our laboratory we observed that a FIX variant with substitutions at the interface of the two EGF-like domains displayed reduced activity towards FX, which was partially due to an increase in \( K_{m} \) [32]. It is further of interest to note that substitution of Ala or Thr, respectively) [35,36]. The fact that replacement of Lys positions P6–P2 (Gly-Ser-Gln-Val-Val versus Asp-Asn-Asn-Leu-Val) had a significant effect on substrate activation [33]. Furthermore, a thrombin variant in which Thr199–204 in blood coagulation factor IX is a cofactor-dependent site involved in the activation of bovine factor X. J. Biol. Chem. 256, 3433–3442


Kolkman, J. A. and Mertens, K. (2000) Insertion loop 256–268 in coagulation factor IXa: the relaxed \( \alpha \)-helix and \( \beta \)-strand. Therefore, it seems conceivable that this interaction involves a residue beyond this region. Although fully compatible with available data, further studies will be needed to verify this view and to identify the FX residue(s) that interact with Lys316.

It has been shown previously that assembly of the FIXa–FVIIIa complex is associated with conformational changes in the active-site region of FIXa [37]. In the present study we demonstrated that the proteolytic activity of FIXa-K316E and FIXa-K316A towards FX was impaired to a lesser extent than substitution by the neutral residue Ala may suggest that Lys316 contacts FX via an electrostatic interaction. Since the P’ region of FX contains no negatively charged amino acids, except for a Glu residue at P’6, it seems conceivable that this interaction involves a residue beyond this region. Although fully compatible with available data, further studies will be needed to verify this view and to identify the FX residue(s) that interact with Lys316.

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REFERENCES


39 Mathur, A. and Bajaj, S. P. (1999) Protease and EGF1 domains of factor IXa play distinct roles in binding to factor VIII. Importance of helix 330 (helix 162 in chymotrypsin) of protease domain of factor IXa in its interaction with factor VIII. J. Biol. Chem. 274, 18477–18486