Coagulation Factor XII Locarno: The Functional Defect Is Caused by the Amino Acid Substitution Arg 353 → Pro Leading to Loss of a Kallikrein Cleavage Site

By Johanna Kremser Hovinga, Johann Schaller, Hans Stricker, Walter A. Wulillemin, Miha Furlan, and Bernhard Lämmlle

The dysfunctional coagulation factor XII (FXII) Locarno was purified from 2 L of the proposita’s plasma. Studies to identify the molecular defect responsible for the lack of amidolytic and proteolytic activity of this FXII variant were performed. Amino acid sequence analysis of peptides obtained from FXII Locarno on activation with either trypsin or plasma kallikrein and dextran sulfate showed an amino acid substitution of Arg 353 by Pro. Thereby, the kallikrein cleavage site at Arg 353-Val 354 is lost. Although trypsin-activated FXII Locarno was fully cleaved at Arg 334-Ans 335 and at Arg 343-Leu 344, neither amidolytic nor proteolytic activity was generated. We conclude that proteolytic cleavage at Arg 343 in the absence of cleavage at Arg 353 is not sufficient to expose the enzymatic active site in FXII Locarno.

© 1994 by The American Society of Hematology.

Materials and Methods

Reagents and Chemicals

Daeethyl aminoethyl (DEAE)-Sephadex A50, SP-Sephadex C50, Sephadex G150 Superfine, CNBr-activated Sepharose 4B, and dextran sulfate (M, 500 kD, sulfur content 17%) were from Pharmacia (Uppsala, Sweden). Nitrocellulose membrane filters (pore size, 0.2 μm) were purchased from Schleicher & Schuell (Keene, NH). The chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide (S-2302) was from Kabi (Molndal, Sweden). Bovine trypsin and soybean negatively charged surfaces as did normal FXII. (4) On exposure of the proposita’s plasma to DS, no proteolytic cleavage of FXII Locarno occurred. (5) On addition of exogenous KK to a mixture of the proposita’s plasma and DS, normal cleavage outside, but only partial cleavage inside the disulfide loop Cys 340-Cys 467 was observed. (6) This proteolytically cleaved FXII Locarno showed neither amidolytic activity towards the chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide, nor proteolytic activity towards the physiologic substrates FXI and PK. Therefore, it was hypothesized that the functional defect in FXII Locarno was caused by a structural alteration affecting the plasma kallikrein-induced cleavage at Arg 353-Val 354.

The present study was performed to identify the structural abnormality underlying the functional impairment of FXII Locarno. The results indicate a replacement of Arg 353 by Pro leading to loss of the plasma KK cleavage site at Arg 353-Val 354 and preventing the generation of αFXIIa on contact activation of plasma.

From the Central Hematology Laboratory, Inselspital, and the Institute of Biochemistry, University of Bern, Bern; and the Ospedale Distrettuale di Locarno, La Carità, Locarno, Switzerland.


Supported by a grant (No 32-36443.92) from the Swiss National Science Foundation.


Address reprint requests to Bernhard Lämmlle, MD, Central Hematology Laboratory, Inselspital, University of Bern, CH-3010 Bern, Switzerland.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.

Fig 1. Schematic model of human factor XII (adapted from Cool and MacGillivray and Pixley and Colman). Amino acids are denoted by standard one-letter code (open circles), numbers refer to amino acid positions. Solid bars indicate (tentative) disulfide bonds based on homologies with other proteins. Bold characters demarcate the proposed domain structures found in human factor XII: fibronectin type II homology (A-B); epidermal growth factor-like regions (B-C and D-E); fibronectin type I homology (C-D); kringle domain (E-F); proline rich connecting region (F-G); and catalytic domain (G-COOH) with active site triad His 393, Asp 442, Ser 544 marked by asterisks. The putative signal peptidase cleavage site is shown by an open arrow and Ile at position 1 is the first amino acid residue of the zymogen. The plasma kallikrein cleavage sites are indicated by solid arrows. Cleavage by plasma kallikrein at Arg 353-Val 354 leads to formation of αFXIIa, and subsequent cleavages at Arg 334-Asn 335 and at Arg 343-Leu 344 generate βFXIIa.

trypsin inhibitor (SBTI) were purchased from Merck (Darmstadt, Germany), and bovine serum albumin, fraction V (BSA) was from Serva (Heidelberg, Germany). Polyvinylidene difluoride (PVDF)-type membranes (ProBlott) and the reagents of Sequanal grade used for N-terminal sequence analysis were from Applied Biosystems (Foster City, CA). All other chemicals were of analytical grade.

Plasma Samples

Citrated blood was obtained by clean venipuncture from the proposita. Plasma was prepared by centrifuging twice at 1,500g and 18°C for 10 minutes each. Plasma samples were stored in polypropylene tubes at −70°C until used. After informed consent, 2 L of the proposita’s plasma for protein purification was obtained by three plasmapheresis sessions, using citric acid/citrate/dextrose (ACD) anticoagulant. The plasmaphereses were kindly performed by Dr D. Castelli (Lugano, Switzerland).

A normal human plasma pool consisting of equal volumes of citrated plasma from 35 healthy male volunteers was stored in small aliquots in liquid nitrogen. Normal human plasma was defined to contain 1 U/mL FXII:C and FXII antigen (FXII:Ag), respectively. Pooled FXII-deficient plasma was prepared by mixing equal volumes of plasma obtained from four individuals with cross-reacting material-negative FXII deficiency.

Anti-FXII Antiserum

The anti-FXII antiserum was a polyclonal antihuman FXII antiserum raised in a rabbit. It was adsorbed with FXII-deficient plasma that had been immobilized on CNBr-activated Sepharose 4B.

Assay of FXII Clotting Activity (FXII:C)

FXII:C was measured by an activated partial thromboplastin time method using congenitally FXII-deficient plasma, as described in detail elsewhere.

Assay of FXII Antigen (FXII:Ag)

FXII:Ag was determined by dot immunobinding assay.

Assay of FXII Amidolytic Activity

Amidolytic activity of purified normal FXII and purified FXII Locarno, activated by either trypsin or KK and DS, was measured as follows. To 800 µL TBS (10 mmol/L Tris, 0.14 mol/L NaCl, pH 7.4), containing 1 mg/mL BSA and 0.1 mg/mL SBTI, 100 µL of 6 mmol/L S-2302 was added and the mixture was prewarmed at 37°C for 5 minutes. Then, 10 µL of the sample to be tested was added and the initial rate of absorbance change at 405 nm was recorded in a spectrophotometer at 37°C. Substrate hydrolysis (micromoles per liter per minute) was calculated using a molar absorption coefficient for p-nitroaniline at 405 nm of 9,800 mol−1 × L × cm−1.

Purification of Normal FXII, FXII Locarno, and PK

Normal FXII was purified from 3 L of a plasma pool of 6 healthy blood donors according to the method of Griffin and Cochrane modified by addition of a final gel filtration step on Sephadex G 150 Superfine. Purified FXII was kept in storage buffer (4 mmol/L
Na-acetate, 2 mmol/L acetic acid, 0.15 mol/L NaCl, pH 5.3) at -70°C and was radiolabeled with \(^{32}P\) by a chloramine T method\(^{15}\) to a specific radioactivity of about 40 \(\mu\)Ci/\(\mu\)g protein. Radiolabeled FXII was stored in 50 mmol/L acetic acid, 0.14 mol/L NaCl, 0.02% NaN\(_3\), pH 5.3, containing 10 mg/mL BSA. FXII Locarno was similarly purified from 2 L of the proposita’s plasma. During purification, FXII-Ag was purified by a supplier to a specific radioactivity of about 40 \(\mu\)Ci/\(\mu\)g protein. Radiolabeled FACTOR XI LOCARNO was freshly prepared from PK just before its use for FXII activation, as follows. To 10 vol of 50 mmol/L Tris, 0.15 mol/L NaCl, pH 7.8, containing 800 \(\mu\)g/mL PK, prewarmed at 37°C, 1 volume of \(\beta\)FXIIa solution (80 \(\mu\)g/mL) in 50 mmol/L Tris, 0.5 mol/L NaCl, pH 7.8 was added and the activation mixture was incubated for 5 minutes at 37°C.

**Preparation of Normal \(\beta\)FXIIa and \(\beta\)FXII Locarno Using Immobilized Trypsin**

Trypsin was immobilized on CNBr-activated Sepharose 4B according to manufacturer’s instructions. Purified normal FXII or FXII Locarno was incubated with immobilized trypsin, in 50 mmol/L Tris, 0.15 mol/L NaCl, pH 7.8, at an enzyme/substrate mass ratio of 2:1. The mixture was agitated end over end for 13 minutes at room temperature. After centrifugation, the supernatant was subjected to ion-exchange chromatography on DEAE-Sephadex A50, which was equilibrated with the same buffer. After extensive washing, \(\beta\)FXIIa or \(\beta\)FXII Locarno was eluted with 50 mmol/L Tris, 0.5 mol/L NaCl, pH 7.8. The peak fraction containing 80 \(\mu\)g/mL \(\beta\)FXIIa showed an amidolytic activity of 51.7 pmol/L/min. For amino acid and sequence analyses, normal \(\beta\)FXIIa and \(\beta\)FXII Locarno were diluted 1:3 with 50 mmol/L Tris, pH 7.8, and rechromatographed on DEAE-Sephadex A50, which was equilibrated with 50 mmol/L Tris, 0.15 mol/L NaCl, pH 7.8. After extensive washing with distilled water, the peptides were eluted with 0.11 mol/L formic acid and subjected to amino acid and sequence analyses. An aliquot of normal \(\beta\)FXIIa was used for high performance liquid chromatography (HPLC; see below).

**Preparation of Normal \(\beta\)FXIIa and \(\beta\)FXII Locarno by KK and DS**

The activation of normal FXII and FXII Locarno with KK/DS was performed in 25 mmol/L Tris, 75 mmol/L NaCl, pH 7.8, at a DS concentration of 12.5 \(\mu\)mol/L. Normal FXII (520 \(\mu\)g/mL) was activated with KK (68 \(\mu\)g/mL) at a KK/DS ratio of 1:7.6. In case of FXII Locarno, the concentration of FXII was 252 \(\mu\)g/mL and that of DS was 33 \(\mu\)mol/L, representing a similar KK/DS ratio. The mixtures were incubated at 37°C for 3 hours. The reaction was stopped by adding one-sixth volume of sample buffer, containing 10% sodium dodecyl sulfate (SDS), and boiling for 5 minutes. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)\(^{25}\) under nonreducing conditions and semi-dry electrophoresis to PVDF membranes followed. After staining with Coomassie blue R-250, the bands corresponding to \(\beta\)FXIIa or \(\beta\)FXII Locarno were cut out and subjected to amino acid sequence analysis.

**Amino Acid Composition and Sequence Analysis**

For amino acid analysis of normal \(\beta\)FXIIa and \(\beta\)FXII Locarno obtained using immobilized trypsin (see above), samples were hydrolyzed in the gas phase with 6 mol/L hydrochloric acid containing 0.1% (by volume) phenol for 24 hours at 115°C under vacuum according to Chang and Knecht.\(^{24}\) The liberated amino acids were reacted with phenylisothiocyanate and the resulting phenylthiocarbamyl amino acids were analyzed by reversed-phase HPLC on a Nova Pak C18 column (4 mm, 3.9 \(\times\) 150 mm; Waters, Milford, MA) in a Hewlett Packard liquid chromatograph with an automatic injection system (Waldbronn, Germany).\(^{26}\) The 0.14 mol/L sodium acetate buffer, pH 6.4, was replaced by corresponding ammonium acetate buffer. N-terminal amino acid sequence analysis was performed using Edman degradation. The final step was performed in a pulsed liquid-phase sequenator from Applied Biosystems (model 477A; Foster City, CA). The released amino acids were analyzed on-line according to the manufacturer’s instructions.

**HPLC Separation of Normal \(\beta\)FXIIa and Peptide Glu 327-Arg 334**

In the amino acid sequence analyses of all independent \(\beta\)FXIIa and \(\beta\)FXII Locarno preparations, obtained by either trypsin or KK/DS activation, we found unexpectedly a peptide belonging to the heavy chain of FXII. In case of trypsin activation, the peptide consisted of the eight amino acids from Glu 327-Arg 334. In case of KK/DS activation, the peptide consisted of nine amino acids (Arg 326-Arg 334). Normal \(\beta\)FXIIa obtained by trypsin activation and eluted with 0.13 mol/L formic acid was dried in a Speed-Vac concentrator and dissolved either in 0.1% trifluoroacetic acid (TFA) or in 0.3 mol/L Tris, 3 mol/L NaCl, pH 7.8. The latter sample was reduced for 6 hours at room temperature in the presence of 10 mmol/L dithiothreitol. Alkylation was performed for 15 minutes at room temperature using 12 mmol/L iodoacetamide. The pH of the solution was then adjusted to about 2 by the additions of 70% TFA. Nonreduced and reduced samples of normal \(\beta\)FXIIa were chromatographed on a reversed-phase Aqauapore-buty1 column (300 Å, 7 μm, 2.1 \(\times\) 100 mm; Applied Biosystems) on a Hewlett Packard 1090 liquid chromatograph. HPLC separation was performed with a linear gradient of 0% to 80% acetonitrile in 0.1% TFA for 60 minutes at a flow rate of 0.3 mL/min. The effluent was monitored by measuring absorbance at 210 and 280 nm. Collected peptide peaks were subjected to amino acid analysis and to sequence determination.

**Functional Studies of Purified Normal FXII and FXII Locarno**

To compare the functional properties of \(\beta\)FXII Locarno with those of normal \(\beta\)FXIIa, all studies were performed in 25 mmol/L Tris, 75 mmol/L NaCl, pH 7.8, if not indicated otherwise.

**Generation of amidolytic activity.** Normal FXII and FXII Locarno were activated with either trypsin or KK/DS at 37°C. Final concentrations in the activation mixtures, with a total volume of 33 μL, were as follows: normal FXII or FXII Locarno, respectively, 252 μg/mL trypsin 12.6 μg/mL, or KK 12.5 μg/mL, and DS 12.5 μg/mL. At predefined time points, amidolytic activity was determined as described above and the remaining sample was subjected to SDS-PAGE\(^{26}\) under both reducing and nonreducing conditions followed by staining with Coomassie blue R-250.

**Prototolysis of purified PK by normal \(\beta\)FXIIa or \(\beta\)FXII Locarno.** FXII or FXII Locarno (4.3 μg) was cleaved by 0.22 μg trypsin at 37°C for 15 minutes in a total volume of 16.5 μL to generate maximum amounts of \(\beta\)FXIIa or \(\beta\)FXII Locarno, respectively. The reactions were stopped by the addition of 3.5 μL of a solution containing 1 mg/mL SBTI. Five microliters of the mixtures containing 1 μg of trypsin-activated normal FXII or FXII Locarno, respectively, was added to 35 μL of a prewarmed (37°C) solution containing 20 μg PK and 286 μg/mL SBTI in 43 mmol/L Tris, 129 mmol/L NaCl, pH 7.8. After incubation at 37°C for 15 minutes, the reaction was stopped by boiling the samples for 5 minutes in the presence of 60 μL SDS-PAGE sample buffer containing 7.5% SDS. Unreduced and reduced aliquots, containing 10 μg of activated PK each, were subjected to SDS-PAGE\(^{26}\) followed by staining with Coomassie blue R-250. A control sample, in which FXII had been replaced by buffer, was analyzed in parallel.
RESULTS

FXII:C and FXII:Ag Values of the Proposita

The proposita’s FXII levels (FXII:C < 0.01 U/mL and FXII:Ag 0.53 U/mL) were in good agreement with the values measured 2 years ago (FXII:C < 0.01 U/mL and FXII:Ag 0.46 U/mL). These laboratory data suggested that the proposita had double heterozygous FXII deficiency, i.e., one allele not coding for any protein and the other coding for a dysfunctional FXII. Unfortunately, this could not be formally confirmed because neither her parents nor her siblings were available for study.

Table 1. Amino Acid Composition of Normal βFXIIa and of βFXII Locarno

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Normal βFXIIa</th>
<th>βFXII Locarno</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>14.4 (14)</td>
<td>13.3 (14)</td>
</tr>
<tr>
<td>Gla</td>
<td>31.4 (29)</td>
<td>30.4 (29)</td>
</tr>
<tr>
<td>Ser</td>
<td>17.7 (20)</td>
<td>18.4 (23)</td>
</tr>
<tr>
<td>Gly</td>
<td>27.9 (26)</td>
<td>28.3 (26)</td>
</tr>
<tr>
<td>His</td>
<td>9.0 (9)</td>
<td>10.1 (9)</td>
</tr>
<tr>
<td>Arg</td>
<td>14.1 (14)</td>
<td>15.1 (14)</td>
</tr>
<tr>
<td>Thr</td>
<td>9.9 (9)</td>
<td>9.6 (10)</td>
</tr>
<tr>
<td>Ala</td>
<td>27.2 (27)</td>
<td>27.4 (27)</td>
</tr>
<tr>
<td>Pro</td>
<td>18.0 (16)</td>
<td>17.8 (17)</td>
</tr>
<tr>
<td>Tyr</td>
<td>8.5 (9)</td>
<td>9.0 (9)</td>
</tr>
<tr>
<td>Val</td>
<td>15.3 (17)</td>
<td>17.0 (17)</td>
</tr>
<tr>
<td>Met</td>
<td>0.7 (1)</td>
<td>1.2 (2)</td>
</tr>
<tr>
<td>Cys</td>
<td>9.4 (14)</td>
<td>9.0 (14)</td>
</tr>
<tr>
<td>Ile</td>
<td>5.1 (6)</td>
<td>5.9 (6)</td>
</tr>
<tr>
<td>Leu</td>
<td>28.9 (29)</td>
<td>30.8 (30)</td>
</tr>
<tr>
<td>Phe</td>
<td>6.0 (6)</td>
<td>6.8 (6)</td>
</tr>
<tr>
<td>Lys</td>
<td>1.3 (1)</td>
<td>1.2 (2)</td>
</tr>
</tbody>
</table>

The amount of protein analyzed was 28 pmol of βFXIIa and 55 pmol of βFXII Locarno. The numbers in parentheses represent the expected values for respective amino acids. The expected amino acid composition of normal βFXIIa was calculated for Asn 335-Arg 343 and Val 354-Ser 596 according to sequence data of Fujikawa and McMullen. The expected composition of βFXII Locarno represents amino acids Asn 335-Arg 343 and Lys 346-Ser 596 (see sequence data).

1) Tryptophan was not determined.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Normal βFXIIa</th>
<th>βFXII Locarno</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>14.4 (14)</td>
<td>13.3 (14)</td>
</tr>
<tr>
<td>Gla</td>
<td>31.4 (29)</td>
<td>30.4 (29)</td>
</tr>
<tr>
<td>Ser</td>
<td>17.7 (20)</td>
<td>18.4 (23)</td>
</tr>
<tr>
<td>Gly</td>
<td>27.9 (26)</td>
<td>28.3 (26)</td>
</tr>
<tr>
<td>His</td>
<td>9.0 (9)</td>
<td>10.1 (9)</td>
</tr>
<tr>
<td>Arg</td>
<td>14.1 (14)</td>
<td>15.1 (14)</td>
</tr>
<tr>
<td>Thr</td>
<td>9.9 (9)</td>
<td>9.6 (10)</td>
</tr>
<tr>
<td>Ala</td>
<td>27.2 (27)</td>
<td>27.4 (27)</td>
</tr>
<tr>
<td>Pro</td>
<td>18.0 (16)</td>
<td>17.8 (17)</td>
</tr>
<tr>
<td>Tyr</td>
<td>8.5 (9)</td>
<td>9.0 (9)</td>
</tr>
<tr>
<td>Val</td>
<td>15.3 (17)</td>
<td>17.0 (17)</td>
</tr>
<tr>
<td>Met</td>
<td>0.7 (1)</td>
<td>1.2 (2)</td>
</tr>
<tr>
<td>Cys</td>
<td>9.4 (14)</td>
<td>9.0 (14)</td>
</tr>
<tr>
<td>Ile</td>
<td>5.1 (6)</td>
<td>5.9 (6)</td>
</tr>
<tr>
<td>Leu</td>
<td>28.9 (29)</td>
<td>30.8 (30)</td>
</tr>
<tr>
<td>Phe</td>
<td>6.0 (6)</td>
<td>6.8 (6)</td>
</tr>
<tr>
<td>Lys</td>
<td>1.3 (1)</td>
<td>1.2 (2)</td>
</tr>
</tbody>
</table>

The expected amino acid composition for normal βFXIIa is that of the βFXIIa light chain (amino acids 335-343) and the βFXIIa heavy chain (amino acids 354-596). The expected amino acid values for βFXII Locarno were calculated for the βFXIIa light chain and the βFXII Locarno heavy chain (amino acids 346-596, see sequence data below). Cysteine was measured as cystine and tryptophan was not determined. The sum of amino acid deviations from the expected compositions was 20.9 of 247 residues (8.5%) for normal βFXIIa, and 22.2 of 255 residues (8.7%) for βFXII Locarno. The presence of two methionine and two lysine residues in βFXII Locarno indicated that the peptide bond 353-354 had not been cleaved. The peptide Glu 327-Gln-Pro-Pro-Ser-Leu-Thr-Arg 334, belonging to the FXII heavy chain, was copurified with normal βFXIIa and βFXII Locarno (see below), but was not taken into account for calculation of the expected amino acid composition.

Amino Acid Sequence of Normal βFXIIa and βFXII Locarno Obtained by Trypsin-Sepharose

Twelve and 24 sequencing cycles were performed to analyze normal βFXIIa and βFXII Locarno, respectively. The analysis of normal βFXIIa provided the two expected amino acid sequences,12 Asn 335-Arg 343, corresponding to the light chain, and Val 354-His 365, representing the amino terminal sequence of the heavy chain of βFXIIa (Table 2). Sequence analysis of βFXII Locarno showed the same light chain (Asn 335-Arg 343). The principal sequence of the heavy chain started at Ser 347, leading up to Ala 370 with a replacement of Arg 353 by Pro. A minor sequence started at Lys 346 and showed the same Arg 353 to Pro substitution.
Table 2. Amino Acid Sequence of Normal βFXIIa and βFXII Locarno

| AA Position | 335 | 336 | 337 | 338 | 339 | 340 | 341 | 342 | 343 | 344 | 345 | 346 | 347 | 348 | 349 | 350 | 351 | 352 | 353 | 354 | 355 | 356 | 357 | 358 | 359 | 360 |... |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| AA sequence according to cDNA* | N   | G   | P   | L   | S   | C   | G   | Q   | R   | L   | R   | K   | S   | L   | S   | S   | M   | T   | R   | V   | V   | G   | G   | L   | V   | A   |... |
| Sequence of βFXIIa produced by trypsin activation† | N   | G   | P   | L   | S   | C   | G   | Q   | R   | L   | R   | K   | S   | L   | S   | S   | M   | T   | R   | V   | V   | G   | G   | L   | V   | A   |... |
| Normal βFXIIa (trypsin activation) | 148 | 103 | 170 | 132 | 82  | 99  | 114 | 146 | 324 | 278 | 219 | 233 | 310 | 251 | 167 |... |
| Normal βFXIIa (KK/DS activation) | 148 | 103 | 170 | 132 | 82  | 99  | 114 | 146 | 324 | 278 | 219 | 233 | 310 | 251 | 167 |... |
| βFXII Locarno (trypsin activation) | 148 | 103 | 170 | 132 | 82  | 99  | 114 | 146 | 324 | 278 | 219 | 233 | 310 | 251 | 167 |... |
| βFXII Locarno (KK/DS activation) | 148 | 103 | 170 | 132 | 82  | 99  | 114 | 146 | 324 | 278 | 219 | 233 | 310 | 251 | 167 |... |

The values under the amino acids indicate the amounts recovered (in picomoles).

* According to Cool et al. and Que and Devie.†
† According to Fujikawa and McMullen.‡
‡ Indicates cycles, where the respective amino acid residue is present in more than one peptide chain.
Fig 3. Separation of reduced and alkylated normal βFXIIa from the octapeptide Glu 327-Arg 334 by HPLC. The retention time of each peak is indicated. The presence of βFXII light chain (LC) in the peak at 7.5 minutes, of the PFXII heavy chain (HC) in the peak at 30.8 minutes, and of the octapeptide Glu 327-Arg 334 (P) in the peak at 13.2 minutes was confirmed by amino acid sequence analysis.

Amino Acid Sequence of Normal βFXIIa and βFXII Locarno Obtained by KK/DS Activation

Sequence analysis of normal βFXIIa was done twice, performing 10 and 20 cycles, respectively. The sequences of Asn 335-Arg 343 and Val 354-Gly 363 or Trp 373 were found (Table 2). Thereby, it was demonstrated that the known trypsin cleavage site at Arg 343-Leu 344 is also a KK cleavage site. In case of βFXII Locarno, 14 cycles were performed and the substitution of Arg 353 by Pro was confirmed. Whereas normal βFXIIa obtained using KK/DS activation showed complete cleavage at Arg 343-Leu 344 and at Arg 353-Val 354, the main sequence in βFXII Locarno starting at Asn 335 led through to Leu 348. The data indicated that KK/DS-induced cleavage of FXII Locarno in the region of Arg 343-Lys 346 was incomplete (see also Fig 5). The following three sequences were recorded in βFXII Locarno: Asn 335-Leu 348, Lys 346-Val 359, and a minor sequence from Ser 347 to Ala 360 (Table 2).

Separation of Normal βFXIIa From the Octapeptide Glu 327-Arg 334

In all five protein preparations subjected to amino acid sequence analysis, a minor sequence of an extra peptide, belonging to the heavy chain domain of FXII, was detected. In preparations of normal βFXIIa and βFXII Locarno produced with immobilized trypsin, the sequence of the peptide started at Glu 327, whereas in those produced with KK/DS, it started at Arg 326. In each case, it was terminated by Arg 334, and thus consisted of the amino acids (Arg 326-) Glu 327-Gln-Pro-Pro-Ser-Leu-Thr-Arg 334. Although this peptide contains no cysteine residue, it was not separated from normal βFXIIa or βFXII Locarno, neither during DEAE-Sephadex chromatography (activation with immobilized trypsin) nor during SDS-PAGE and electroblotting (activation with KK/DS). We were also unable to separate the octapeptide from the unreduced normal βFXIIa using reversed-phase HPLC. However, separation from βFXIIa was achieved by HPLC of the reduced and alkylated βFXIIa (Fig 3). Amino acid sequence analysis showed the presence of the octapeptide Glu 327-Arg 334 in the peak at 13.2 minutes, of βFXII light chain in the peak at 7.5 minutes, and of the βFXII heavy chain in the peak at 30.8 minutes (Fig 3).

Functional Studies of Purified FXII Locarno

Generation of FXII amidolytic activity on proteolytic activation. The amidolytic activity generated by trypsin activation of normal FXII increased rapidly, reaching a maximum (49 μmol/L/min) at an activation time of 15 minutes, and decreased slightly upon prolonged activation, probably because of further digestion of βFXIIa (Fig 4). Generation of FXII amidolytic activity by KK/DS activation of normal FXII was delayed, reaching a plateau (42 μmol/L/min) after an activation time of 2 hours (Fig 4). Maximum amidolytic activity of FXII Locarno after trypsin activation for up to 2 hours (0.2 μmol/L/min) or after KK/DS activation for up to 3 hours (0.4 μmol/L/min) was less than 1% of that displayed by activated normal FXII. SDS-PAGE analysis of FXII acti-

Fig 4. Amidolytic activity of activated normal FXII and FXII Locarno towards S-2302. Normal FXII and FXII Locarno were incubated with either trypsin or KK and DS at 37°C. After various time intervals, aliquots of the activation mixtures were tested for amidolytic activity against S2302 in the presence of SBTI. Amidolytic activity during trypsin (O) or KK/DS (V) activation of normal FXII, and during trypsin (C) or KK/DS (V) activation of FXII Locarno.
Proteolysis of purified PK by normal βFXIIa or βFXII Locarno. Activation with trypsin in the fluid phase led to complete cleavage of both normal FXII and FXII Locarno, generating similar amounts of βFXIIa and βFXII Locarno; cleavage of FXII Locarno with KK/DS, however, was incomplete (Fig 5).

In our laboratory, we had previously characterized the functional defects of two abnormal FXII variants using trypsin-activated FXII Locarno (Fig 6, lanes d) or with a trypsin-buffer control sample (Fig 6, lanes e), showing that trypsin was efficiently inhibited by SBTI.

**DISCUSSION**

In recent years, several dysfunctional FXII variants have been discovered in subjects whose plasma lacked FXII:C despite the presence of FXII-like antigen.\(^2\)\(^9\) So far, the structural defect of only one of the six reported FXII variants has been unraveled. In FXII Washington D.C.,\(^2\) the substitution of Cys 571 by Ser disrupts the disulfide bridge Cys 540-Cys 571 near the active site Ser 544, leading to the loss of enzymatic activity.

In our laboratory, we had previously characterized the functional defects of two abnormal FXII variants using...
whole plasma. Whereas the functional defect in FXII Bern (displaying normal surface binding and normal KK cleavage pattern, and lacking proteolytic activity) is similar to that of FXII Washington D.C., the defect in FXII Locarno is different. Based on studies using whole plasma, an alteration of the KK cleavage site at Arg 353-Val 354 was predicted.

In the present study, FXII Locarno was purified from the proposita’s plasma and the structure and function of βFXII Locarno generated by cleavage with either immobilized trypsin or KK/DS were compared with those of normal βFXIIa. The amino acid sequence analysis of trypsin-induced βFXII Locarno showed a substitution of Arg 353 by Pro (Table 2). This substitution was confirmed by sequence analysis of KK/DS-cleaved FXII Locarno. Because the genetic codon for Arg 353 was determined as CGC, most likely a single-base mutation resulting in the codon CCC has occurred in the proposita’s genomic DNA encoding FXII. The peptide bond Arg 343-Leu 344 in FXII Locarno as well as in normal FXII was completely cleaved by trypsin. In contrast to normal FXII, FXII Locarno showed incomplete KK/DS-induced cleavage of this peptide bond. This is in good agreement with the impaired KK-induced cleavage inside the disulfide loop Cys 340-Cys 467 of FXII Locarno in whole plasma experiments. It seems likely that the peptide bond Arg 343-Leu 344 can only be cleaved efficiently by KK after prior cleavage of Arg 353-Val 354.

The nonapeptide or octapeptide Arg 326 (Glu 327)-Arg 343, derived from the FXII heavy chain, was found in all five preparations of normal βFXIIa or βFXII Locarno obtained by trypsin-induced cleavage and DEAE-Sephadex chromatography or, even more surprisingly, by activation with KK/DS followed by SDS-PAGE and electrophoresis on PVDF membranes. Obviously, a very strong association exists, because separation of the octapeptide and βFXIIa was only achieved by HPLC of the reduced and alkylated βFXIIa sample (Fig 3). As shown by amino acid sequence analyses, this peptide was not part of an alternative βFXIIa light chain (amino acids Arg 326 [Glu 327] Arg 343), because its sequence ended in every case at Arg 334.

Further studies are required to elucidate the nature, localization, and significance of the binding interaction between the octanona-peptide and βFXIIa.

On activation of purified FXII Locarno by either trypsin or KK/DS, the generated βFXII Locarno developed less than 1% of amidolytic activity towards the chromogenic substrate S-2302 as compared with identically generated normal βFXIIa (Fig 4). Whereas in case of activation with KK/DS incomplete cleavage occurred within the disulfide loop Cys 340-Cys 467 in FXII Locarno (Fig 5 and Table 2), βFXII Locarno obtained by trypsin activation was completely cleaved at Arg 343-Leu 344 (Table 2). Despite complete trypsin-induced cleavage at Arg 334-Asn 335 and at Arg 343-Leu 344, βFXII Locarno showed neither amidolytic (Fig 4) nor proteolytic activity towards its physiologic substrate PK (Fig 6).

In conclusion, studies of the variant FXII Locarno (Arg 353 → Pro) lacking the KK cleavage site Arg 353-Val 354 provided evidence that cleavage exclusively at Arg 343, though only 10 amino acids apart from Arg 353, is not sufficient and cleavage at Arg 353 is essential to expose the catalytic active site in FXII.

REFERENCES

From www.bloodjournal.org by guest on April 19, 2017. For personal use only.


Coagulation factor XII Locarno: the functional defect is caused by the amino acid substitution Arg 353-->Pro leading to loss of a kallikrein cleavage site

JK Hovinga, J Schaller, H Stricker, WA Wuillemin, M Furlan and B Lammle