Pathways in the Activation of Human Coagulation Factor X

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Purified human Factor X (apparent mol.wt. 72000), which consists of two polypeptide chains (mol.wt. 55000 and 19000), was activated by both Russell's-viper venom and the purified physiological activators (Factor VII/tissue factor and Factor IXa/Factor VIII). They all convert Factor X to catalytically active Factor Xa (mol.wt. 54000) by cleaving the heavy chain at a site on the N-terminal region. In the presence of Ca2+ and phospholipid, the Factor Xa formed catalyses (a) the cleavage of a small peptide (mol.wt. 4000) from the C-terminal region of the heavy chain of Factor Xa, resulting in a second active form (mol.wt. 50000), and (b) the cleavage of a peptide containing the active-site serine residue (mol.wt. 13000) from the C-terminal region of the heavy chain of Factor X, resulting in an inactivatable component (mol.wt. 59000). A nomenclature for the various products is proposed.

Factor X is a plasma glycoprotein, which consists of two polypeptide chains. [The nomenclature for the various coagulation factors is that recommended by an international committee (Wright, 1959). For the various molecular forms of Factors X and Xa the nomenclature of Fujikawa et al. (1974) is used.] It is involved in both the intrinsic and the extrinsic blood-coagulation pathways. During the coagulation process, Factor X is converted to Factor Xa (EC 3.4.21.6), a serine proteinase catalysing the conversion of prothrombin to thrombin (Davie & Fujikawa, 1975; Suttie & Jackson, 1977).

The mechanism of activation of bovine Factor X by (a) a protease from Russell's-viper venom, (b) the extrinsic activator (Factor VII and tissue factor) and (c) the intrinsic activator (Factor VIII and Factor IXa) has been studied extensively (Jesty & Esnouf, 1973; Jesty & Nemerson, 1974; Jesty et al., 1974, 1975; Fujikawa et al., 1972, 1974, 1975). These activators hydrolyse a specific Arg-Ile bond in the N-terminal region of the heavy chain of Factor X, giving rise to a glycopeptide and an active enzyme, Factor Xα. In the presence of phospholipid, Factor Xα can be converted to another active form, Factor Xαβ, by the autocatalytic removal of a small peptide from the C-terminal region of the heavy chain. Under some conditions, these two proteolytic cleavages can occur in the reversed order: removal of the C-terminal peptide then gives rise to an inactive intermediate, Factor Xβ, which can be converted to Factor Xαβ by the subsequent removal of the N-terminal glycopeptide.

The mechanism of activation of human Factor X, however, has been much less investigated. Di Scipio et al. (1977b) described the activation of Factor X by the Factor X-activating proteinase from Russell's-viper venom. Their results showed the presence of two sites of cleavage on the heavy chain of human Factor X, suggesting a close resemblance between the mechanisms of activation of human and bovine Factor X.

In this paper we report our investigations on the molecular events that occur during the activation of human Factor X by Russell's-viper venom and by the purified physiological activators involved in the intrinsic and extrinsic pathway. Conversion of Factor X was followed by amidolytic assays and SDS/polyacrylamide-gel electrophoresis. The results demonstrate the presence of three sites of cleavage on the heavy chain of human Factor X. The cleavage at one of these sites is responsible for the removal of the active-site serine residue from the Factor X molecule.

Experimental

Materials

DEAE-Sephadex A-50, Sephadex G-75, Sepharose 4B and CNBr-activated Sepharose 4B were products of Pharmacia Fine Chemicals, Uppsala,
Sweden. Heparin was purchased from Organon, Oss, The Netherlands. Trasylol (aprotinin) and benzamidine hydrochloride were products of Bayer, Leverkusen, West Germany, and Aldrich Europe, Beerse, Belgium respectively. Hirudin, soya-bean trypsin inhibitor, *Echis carinatus* venom, L-α-phosphatidyl-L-serine and L-α-phosphatidylcholine (type I-H) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. SDS, acrylamide and dithiothreitol were purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A.; *NN'*-methylenebisacrylamide was from Merck-Schuchart, Hohenbrunn, West Germany; Coomassie Brilliant Blue R250 was from Serva, Heidelberg, West Germany; molecular-weight markers (range 14 300–71 500) were from BDH Chemicals, Poole, Dorset, U.K. *NN'*-Tetramethylethylenediamine and Dip-F were products of Koch–Light Laboratories, Colnbrook, Bucks., U.K. [*H]*Dip-F (specific radioactivity 3.0Cl/mmoll) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Russell's-viper venom was obtained from Wellcome Reagents, Beckenham, Kent, U.K. Compounds S-2222, *N*-Bz-Ile-Gly-Arg-p-nitroanilide hydrochloride and S-2238, (H-D-Phe-Pip-Arg-p-nitroanilide dihydrochloride) were products of Kabi Diagnostic, Stockholm, Sweden. All other chemicals were analytical-grade products from Merck, Darmstadt, West Germany or BDH Chemicals.

Heparin–Sepharose type I was prepared as described by Andersson et al. (1975) (heparin and CNBr were added simultaneously to the Sepharose 4B). Heparin–Sepharose type II was prepared by coupling heparin to CNBr-activated Sepharose 4B (20 mg/g of activated Sepharose) by following the instructions of the manufacturers. This method was also used for coupling Russell's-viper venom to Sepharose 4B (2 mg/g of activated Sepharose).

**Analytical methods**

**Assays for coagulation factors.** Factor IX activity was assayed as a kaolin-activated partial thromboplastin in plasma of a patient with severe Factor IX deficiency (Veltkamp et al., 1968). Activities of Factors II, VII and X were determined in one-stage assays (with human brain thromboplastin as the activator) by using artificially depleted reagents prepared as described by Koller et al. (1951), Bertina et al. (1978) and Hemker et al. (1972). Very low concentrations of Factor II were assayed by activating it with *E. carinatus* venom and measuring the released thrombin by its amidolytic activity towards the chromogenic peptide substrate S-2238 (Bertina et al., 1979). Factor X antigen was measured in an electroimmunoassay, by using a specific rabbit anti-(human Factor X) serum, essentially as described for Factor IX by Bertina & Van der Linden (1977). One unit of coagulant activity or antigen equals the amount of activity or antigen present in 1 ml of pooled normal plasma. Amidolytic assays for Factor Xa and Factor X were performed by using the chromogenic substrate S-2222, by modifications of the methods of Aurell et al. (1977) and Bergström & Egberg (1978) respectively. For the determination of Factor Xa, samples of 50 μl were added to cuvettes containing 100 μl of buffer (40 mM-CaCl₂/0.8 mM-NaCl/0.4 mM-Tris/HCl, pH 7.5), 650 μl of water and 50 μl of S-2222 solution (3.3 mm in water). For the determination of Factor X, 50 μl samples were added to cuvettes containing 100 μl of buffer, 600 μl of water and 50 μl of Russell's-viper venom (0.2 mg/ml in water). After incubation for 2 min at 37°C, 50 μl of substrate solution was added. The initial rates of hydrolysis of compound S-2222 were measured by recording the A₄₀₅ in spectrophotometers (PM6, Zeiss, Oberkochen, West Germany or 1101M, Eppendorf, Hamburg, West Germany) equipped with cuvette holders thermostatically controlled at 37°C. The concentrations of Factor X and Factor Xa were expressed in nkat/ml. Under the assay conditions used, the Factor X concentration in pooled normal plasma (1 unit/ml) equals 3.1 nkat/ml.

**SDS/polyacrylamide-gel electrophoresis.** This was performed by the method described by Weber & Osborn (1969). Unless stated otherwise, the gels were 7.5% (w/v) in acrylamide. Samples (approx. 10 μg of protein) were prepared for electrophoresis by incubation for 1 h at 56°C in the presence of 4.3 mM-urea, 1% (w/v) SDS and, when samples had to be reduced, 20 mM-dithiothreitol. Routinely, no EDTA was present in the samples; however, it was always checked whether identical protein patterns were obtained upon addition of EDTA (20 mM) to the samples before electrophoresis. Electrophoresis (6 h at 8 mA/gel at 30°C) was performed in the phosphate-buffered system described by Weber & Osborn (1969). After electrophoresis, gels were stained for protein with Coomassie Brilliant Blue R250 (0.25% (w/v) in water/methanol/acetic acid (5:5:1, by vol.)) or for carbohydrate by the periodic acid/Schiff method as described by Fairbanks et al. (1971). After destaining, densitometric records were obtained by using a type 1101M spectrophotometer equipped with a type 2602 scanning device (Eppendorf). The gels were scanned at 623 nm or 546 nm when stained for protein or carbohydrate respectively. Molecular weights were estimated from a linear semilogarithmic plot relating molecular weight to electrophoretic mobility by the BDH molecular-weight markers (range 14 3000–71 500).

**Protein determination.** This was performed by measuring the A₂₈₀ by using A₁₆₅₄, 11.6 for purified human Factor X (Di Scipio et al., 1977a) and A₁₆₅₄, 10 for all other proteins.

**Lipid phosphorus determination.** This was per-
formed after HClO₄ combustion, as described by Bottcher et al. (1961).

Radioactivity measurements. These were performed in a model 3380 Tri-Carb liquid-scintillation spectrometer (Packard Instrument, Brussels, Belgium). Polyacrylamide-gel slices of 1.2 mm thickness were solubilized in 300μl of 30% (v/v) H₂O₂ by heating at 50°C for 18 h. After cooling, 10 ml of scintillation fluid (Packard Pico Fluor 30) was added. ³H radioactivity was counted with an efficiency of 40%.

Isolation of human Factor X

Human Factor X was purified in a four-step method including barium citrate adsorption and elution, DEAE-Sephadex gradient elution, heparin–Sepharose chromatography and heparin–Sepharose gradient elution. Human cryosupernatant, obtained from citrated plasma by removal of the proteins precipitating at 4°C, was mixed with Trasylol (5 Kalli- krein inhibitor units/ml), soya-bean trypsin inhibitor (0.1 mg/ml), heparin (2 i.u./ml) and 10 mM-benzamidine hydrochloride. Solid BaCl₂ (15 g/l) was then added gradually at 4°C under continuous stirring. After 30 min the insoluble barium citrate was separated from the supernatant plasma by centrifugation (15 min at 5500 g). The precipitate was washed with one-third of the original volume of 0.15 M-BaCl₂ and distilled water, and eluted with 0.33 vol. of 0.15 M-sodium citrate buffer (pH 7.5). Washing and eluting solutions always contained 6 mM-benzamidine, soya-bean trypsin inhibitor (0.1 mg/ml) and Trasylol (5 Kallikrein inhibitor units/ml). Eluted proteins were dialysed overnight against 25 vol. of 25 mM-sodium citrate buffer (pH 8.0) containing 3 mM-benzamidine, concentrated by ultrafiltration (PM10 membrane, Amicon Europe B.V., Oosterhout, The Netherlands), and applied to a column (2.6 cm x 12 cm) of DEAE-Sephadex A-50, previously swollen and equilibrated with 30 mM-sodium citrate/3 mM-benzamidine (pH 8.0, conductivity 7 mS). The column was washed with about 180 ml of equilibration buffer, and proteins were eluted with a linear gradient of KCl in the same buffer (0–0.5 M; 2 x 800 ml). Factors II, IX and X are eluted at about 37 mS, whereas Factor VII is eluted at about 25 mS. Fractions containing Factor X activity were pooled, dialysed against 1 mM-benzamidine/0.1 M-NaCl/0.02 mM-sodium citrate/0.05 M-Tris/HCl, pH 7.4, and concentrated to about 20 ml. The Factor X concentrate was then applied to a column (2.6 cm x 26 cm) of heparin–Sepharose (type I) to remove Factor IX. Factor X and II activities pass through, whereas the Factor IX activity is retarded considerably (Bertina & Veltkamp, 1978). Factor X-containing fractions were pooled and dialysed against 5 mM-KCl/10 mM-triethanolamine/HCl, pH 6.35. After the addition of CaCl₂ (final concentration 3 mM) the dialysis residue was applied to a column (1.6 cm x 22 cm) of heparin–Sepharose (type II) previously equilibrated in the same buffer. Factor II activity passes through, whereas Factor X is eluted in a linear gradient of 5–500 mM-KCl (2 x 200 ml) at a conductivity of about 20 mS. Fractions containing Factor X activity were pooled, dialysed against 0.1 M-NaCl/0.05 M-Tris/HCl, pH 7.5, and stored at -20°C.

Preparation of other coagulation factors

Human Factor VII. This was prepared from the Factor-VII-containing fractions obtained after the DEAE-Sephadex gradient elution (see Factor X isolation). It was purified further by heparin–Sepharose (type II) gradient elution and Sephadex G-75 gel chromatography (R. M. Bertina & G. H. J. Tiedemann-Alderkamp, unpublished work). The Factor VII used in the experiments described in this study is slightly activated (750 units/mg), migrates as one single band on SDS/polyacrylamide-gel electrophoresis (mol.wt. 54,400) and does not contain detectable amounts of Factor II, thrombin, Factor Xa or Factor IX.

Human brain thromboplastin. This was prepared as described by Owren (1949); for the experiments described in Fig. 6 a thromboplastin fraction was prepared that sedimented on centrifugation at 20000 g for 30 min; after ten washes with 0.9% (w/v) NaCl the thromboplastin was suspended in about one-tenth of the original volume.

Human Factor VIII. This was prepared by gel chromatography on Sepharose 4B of freshly prepared cryoprecipitate (precipitate obtained after thawing frozen citrated plasma at 4°C). The Factor VIII coagulant activity (specific activity about 35 units/mg) is eluted in the void volume; after reduction, the protein migrates as one single band on 5% (w/v) SDS/polyacrylamide gels (mol.wt. 225,000).

Human Factor IXa. This was prepared by activation of 145 μg of human Factor IX (Bertina & Velt- kamp, 1978) with about 4 μg of a Factor XIa preparation (Osterud & Rapaport, 1977) in the presence of CaCl₂ (5 mM) for 24 h at room temperature in a plastic tube. Factor XIa was then removed by transferring the reaction mixture into a glass tube. The Factor IXa prepared this way consists of a two-chain molecule with an apparent mol.wt. of 47,000.

Phospholipid (‘cephalin’). This was prepared from human brain as described by Milstone (1950). The resulting phospholipid suspension was extracted twice in a chloroform/methanol/water system (6:3:1, by vol.). Purified phospholipids were stored as stock solutions in chloroform at -20°C under N₂. For use in the experiments, liposomes were prepared by removing the solvent in a rotating evaporator and by dispersing the dry lipid film in 0.1 M-
NaCl/0.05 M-Tris/HCl, pH 7.5 (0.2 ml/μmol of phosphorus) by vigorous mixing on a vortex-mixer for 1 min.

Results

Isolation of Factor X

Table 1 summarizes the isolation of human Factor X as described in the Experimental section. In steps 3, 4 and 5, Factor X is separated from Factors VII, IX and II respectively. The final preparation contained no detectable thrombin, Factor Xα, Factor VII or Factor IX activity; the presence of trace amounts of Factor II (<0.0025 unit/unit of Factor X) cannot be excluded. For the specific activity of the final Factor X preparation two different values were obtained: (a) 46 units/mg by the one-stage Factor X-coagulation assay, with Factor VII and thromboplastin as activator; (b) 130 units/mg by two-stage amidolytic assay, with Russell's-viper venom as activator. The latter value, which could be confirmed by the electroimmunoassay for Factor X, is similar to the values obtained by Kosow (1976) and Di Scipio et al. (1977a) using a coagulation assay with Russell's-viper venom as activator; a specific activity of 130 units/mg corresponds to a 9000-fold purification of Factor X when compared with pooled normal plasma. The reason for the low specific activity obtained in our coagulation assay might be that, owing to aggregation of purified Factor X, the initial rate of Factor X activation by Factor VII and thromboplastin is decreased compared with Factor X in normal plasma. On SDS/polyacrylamide-gel electrophoresis (see Fig. 1), purified Factor X migrates as a single band with an apparent mol.wt. of 72000; after reduction, more than 95% of the protein appears in two bands of mol.wts. of 55000 and about 19000. A rabbit antibody prepared against the purified Factor X gave one precipitation arc against normal plasma, while no pre-

activation occurred against plasma of a patient with congenital Factor X deficiency (Factor X < 0.01 unit/ml).

Activation of Factor X by Russell's-viper venom

Fig. 2(a) shows the development of amidolytic activity during the activation of Factor X by Russell's-viper venom in the presence of Ca²⁺. Analysis of the incubation mixture by SDS/polyacrylamide-gel electrophoresis shows that, parallel to the appearance of amidolytic activity, a major band

![Image](relative_mobility)

Fig. 1. Analysis of purified human Factor X by SDS/polyacrylamide-gel electrophoresis

(A) 10 μg of non-reduced Factor X; (B) 10 μg of reduced Factor X.

Table 1. Isolation of Factor X from human plasma

<table>
<thead>
<tr>
<th>Step</th>
<th>Factor X activity</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purification factor</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cryosupernatant</td>
<td>2400</td>
<td>83000†</td>
<td>0.029</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Barium citrate adsorption</td>
<td>840*</td>
<td>910†</td>
<td>0.92*</td>
<td>32*</td>
</tr>
<tr>
<td>3.</td>
<td>DEAE-Sephadex A-50 gradient elution</td>
<td>1140</td>
<td>300†</td>
<td>3.8</td>
<td>130</td>
</tr>
<tr>
<td>4.</td>
<td>Heparin-Sepharose I chromatography</td>
<td>770</td>
<td>190†</td>
<td>4.1</td>
<td>140</td>
</tr>
<tr>
<td>5.</td>
<td>Heparin-Sepharose II</td>
<td>360</td>
<td>7.8‡</td>
<td>46§</td>
<td>1590</td>
</tr>
</tbody>
</table>

* Minimal values (because of interference of traces of heparin with the Factor X-coagulation assay).
† Protein was determined by assuming A₄₀₀nm = 10.
‡ Protein was determined by assuming A₂₈₀nm = 11.6 (Di Scipio et al., 1977a).
§ A specific activity of 130 units/mg was obtained by a two-stage amidolytic assay with Russell's-viper venom as activator (see the text).
ACTIVATION OF HUMAN FACTOR X

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Fig. 2. Activation of Factor X by Russell's-viper venom

Factor X (13.0 ml; 115 µg/ml in 0.1 M NaCl/0.05 M Tris/HCl, pH 7.5) was incubated with Sepharose-coupled Russell's-viper venom (220 µl of packed gel) in the presence of CaCl₂ (10 mM) at 25°C. (a) At the times indicated, samples were removed from the incubation mixture. After removal of the Sepharose-coupled venom by centrifugation (1 min at 8000 g), the supernatants were assayed for Factor Xa activity. (b) SDS/polyacrylamide-gel electrophoresis was performed on the non-reduced supernatants of the samples removed after 0, 0.5, 1.5, and 5 h [patterns (A), (B), (C) and (D) respectively]. After 5 h the reaction was stopped by removing the Sepharose-coupled venom. Part of this preparation was incubated further with phospholipid (cephalin, 0.5 µmol of phosphorus/mg of Factor Xa) for 3 h on ice [electrophoretic pattern (E)].

appeared with an apparent mol wt of 54 000 (Fig. 2b). A smaller band appeared that had a mol wt of 39 000. After 5 h of incubation, a small additional band (mol wt. 50 000) became visible as a shoulder on the 54 000-mol wt. band (pattern D in Fig. 2b). At this time, the conversion of Factor X to Factor Xa was nearly complete. This preparation was used as source of Factor Xa in the experiments described below. Pattern (E) in Fig. 2(b) shows that incubation of this preparation (still containing Ca²⁺) with phospholipid caused a nearly complete shift of the Factor Xa band from mol wt. 54 000 to 50 000. After this incubation, 70% of the amidolytic activity was recovered. This indicates that the component with mol wt. 50 000 represents another form of Factor Xa.

Conversion of Factor X by Factor Xa

To study possible feedback reactions of Factor Xa on Factor X during activation reactions, these two factors were incubated in the presence of Ca²⁺ and phospholipid. Fig. 3(a) shows the results of the amidolytic assays of samples from the incubation mixture. The assays for Factor Xa showed that no amidolytic activity developed during the incubation. However, the assays for Factor X showed a sharp decrease in the concentration of activatable Factor X. Fig. 3(b) shows the analysis of samples from the same incubation mixture by SDS/polyacrylamide-gel electrophoresis. Factor X was initially converted to a product with an apparent mol wt. of 59 000 (Fig. 3b, pattern B). On further incubation, bands appeared with mol wts. of 39 000, 24 000 and 16 000. After incubation for 22 h, no intact Factor X was present anymore (Fig. 3b, pattern D).

Essentially no degradation of Factor X could be detected, either when Factor Xa, phospholipid or Ca²⁺ were omitted from the incubation mixture or when soya-bean trypsin inhibitor, an inhibitor of Factor Xa, was included in the complete incubation mixture. Addition of hirudin, an inhibitor of thrombin, did not influence the degradation of Factor X; this excludes the possibility that the conversions observed are due to thrombin, which might have been formed from trace amounts of Factor II contaminating the Factor X preparation. Replacement of the cephalin by a mixture of pure phosphatidylcholine and phosphatidylserine (molar ratio 1:1) did not in-
fluence the degradation pattern; this excludes the human brain cephalin preparation as the possible source of contaminating proteolytic activity.

These results demonstrate that Factor Xa can convert Factor X to components with mol.wts. of 59 000 and 39 000, and to smaller products, all without amidolytic activity towards the substrate S-2222. Furthermore, the assays for Factor X indicate that none of these products, including the 59 000-mol.wt. component, can be converted to an active form by the Russell's-viper venom used in the assay (see Fig. 3b, pattern D).

Activation of Factor X in the presence of phospholipid

In the experiments described above, the conversions of Factor X by an activator, Russell's-viper venom, and by the product of the activation reaction, Factor Xa, have been studied separately. In the following experiment, these two processes were combined by incubating Factor X with Russell's-viper venom in the presence of Ca²⁺ and phospholipid.

Fig. 4 shows the analysis by SDS/polyacrylamide-gel electrophoresis of the reaction mixture after 30 min of incubation. Pattern (A) shows that the major products formed from Factor X (mol.wt. 72 000) are the components with mol.wts. 54 000 and 50 000, which have been shown to represent two forms of Factor Xa (cf. Fig. 2b, patterns D and E). The other products, the components with mol.wts. 59 000, 39 000, 24 000 and 16 000, have been shown to represent inactive products (cf. Fig. 3b). Pattern (C) shows that, after reduction, a comparable electrophoretic pattern is obtained. The components with mol.wts. 72 000, 59 000, 54 000 and 50 000 are located now at positions corresponding to mol.wts. of 55 000, 41 000, 35 000 and 31 000 respectively. In all these cases the difference between the molecular weights of the non-reduced and reduced molecules is about 19 000, the molecular weight of the light chain (see Fig. 1, pattern B). It is not clear whether the molecular weight of the heavy chain of the 39 000-mol.wt. component is about 21 000, because also in the non-reduced pattern fragments were detected in this region. When the gels were stained for carbohydrate (patterns B and D), all stainable carbohydrate appeared to be associated with intact Factor X and the 59 000-mol.wt. component or with the heavy chains of these molecules. No carbohydrate was detected on Factor Xa.

These data show that the components formed by the combined action of Russell's-viper venom and Factor Xa on Factor X result from proteolytic cleavages in the heavy chain only. Upon conversion

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Fig. 3. Conversion of Factor X by Factor Xa

Factor X (50 µg) was incubated with Factor Xa (2 µg) in the presence of CaCl₂ (5 mM) and phospholipid (cephalin; 0.05 µmol of phosphorus) in 0.1 M NaCl/0.05 M Tris/HCl, pH 7.5 at 37°C; the final volume was 500 µl. (a) At the times indicated, samples from the incubation mixture were assayed for Factor Xa (●) and Factor X (○). (b) Non-reduced samples from the incubation mixture, containing 20 mM-EDTA, were analysed by SDS/polyacrylamide-gel electrophoresis [patterns (A), (B), (C) and (D): samples after 0, 1, 4 and 22 h of incubation respectively].
of Factor X to Factor Xa most of the carbohydrate is lost, whereas upon formation of the 59 000-mol.wt. component most of the carbohydrate is conserved. In human Factor X the major portion of the carbohydrate is known to be located on the N-terminal portion of the heavy chain (Di Scipio et al., 1977b). Therefore formation of the relatively carbohydrate-rich 59 000-mol.wt. component from Factor X can be explained by the release of a carbohydrate-poor peptide with a calculated mol.wt. of 13 000 from the C-terminal region of the heavy chain. In parallel with the formation of the 59 000-mol.wt. component, the amidolytic activity generated in the Factor X assay decreases (see Fig. 3a). This suggests that the released 13 000-mol.wt. peptide contains a part of the Factor X molecule that is essential for the catalytic activity. A peptide of mol.wt. 13 000 consists of about 100 amino acids. As is known from the primary structure of bovine Factor X (Titani et al., 1975), the active-site serine is the 75th amino acid, as counted from the C-terminus. If the same is assumed to be true for human Factor X, the 13 000-mol.wt. peptide would contain the active-site serine residue. This hypothesis can be tested by labelling Factor X in its active-site serine and by analysing the degradation products of this Factor X on the presence of the labelled serine residue.

Degradation of $[^{3}H]$Dip-Factor X by Factor Xa

Figs. 5(a) and 5(b) show the analysis by SDS/polyacrylamide-gel electrophoresis of human Factor X after incubation with $[^{3}H]$Dip-F. In preliminary experiments performed with unlabelled Dip-F, it was found that under the experimental conditions described about 25% of the amidolytic activity in the Factor X assay is lost, whereas in a control incubation in the absence of Dip-F all activity is recovered. This confirms the incorporation of Dip into the active-site serine residue of thezymogen form of human Factor X, as described for bovine Factor X by Kerr et al. (1978). Comparison of the protein pattern (Fig. 5a) with the distribution of the radioactivity (Fig. 5b) in the reduced samples shows that about 85% of the $[^{3}H]$Dip had been incorporated into the heavy chain. Some $[^{3}H]$Dip (about 15%) was found to be associated with a peptide of 17 000 mol.wt.; on electrophoresis of non-reduced samples, all $[^{3}H]$Dip was found in the position of the intact Factor X (results not shown); it is likely then that, because of the long incubation time required, some radioactivity had been incorporated into the light chain. The specific radioactivities of the $[^{3}H]$Dip-F and the $[^{3}H]$Dip-Factor X obtained were $2.4 \times 10^{4}$ d.p.m./nmol and $9.2 \times 10^{4}$ d.p.m./mg respectively. By assuming a value of 59 000 for the molecular weight of human Factor X (Di Scipio et al., 1977b), this implies an incorporation of 0.22 mol of Dip/mol of Factor X. As shown in Fig. 5(c), incubation of $[^{3}H]$Dip-Factor X with Factor Xa in the presence of Ca$^{2+}$ and phospholipid resulted in the appearance of two new protein bands, corresponding to a peptide of mol.wt. 13 000 and to the heavy chain of the 59 000-mol.wt. component (cf. Fig. 3b, pattern B). About 80% of the radioactivity present in these products is associated with the 13 000-mol.wt. peptide (Fig. 5d). This demonstrates that Factor Xa cleaves the heavy chain of Factor X, releasing a peptide of mol.wt. 13 000, which contains the active-site serine residue.

Activation of Factor X by the physiological activators

To answer the question whether upon activation of human Factor X by its physiological activators the same conversions occur as described above, Fac-
tor X was activated by the extrinsic and intrinsic activator. In these experiments low concentrations of activating proteins were used, both to prevent interference with the Factor X-derived bands on the SDS/polyacrylamide-gel patterns, and to enable detection of the initial events.

Fig. 6 shows the activation of Factor X by Factor VII and thromboplastin in the presence of Ca\(^{2+}\) (the extrinsic activator). In preliminary experiments, the rate of Factor Xa formation was found to depend on the Factor VII concentration, whereas no Factor Xa was formed when either Factor VII or thromboplastin were omitted. Fig. 6(b) shows the analysis of samples from the incubation mixture by SDS/polyacrylamide-gel electrophoresis. The component with apparent mol.wt. 55000 (see pattern B) corresponds to the form of Factor Xa with mol.wt. 54000 (cf. Figs. 2b and 4). The conversion of this form to the lower-molecular-weight form in the presence of phospholipid was also observed in the present experiment: after 1h a small band was visible at mol.wt. 51000, which became more intense than the 55000-mol.wt. band on further incubation.

In agreement with the relatively low intensity of these two bands, only low amidolytic activity developed during the experiment (Fig. 6a). After 3h of incubation, the rate of development of Factor Xa activity was decreased, possibly because of inactivation of activated Factor VII by Factor Xa, as has been reported by Radcliffe & Nemerson (1975). At the same time, the electrophoretic pattern (Fig. 6b, pattern D) shows an additional band at mol.wt. 60000, corresponding to the inactivatable 59000-mol.wt. component in Figs. 3(b), 4 and 5. Also the remaining bands, at mol.wts. 40000 and 18000 have been observed in the previous experiments.

Fig. 7 shows the activation of Factor X by Factor IXa and Factor VIII in the presence of Ca\(^{2+}\) and phospholipid (the intrinsic activator). In preliminary experiments, no detectable Factor Xa was formed in the absence of Ca\(^{2+}\), phospholipid or Factor IXa, whereas omission of Factor VIII (not previously activated by thrombin) had only little effect on the initial rates of Factor Xa production and Factor X degradation. Fig. 7(a) shows that during the experiment Factor Xa activity increased slowly until it

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**Fig. 5. Degradation of \[^{[3]H}\]Dip-Factor X by Factor Xa**

\[^{[3]H}\]Dip-Factor X was prepared by incubation of 450\(\mu\)l of Factor X (880\(\mu\)g/ml in 0.1M-NaCl/0.05M-Tris/HCl, pH 7.5) with 4.5\(\mu\)l of 1M-Dip-F (in propan-2-ol) and 100\(\mu\)l of \[^{[3]H}\]Dip-F (0.5mCi; 0.17\(\mu\)mol in propylene glycol) at 25°C. After 24h, 450\(\mu\)l of 0.1M-NaCl/0.05M-Tris/HCl, pH 7.5, was added; this mixture was dialysed exhaustively against the same buffer. Reduced samples (100\(\mu\)l) from this preparation (containing 20mm-EDTA) were analysed by SDS/polyacrylamide-gel electrophoresis on 10% (w/v) gels. The gels were stained for protein (a) or sliced and analysed for radioactivity as described in the Experimental section (b). In a final volume of 350\(\mu\)l, 90\(\mu\)g of \[^{[3]H}\]Dip-Factor X (specific radioactivity 9.2 \times 10^{8} d.p.m./mg) was incubated with Factor Xa (3.5\(\mu\)g) in the presence of phospholipid (cephalin, 0.1\(\mu\)mol of phosphorus) and CaCl$_2$ (10mM) at 37°C. After 4h of incubation, 140\(\mu\)l from the reaction mixture was analysed for protein (c) and radioactivity (d) as described above.
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Fig. 6. Activation of Factor X by the extrinsic activator

Factor X (1.0 ml; 90 μg/ml in 0.1 M NaCl/0.05 M Tris/HCl, pH 7.5) was incubated with 10 μl of thromboplastin preparation, 40 μl of partially activated Factor VII (0.08 μg of protein) and 25 μl of 0.2 M CaCl₂ at 37°C. (a) At the times indicated the incubation mixture was assayed for Factor Xa. (b) SDS/polyacrylamide-gel electrophoresis was performed on non-reduced samples after incubation for 0, 1, 1.5 and 3 h [patterns (A), (B), (C) and (D) respectively].

decreased again after 2 h of incubation. The assays for Factor X, however, demonstrated a sharply decreasing Factor X concentration during the incubation, comparable with that shown in Fig. 3(a). After 6 h the amidolytic activities in the Factor X and Factor Xa assays were equal, suggesting that no Factor X was left for activation by the Russell's-viper venom used in the Factor X assay. Similar results were obtained from the SDS/polyacrylamide-gel patterns (Fig. 7b). Parallel to the decreasing activity in the Factor X assays, the Factor X band, at mol.wt. 72000, moved to two major bands at mol.wts. 60000 and 40000 (cf. Fig. 3b). When Factor Xa activity was maximal (after 1 and 2 h of incubation), the gel patterns (C and D) show a minor band at mol.wt. 51000, which suggests that this band represents the 50000-mol.wt. form of Factor Xa (cf. Fig. 2b, pattern E). As in the experiment described in Fig. 3, at the end of the incubation all protein had accumulated in three major bands, at mol.wts. of 40000, 24000 and 17000.

These results show that when human Factor X is activated by its physiological activators, essentially the same products are formed as on activation by Russell's-viper venom in the presence of Ca²⁺ and phospholipid (Fig. 4). This suggests that Factor X is converted to Factor Xa by the physiological activators in the same way as by Russell's-viper venom, whereas the other, inactive, products are formed by the subsequent action of the product of the activation reaction, Factor Xa.

Discussion

By use of the electrophoresis system described in the Experimental section, a mol.wt. of 72000 was found for human Factor X. However, it has to be realized that molecular-weight values strongly depend on the method of determination. Molecular-weight values for human Factor X have been reported of between 59000, based on amino acid and carbohydrate composition (Di Scipio et al., 1977b), and 75000, as found by Kosow (1976) using SDS/polyacrylamide-gel electrophoresis. In the present study, Factor X and the Factor X-derived components have been characterized according to their apparent molecular weights.
In our experiments, Factor X was converted to active forms of mol.wts. 54000 and 50000, to inactive components of mol.wts. 59000 and 39000, and to smaller fragments. These conversions have been shown to be caused by proteolytic cleavages at three sites on the heavy chain (Fig. 4). The location of these sites, which we called the α-, β- and γ-sites, is shown in Fig. 8. The first two sites have been recognized earlier in bovine Factor X (Fujikawa et al., 1972, 1974, 1975; Jestly et al., 1974, 1975) and human Factor X (Di Scipio et al., 1977b), and have been shown to be located on the N-terminal region (the α-site) and the C-terminal region (the β-site) of the heavy chain. The cleavage of Factor X at the third site, the γ-site, has not been reported earlier. The location of this site on the C-terminal region of the heavy chain has been demonstrated in two ways: (a) the fragment released does not contain major amounts of carbohydrate (see Fig. 4), which are known to be located on the N-terminal region of the heavy chain (Di Scipio et al., 1977b), and (b) the fragment released contains the active-site serine residue (see Fig. 5), which is known to be located on the C-terminal region of the heavy chain (Titani et al., 1972, 1975; Di Scipio et al., 1977b).

According to our nomenclature proposal (see Fig. 8), the various active (Xa) and inactive (X) molecular forms are indicated with α, β and/or γ as (a) prefix(es), according to the proteolytic cleavage(s) by which they are formed. In our model, the first active form of Factor X, with mol.wt. 54000, is formed by proteolytic cleavage of the α-site in intact Factor X by Russell’s-viper venom or the physiological activators (Figs. 2b, 4 and 6b). The products of this cleavage are Factor αXa and an N-terminal glycopeptide, fragment-α (Fα). In the presence of phospholipid, Factor αXa is converted to a second active form, with mol.wt. 50000, by the cleavage of the β-site by Factor Xa (Figs. 2b, 4, 6b and 7b), giving rise to Factor αβXa and a C-terminal peptide, fragment-β (Fβ). The conversion of Factor X to the inactivatable component of mol.wt. 59000 (Figs. 3b, 4, 5, 6b and 7b) is due to the cleavage of the γ-site by Factor Xa. In our nomenclature the products of this cleavage are called Factor γX and fragment-γ (Fγ). With Fγ also the active-site serine residue is released; this explains why Factor γX cannot be converted to an active form by Russell’s-viper venom. The inactive component with mol.wt. 39000 (Fig. 3b, patterns C and D; Fig. 7b, pattern E) is negative on staining for carbohydrate (Fig. 4). Therefore this component may be formed from Factor γX by release of a glycopeptide with a mol.wt. of about 20000. This suggests that this conversion re-
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Fig. 8. Schematic representation of the human Factor X molecule

The location of the sites of cleavage is discussed in the text; the position of the active-site serine is marked with a circle. The nomenclature proposed for the various Factor X-derived components is indicated.

quires cleavage of the α-site by Factor Xa or by the physiological activators or Russell's-viper venom. In our nomenclature this component should be called Factor αγX. Alternatively, this compound may be formed from factors αXa and αβXa by cleavage of the γ-site, releasing fragments called Fγ and Fβγ.

In our experiments, fragments were detected in positions corresponding to mol.wts. of about 24000 and 16000. The fragments released in our model, Fa, Fβ, Fβγ and Fγ, have calculated mol.wts. of 18000, 4000, 9000 and 13000 respectively. Because glycoproteins have relatively low mobilities on SDS/polyacrylamide-gel electrophoresis (Segrest et al., 1971), it can be expected that for the glycopeptide Fa a higher molecular weight is found than the value of 18000 that can be calculated as the difference in molecular weight between factor X and factor αXa. Therefore the 24000-mol.wt. band may be attributed to fragment Fa. The other fragments migrate in the front in our electrophoresis system (7.5% w/v polyacrylamide gels), and are found in the 16000-mol.wt. position. The calculated mol.wt. of 13000 for fragment Fγ could be confirmed by using 10% (w/v) polyacrylamide gels (Fig. 5).

Factors βX, αXa and αβXa in our nomenclature correspond to Factors Xβ, Xαa and Xαβ in the nomenclature of Fujikawa et al. (1974) and Di Scipio et al. (1977b) for bovine and human Factor X respectively. According to the proposal of Jesty et al. (1974, 1975) for bovine Factor X, these components are called Intermediate-1, α-Xa and β-Xa. These authors also identified an additional component, Intermediate-2, which was formed in a relatively insignificant pathway by the cleavage of the heavy chain at a third site. Because this site was identified between the α-site and the N-terminus, it is different from the γ-site shown in Fig. 8.

In the bovine system, Factor αβXa can also be formed in a pathway other than that described above: Factor X is cleaved by Factor Xa at the β-site, and the resulting Factor βX is converted to Factor αβXa by the subsequent cleavage of the α-site by an activator or by Factor Xa (Fujikawa et al., 1974; Jesty et al., 1974). In the human system, however, the major product of the action of Factor Xa on Factor X is Factor γX, which, because it lacks the active-site serine residue, cannot be converted to an active product by the subsequent cleavage of the α-site. Preliminary experiments on the question why the action of Factor Xa on Factor X is different in the human and the bovine system suggest a structural difference between human and bovine
Factor X rather than a difference in substrate specificity between human and bovine Factor Xa.

Recent kinetic studies have shown that the $K_m$ of Factor X for its activation by the extrinsic activator (Silverberg et al., 1977) and the intrinsic activator (Brown et al., 1978) is higher than the plasma concentration. With respect to this finding, the cleavage of Factor X at the $γ$-site by Factor Xa might be a regulatory principle. Theoretically, the formation of Factor $γX$ can then influence the rate of Factor Xa formation both by decreasing the Factor X concentration and by introducing competition between Factor $γX$ and Factor X for cleavage of the $α$-site by the intrinsic and extrinsic activator.

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