The Mechanism of Activation of Factor X

KINETIC CONTROL OF ALTERNATIVE PATHWAYS LEADING TO THE FORMATION OF ACTIVATED FACTOR X*

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SUMMARY

The initial step in the activation of Factor X by a tissue factor-Factor VII complex has been shown to involve hydrolysis of the same peptide bond as is hydrolyzed by Russell's viper venom. The product, designated $\alpha$-X$a$, undergoes autocatalytic conversion to a lower molecular weight form of equal coagulant activity, $\beta$-X$a$. The rate of this conversion is markedly enhanced by phospholipid which is present as the lipid component of tissue factor.

An alternative pathway of activation, initiated by X$a$, has been demonstrated. This involves the conversion of Factor X to an enzymically inactive intermediate, designated I$\alpha$. This intermediate may then be activated directly to $\beta$-X$a$ at rates identical with the activation of Factor X, by either the tissue factor-Factor VII complex or Russell's viper venom. A third, apparently minor, pathway of activation of Factor X to $\beta$-X$a$ has been demonstrated that is catalyzed entirely by tissue factor. This intermediate may then be activated directly to $\beta$-X$a$. A second intermediate appears during this reaction, but whether it is an obligatory intermediate is not known.

NH$_2$-terminal amino acid sequence data suggest that the conversion by X$a$ of Factor X to I$\alpha$, and of $\alpha$-X$a$ to $\beta$-X$a$, probably involve the release of COOH-terminal peptides. The forms of $\beta$-X$a$ produced from I$\alpha$ and $\alpha$-X$a$ are apparently identical.

These pathways are under kinetic control; at high rates of activation the direct pathway to $\alpha$-X$a$ and $\beta$-X$a$ predominates, whereas at low rates the X$a$ formed initially by the direct pathway feeds back and converts Factor X to I$\alpha$, thus initiating an alternative set of reactions. The final yield of X$a$ is also shown to be a function of the concentration of the tissue factor-Factor VII complex. It is shown that this complex is rapidly inactivated by its product, X$a$, and we propose this as a mechanism of control of the tissue factor pathway.

In a previous paper (1) we presented data on the activation of factor X by the tissue factor-Factor VII complex (TF-VII). Analysis of the reaction products by sodium dodecyl sulfate gel electrophoresis showed the formation of a final product and two intermediates, one of which, we concluded, also had X$a$ activity. Further, we concluded that the changes in apparent molecular weights of these species could be accounted for by cleavages of the heavy chain of Factor X. In contrast to this complex activation sequence, activation of Factor X by Russell's viper venom produced a single form of X$a$ and no intermediates. This X$a$ of identical apparent molecular weight with one of the products of the tissue factor activation. The observation that the venom initially produces a single species of X$a$ is in agreement with the data of Esnouf and Williams (2) and Fujikawa et al. (3). The latter investigators isolated a single NH$_2$-terminal activation peptide derived from the heavy chain of Factor X.

In this paper we describe alternative pathways of the activation of Factor X. In agreement with others, we find that Russell's viper venom, and under certain conditions TF-VII, produce identical forms of X$a$, presumably due to the cleavage of a single peptide bond in the heavy chain of the zymogen. This form of X$a$ has been shown to be rapidly converted autocatalytically to a form of lower molecular weight in the presence of lipid. The same conversion occurs more slowly in the absence of lipid. We also describe an alternative pathway initiated by the action of X$a$ on Factor X to produce an intermediate. Activation of this intermediate by either TF-VII or the venom produces the lower molecular weight form of Factor X$a$. A third, apparently minor, pathway is described in which Factor X$a$ activates Factor X to the smaller form of X$a$.

Recently, Radcliffe and Barton (4) examined activated Factor X produced by the intrinsic pathway, Russell's viper venom, prolonged incubation of Factor X in 25% sodium citrate, and by the action of tissue factor. These investigators concluded that the forms of X$a$ produced by each of these methods had identical molecular weights and NH$_2$-terminal amino acids. Our observation that the higher molecular weight form of X$a$ is autocatalytically converted to the lower molecular weight form in the absence of lipid explains this discrepancy.

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1 The abbreviations used are: X$a$, activated Factor X; TF-VII, tissue factor-Factor VII complex; DFP, diisopropylphosphorofluoridate; DIP-, diisopropylphosphoro-; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
EXPERIMENTAL PROCEDURE

Materials

Sodium dodecyl sulfate, disopropylphosphorofluoridate, Coomassie blue, Tris (‘trizma base’), ovalbumin, Russell’s viper venom, and Factors VII and X-deficient plasma were obtained from Sigma, St. Louis, Mo. [3H]DFP was a product of Amer sham-Searle and used as a solution 2 mCi per ml in dry 2-propanol, nonradioactive DFP being added to give a final concentration of 0.1 mM. Guanidinium hydrochloride (ultrapure) was a product of Heico, Delaware Water Gap, Pa. Acrylamide and N,N'-methylenebisacrylamide (Eastman) were recrystallized from chloroform and acetone, respectively. DEAE-Sephadex A-50, SP-Sephadex C-50, and Sephadex G-100 were products of Pharmacia. All other chemicals were reagent grade products of Fisher or Baker.

Benzamidine-Sepharose was prepared by the method of Jesty and Nemerson (1). Factor VII-deficient bovine plasma was prepared by the method of Nemerson and Clyne (5). Tissue factor apoprotein was prepared by a modification of the method of Nemerson and Pittick (6) and stored at 50% glycerol at 20°C. A chloroform extract of human brain (‘cephalin’) was prepared by the method of Bell and Alton (7), and contained 500 μg of phosphorus per ml and was used as a source of mixed phospholipids.

Methods

Assays—Factor VII was assayed by the method of Nemerson and Clyne (6); Factor X was assayed by the method of Denson (9) with Factors VII- and X-deficient plasma; activated Factor X was assayed by the method of Jobin and Esmouf (10), except that Factors VII and X-deficient plasma was used; and the coagulant factor in Russell’s viper venom was assayed by the method of Williams and Esmouf (11). Normal citrated bovine plasma is defined as containing 100 units of Factor VII and Factor X per ml. One unit of Xα activity was defined as containing the activity of 1 μg of crude venom.

Determination of Protein—Protein concentrations were estimated by measurement of absorbance at 280 nm. A280 for Factor X in 0.6 (19), and it was arbitrarily assumed that the value of 1 (see “Results”) was the same. A280 for Xα-Xα was taken as 9.4 (13). Other proteins are quoted in terms of A280 units.

Sodium Dodecyl Sulfate Gel Electrophoresis—Electrophoresis was carried out in 1.5-mm vertical gel slabs that held up to 50 samples, each loaded 25 μl with acrylamide and 0.2% N,N’-methylenebisacrylamide, and the gel and tank buffer was 20 mM sodium phosphate, pH 7.2, containing 0.1% sodium dodecyl sulfate. After casting, the gels were removed from the glass plates and washed twice for at least 20 hours with 2 liters of the gel buffer. They were then replaced in the plates as before for use. The sample was prepared by heating to 100°C for 5 min in 10 mM sodium phosphate, pH 7.2-2% sodium dodecyl sulfate-5% 2-mercaptoethanol. A sample, 0.1 ml up to 2 mg per ml, containing Pyronin Y, 10 μg per ml, and 10% glycerol, was applied to each well and overlayed with the tank buffer. Electrophoresis was started at 5 to 10 mA, until the dye band had moved about 2 cm, and then continued overnight at 20 mA. In order to maintain the correct pH value, the buffer was recirculated by a pump and overflow system. After electrophoresis, one glass plate was removed and the gel covered with cellophane wrap. After 30 min at 4°C, the protein bands were clearly visible against a black background as precipitates of the sodium dodecyl sulfate against a background of lighter precipitation. The bands required were then cut out directly and the gel sliced into small slivers. The protein was eluted by the method of Weiner et al. (16).

NH₂-terminal Sequence Determination—Lipopolysaccharides of the protein bands were isolated by the subtractive Edman procedure of Weiner et al. (16) to determine the first and second NH₂-terminal amino acids. The dinitrophenylated (17) amino acids were identified by chromatography on polyamide sheets (18) and allowed to stand in 14N7H₄ methanol-70% acetic acid at 35°C with agitation for 4 hours, then for 16 hours at 4°C. Gel was then rechromatographed on DEAE-Sephadex at 4°C with an ice-cold water and centrifuged down again. The washed precipitate was then rechromatographed at 4°C for 20 min at 4°C, then for 16 hours at 4°C. The precipitate was then washed quickly with 150 ml of ice-cold water and centrifuged down again. The washed precipitate was suspended well in 40 ml of 35% saturated ammonium sulfate (pH adjusted to 7.2 with NH₄OH after addition), and stirred for 15 min at 4°C. After centrifuging out the precipitate (10,000 x g for 15 min) the supernatant was made 65% saturated with respect to ammonium sulfate (pH again adjusted to 7.2) and allowed to stand for 30 min at 4°C. The precipitate was then centrifuged down (10,000 x g for 30 min) and taken up in 50 ml of 0.1 mM NaCl-25 mM Tris, pH 7.5. The solution was then treated with 1 ml of 1 M DFP (in 2-propanol) and allowed to stand for 1 hour at room temperature before dialysis against 0.24 M NaCl-0.1 mM Tris, pH 7.5, overnight at 4°C. The dialyzed Factor X was then rechromatographed on DEAE-Sephadex A-50 at 4°C as described by Esmouf et al. (12). The Factor X and Xα peaks were pooled together, concentrated to about 10 ml of 12 mM sodium citrate, ultracentrifugation through an XM-100 membrane (Amicon) replaced the gel filtration step. The specific activity of the final product was about 600,000 units per mg, and it was stored at a concentration of 0.1 mg per ml in 50% glycerol-50 mM NaCl-25 mM Tris, pH 7.5, at -20°C.

Factor X was also prepared from the barium sulfate eluate by a modification of the method of Jesty and Nemerson (1), except that to remove high molecular weight contaminants, ultracentrifugation through an XM-100 membrane (Amicon) replaced the gel filtration step. The specific activity of the final product was about 600,000 units per mg and was stored at a concentration of 0.1 mg per ml in 50% glycerol-50 mM NaCl-25 mM Tris, pH 7.5, at -20°C.

Synthesis of Venom-Sepharose—The coagulant fraction of Russell’s viper venom was coupled to Sepharose 4B by the methods of Cuatrecasas (19); 5 ml of packed Sepharose 4B was activated with 0.5 g CNBr. The washed activated Sepharose, in 0.1 M sodium bicarbonate, pH 8, was then added to 2.2 A units of the venom fraction in 5 ml of the same buffer, and the mixture was shaken for 4 hours at 22°C, then for 16 hours at 4°C. The resulting material was washed with, and then suspended in 0.1 M NaCl-50 mM Tris, pH 7.5.

Purification of Factors VII and X—Factor VII was purified from a barium sulfate eluate by the method of Clyne and Nemerson (12), except that to remove high molecular weight contaminants, ultracentrifugation through an XM-100 membrane (Amicon) replaced the gel filtration step. The specific activity of the final product was about 600,000 units per mg and was stored at a concentration of 0.1 mg per ml in 50% glycerol-50 mM NaCl-25 mM Tris, pH 7.5, at -20°C.

Preparation of Tissue Factor—Factor VII complex—Tissue factor apoprotein (0.2 mg in 2 ml) was recombined with mixed brain phospholipids by the method of Nemerson and Pitlick (6) and diluted to 10 ml with 30 mM Tris, pH 8. Factor VII (1000 units) was then added, and the mixture was incubated for 1 hour at room temperature before dialysis against 0.24 M NaCl-0.1 mM Tris, pH 7.5, overnight at 4°C. The dialyzed Factor X was then rechromatographed on DEAE-Sephadex A-50 at 4°C as described by Esmouf et al. (12). The Factor X and Xα peaks were pooled together, concentrated to about 10 ml of 12 mM sodium citrate, ultracentrifugation through an XM-100 membrane (Amicon) replaced the gel filtration step. The specific activity of the final product was about 600,000 units per mg and was stored at a concentration of 0.1 mg per ml in 50% glycerol-50 mM NaCl-25 mM Tris, pH 7.5, at -20°C.

Factor X was also prepared from the barium sulfate eluate by a modification of the method of Esmouf et al. (12). The Factor X pool from DEAE-Sephadex chromatography of an eluate made from admixture of 3% human serum was used for recombination with mixed brain phospholipids, following the method of Nemerson and Pitlick (6) and stored in 50% glycerol at -20°C. Tissue factor apoprotein was prepared by a modification of the method of Nemerson and Clyne (5). Other proteins prepared by a modification of the method of Nemerson and Clyne (5). Other proteins were prepared by a modification of the method of Nemerson and Clyne (5). Other proteins were prepared by a modification of the method of Nemerson and Clyne (5).
5616 units) and 0.5 ml of 0.1 M CaCl₂ were then added and the mixture was incubated for 20 min at 37°C. The complex was then centrifuged down (120,000 × g for 1 hour), and suspended in 0.4 ml 0.1 M NaCl-50 mM Tris-Cl, pH 7.5.

RESULTS

Analysis by sodium dodecyl sulfate gel electrophoresis of the activation of Factor X by TF-VII shows the appearance of three major bands derived from the heavy chain of the zymogen (Fig. 1). Two of these are labeled by [³²P]DFP (gel not shown, cf. Fig. 2) and are designated α-Xₐ and β-Xₐ (heavy chains). The third new band, which does not incorporate [³²P]DFP (Fig. 6B), appears just below the heavy chain of Factor X (Fig. 1B) and is called Intermediate (I).

During the course of activation, α-Xₐ appears first, followed by β-Xₐ (Fig. 1A), which suggests the following pathway:

\[ X \rightarrow \alpha-Xₐ \]  
\[ \alpha-Xₐ \rightarrow \beta-Xₐ \]  

At lower rates, however, Intermediate appears (Fig. 1B), and eventually disappears; this disappearance does not seem to involve the formation of α-Xₐ. Therefore, the following pathway is also proposed:

\[ X \rightarrow \text{Intermediate} \]  
\[ \text{Intermediate} \rightarrow \beta-Xₐ \]  

Each of these species was isolated, enabling us to study each reaction separately and elucidate the mechanisms of activation.

Reaction 1: Factor X \(\rightarrow\) α-Xₐ—The action of Russell's viper venom on Factor X has been clearly shown by others to involve the cleavage of a single peptide bond leading to the release of an activation peptide and the formation of activated Factor X (3). This species of Xₐ migrates identically on sodium dodecyl sulfate gels to a-Xₐ (Figs. 1 and 2) formed during activation by TF-VII. From this observation we conclude that TF-VII, as well as venom, can catalyze Reaction 1.

Reaction 2: α-Xₐ \(\rightarrow\) β-Xₐ—It is clear from the patterns shown in Fig. 1A that during the reaction with TF-VII, α-Xₐ is rapidly converted to β-Xₐ. This conversion could be the result of further proteolysis catalyzed by either TF-VII or α-Xₐ itself. To test these alternatives we prepared α-Xₐ to use as a potential substrate for these enzymes. Initially, Factor X was activated with venom, and the Xₐ purified by the method of Jesty and Esnouf (13). During this procedure, however, the α-Xₐ was completely converted to β-Xₐ, showing the need for a faster procedure. Accordingly, the venom fraction was coupled to Sepharose 4B, and incubated with Factor X to produce α-Xₐ (Fig. 3A). The reaction was temporarily stopped by the addition of sodium citrate, and the venom-Sepharose was removed by centrifugation. Upon

![Fig. 1. Activation of Factor X by tissue factor-Factor VII.](http://www.jbc.org/)

![Fig. 2. Incorporation of [³²P]DFP by α-Xₐ and β-Xₐ.](http://www.jbc.org/)
FIG. 3. The autocatalytic conversion of \( \alpha-X_\alpha \) to \( \beta-X_\alpha \). A, a sample (1.07 ml) of Factor X (0.59 mg per ml in 0.1 M NaCl-50 mM Tris-Cl, pH 7.5) was incubated at 37° with 0.6 ml Russell’s viper venom-Sepharose suspension and a zero time sample was removed (50 pl). CaCl\(_2\) (0.1 M, 0.1 ml) was then added to start the reaction. Samples (50 pl) were removed at 1-min intervals into 50 pl of 4% sodium dodecyl sulfate-10 M urea-10% 2-mercaptoethanol for electrophoresis. The reaction was stopped at 4 min by the addition of 70 pl of 0.18 M sodium citrate, and the venom-Sepharose was removed by centrifugation (500 \( \times \) g, 1 min). The supernatant containing \( \alpha-X_\alpha \) was then incubated in the absence and presence of phospholipid. B, a quantity (20 pl) of buffered saline (0.1 M NaCl-50 mM Tris-Cl, pH 7.5) was added to 0.5 ml of the mixture at 37°, and the reaction was restarted with 20 pl of 0.15 M CaCl\(_2\). Samples (50 pl) were removed at the times shown (in minutes) and added to 50 pl of 4% sodium dodecyl sulfate-10 M urea-10% 2-mercaptoethanol and heated at 100° for 3 min before electrophoresis. C, as in B, except that 20 pl of “cephalin” replaced the buffered saline. The assay of suitable dilutions (1:20,000) of samples removed after the initial activation (4 min, A) and at the end of the further incubations (30 min, B and C) showed the X\(_\alpha\) activity to be unchanged over the course of incubation, both in the presence and absence of phospholipid.

The mixture of standards (8) is described in the legend of Fig. 1.

Reactions \( 3, 5, \) and \( 6: X \rightarrow \text{Intermediate}; \) and Autocatalytic Formation of \( \alpha-X_\alpha \) and \( \beta-X_\alpha \) in the Presence of Ca\(^{2+}\) and phospholipid. In order to determine whether TF-VII can also catalyze this reaction, DIP-\( \alpha-X_\alpha \) was prepared for use as a substrate for both TF-VII and \( \alpha-X_\alpha \). \( \alpha-X_\alpha \) was prepared with venom-Sepharose as before (see Fig. 3) and immediately incubated with 20 mM DFP for 2 hours at room temperature. Excess DFP and hydrolysis products were removed by dialysis overnight against 1 mM sodium citrate in 30 mM sodium acetate, pH 6. Even under these conditions approximately 20% conversion to \( \beta-X_\alpha \) occurs, showing the incomplete inhibition of \( \alpha-X_\alpha \). The addition of Ca\(^{2+}\) + phospholipid to DIP-\( \alpha-X_\alpha \) results in further slight conversion to \( \beta-X_\alpha \) in 45 min (Fig. 4A), confirming the incomplete inhibition of \( \alpha-X_\alpha \) by DFP (21). The addition of active \( \alpha-X_\alpha \) + Ca\(^{2+}\) + phospholipid, at a DIP-\( \alpha-X_\alpha \):X\(_\alpha\) ratio of 5:1, results in complete conversion to \( \beta-X_\alpha \) (Fig. 4B); whereas incubation with TF-VII yielded no additional \( \beta-X_\alpha \) beyond that observed in the control (Fig. 4C). Thus the enzyme which catalyzes the conversion of \( \alpha-X_\alpha \) to \( \beta-X_\alpha \) has been unequivocally shown to be Factor X\(_\alpha\). In other experiments (data not shown) \( \alpha-X_\alpha \) and \( \beta-X_\alpha \) were found to be equally effective in catalyzing this reaction.

Activated Factor X was prepared by activation with viper venom and was purified chromatographically (13). During the course of the purification, the \( \alpha-X_\alpha \) was converted completely to \( \beta-X_\alpha \). Factor X was then immediately incubated with \( \beta-X_\alpha \) and Ca\(^{2+}\) (X: \( \beta-X_\alpha \) ratio of 50:1) at room temperature with and without phospholipid. The mixture without phospholipid showed only a slight
FIG. 4. Comparison of the action of α-Xₐ + lipid and TF-VII on DIP-α-Xₐ. α-Xₐ was prepared by the action of Russell's viper venom-Sepharose on Factor X as described in Fig. 3A except that the buffer was 0.1 M Tris-Cl, pH 7.5. DFP (1% volume, 1 M in 2-propanol) was then added and the mixture was left for 1 hour at 22°C. A further 1% volume of 1 M DFP was added and incubation was continued for 1 hour at 22°C before dialysis overnight at 4°C against 1 mM sodium citrate-30 mM sodium acetate, pH 6. Before use, this DIP-α-Xₐ solution was added to a 1% volume of 1 M NaCl-0.5 M Tris-Cl, pH 7.5. A, a sample (0.4 ml) of DIP-α-Xₐ (0.42 mg per ml) was incubated at 37°C with 20 μl of “cephalin” and 80 μl of 0.1 M NaCl-50 mM Tris-Cl, pH 7.5 (buffered rise in Xₐ activity (Fig. 5) and no detectable change in the Factor X by sodium dodecyl sulfate gel electrophoresis. In contrast, the mixture containing lipid showed early conversion of Factor X to Intermediate (I₁) within 3 hours, followed by the appearance of a band not previously seen (intermediate in mobility between α-Xₐ and β-Xₐ), and designated I₂, and finally the appearance of β-Xₐ (Fig. 6A), which was identified by assay (Fig. 5). The sigmoid form of the activation curve is consistent with the autocatalytic nature of this formation of β-Xₐ from Factor X. Treatment of the samples from the reaction mixture with [³²P]DFP followed by autoradiography showed clearly that Factor X, I₁, and I₂ do not incorporate significant amounts of DFP, whereas β-Xₐ (heavy chain) is heavily labeled (Fig. 6B). This strongly suggests that I₂ is not enzymically active. It does, however, still appear to be a result of cleavage of the heavy chain: electrophoresis of overloaded gels shows the mobility of the light chain to be unchanged.

These data suggest two additional reactions in the activation pathway of Factor X:

\[ I₁ → I₂ \]  \hspace{1cm} (5)
\[ I₂ → β-Xₐ \]  \hspace{1cm} (6)

While Reaction 5 clearly occurs during the reaction of Xₐ with Factor X, Reaction 6 must be considered tentative until I₂ is isolated and can be studied directly.

Reaction 4: Intermediate (I₁) → β-Xₐ—We have noted repeatedly that the final yield of Factor Xₐ from Factor X in activations by TF-VII is dependent on the rate of activation and that in slower activations I₁ accumulates. It was therefore possible that I₁, produced from Factor X by the action of Factor Xₐ + lipid, might be refractory to activation by TF-VII. I₁ was therefore prepared by the action of β-Xₐ + lipid on Factor X and separated from β-Xₐ by chromatography on benzamidine-Sepharose by the use of a linear gradient (Fig. 7) of NaCl for elution. This material contains a small proportion of I₂. β-Xₐ is not eluted from the column under these conditions, but may be eluted by a gradient of guanidine hydrochloride (Fig. 7). Single stage assay for Factor X activity with Russell’s viper venom (9) showed reasonably constant specific activity across the I₁ peak (Fig. 7), at about 10,000 units per A unit. This is very close to the specific activity of pure Factor X observed by us and others (18, 22-24). The rates of activation of Factor X and I₁ by TF-VII were then compared (Fig. 8) and it is seen that at the two activation rates shown the initial rates are similar for Factor X and I₁, indicating that I₁ is not refractory to activation by TF-VII. Sodium dodecyl sulfate gel electrophoresis of the fast activations of Factor X and I₁, by TF-VII are shown in Fig. 9, A and B, and it is noticeable that in the activation of I₁ (B), only β-Xₐ is formed. This is also true of the activation of I₁ by the venom fraction (Fig. 9C); in the activation of Factor X, the
Fig. 5. The activation of Factor X by Xα. A sample (0.48 ml) of Factor X (0.44 mg per ml in 0.1 M NaCl-50 mM Tris-Cl, pH 7.5) was incubated at room temperature with 25 μl of "cephalin" (+lipid) and 10 μl of β-Xα (0.45 mg per ml); a portion (20 μl) of CaCl₂ (0.15 M) was added to start the reaction. In the sample without lipid (−lipid) 25 μl of buffered saline replaced the "cephalin." Samples (5 μl) were removed at the times shown and added to 0.5 ml of 12 mM sodium citrate. This was further diluted (1/10 to 1/50) for assay of Xα into 0.01% ovalbumin-0.1 M NaCl-50 mM Tris-Cl, pH 7.5.

Fig. 6. The activation of Factor X by Xα. Samples (50 μl) were removed from the incubation mixture described in the legend of Fig. 5 (+lipid) at the times shown and added to 5 μl of 0.18 M sodium citrate. A portion (1 μl) of [32P]DFP (2 mCi per ml, 0.1 M) was added to each sample and incubated for 1 hour at 22°; the reaction was stopped by the addition of 50 μl of 4% sodium dodecyl sulfate-10 mM urea-10% 2-mercaptoethanol, and heated at 100° for 3 min before electrophoresis of the samples. The mixture of standards (S) is described in Fig. 1. The gel was stained (Protein) and then dried before autoradiography (32P).

Fig. 7. The purification of I₁. Factor X, 18 mg in 35 ml of 5 mM CaCl₂-0.1 M NaCl-50 mM Tris-Cl, pH 7.5, was incubated for 5 hours at 22° with 2 ml of "cephalin" and 0.5 ml of β-Xα (0.72 mg per ml). The reaction was stopped by the addition of 2 ml of 0.5 M EDTA, pH 7.5, and the phospholipid was removed by centrifugation (120,000 × g, 1 hour). The supernatant was freed of low molecular weight material by repeated concentration and dilution on a PM-10 ultrafiltration membrane (Amicon) with 5 mM EDTA-0.1 M NaCl-50 mM Tris-Cl, pH 7.5. The resulting solution was applied to a column (1.2 × 15 cm) of benzamidine-Sepharose at 15 ml per hour. Protein was then eluted (-----) with a linear 300-ml gradient, 0.2 to 0.5 M NaCl, in 50 mM Tris-Cl (pH 7.5). Fractions (4 ml) were collected and assayed for Factor X activity (Ο) and for Xα; no Xα activity was detected in the fractions. The fractions containing I₁ were pooled as shown (↑↑↑). The column was then washed with 100 ml of 0.1 M NaCl-50 mM Tris-Cl, pH 7.5, (↓↓↓) and re-eluted with a linear 200-ml gradient, 0.1 to 0.9 M guanidine HCl, in 50 mM Tris-Cl (pH 7.5). The fractions shown (↑↑↑) were pooled; this material was largely β-Xα, of specific activity about 0.6 unit per μg.

Fig. 8. The activation of Factor X and Xα by TF-VII. A, samples (0.5 ml) of Factor X (Ο) and Xα (Ο) (0.66 mg per ml in 0.1 M NaCl-50 mM Tris-Cl, pH 7.5) were each incubated at 37° with 0.2 ml TF-VII (Factor VII at 1000 units per ml), and 20 μl of 0.15 M CaCl₂ were added to each mixture to start the reactions. Samples (5 μl) were removed at the times shown into 1 ml of 12 mM sodium citrate. These were further diluted (1/20 to 1/500) in 0.01% ovalbumin-0.1 M NaCl-50 mM Tris-Cl, pH 7.5, for assay of Xα. B, samples (0.5 ml) of Factor X (Ο) and Xα (Ο) (0.66 mg per ml in same buffer) were each incubated at 37° with 0.2 ml of TF-VII (Factor VII at 150 units per ml) and 20 μl of 0.15 M CaCl₂ were added to each mixture to start the reactions. Samples (5 μl) removed at the times shown were diluted as in A for assay of Xα activity.

venom fraction produces only α-Xα. These experiments prove that β-Xα can be formed directly from I₁, and that I₁ → α-Xα is forbidden.

A proposed scheme for the interrelation of these pathways is shown in Fig. 10. Inasmuch as I₁ is activated at the same rate as factor X by TF-VII, its appearance in slow activations cannot explain low yields of Xα at low activation rates. Another reason
FIG. 9. The activation of Factor X (A) and I1 (B) by TF-VII, and of I1 by Russell’s viper venom (C). Samples (50 µl) removed at the times shown from the activation mixtures of Factor X (A) and I1 (B) described in Fig. 8A were added to 50 µl of 4% sodium dodecyl sulfate-10 M urea-10% 2-mercaptoethanol and heated at 100° for 3 min before electrophoresis. C, a sample (0.55 ml) of I1

for the low yield of Xa at slow rates of activation might be the inactivation of TF-VII by Factor Xa. As it was impossible to assay TF-VII activity in the presence of Xa by a one-stage assay (1), the complex was incubated at high concentration (Factor VII at about 800 units per ml) with β-Xa (30 µg per ml). This concentration of Xa corresponds to approximately 10% conversion of Factor X in our activation mixtures (e.g. Fig 1); however the TF-VII concentration is about five times that used in fast activations (e.g. Fig 1A). Samples were removed from the incubation mixture and added to 5 µl of 60 mM sodium citrate on ice, and then used to activate Factor Xa. The initial rates of Xa formation were measured (Fig. 11A) and a curve derived for the inactivation of TF-VII (Fig. 11B). In the absence of Xa, the TF-VII is stable over the time course studied (1). Thus it is clear that under these conditions, TF-VII activity is rapidly destroyed by the action of Factor Xa.

NH2-terminal Sequences of I1, α-Xa, and β-Xa—The conversion of Factor X to I1 and to α-Xa and β-Xa has been shown to involve reduction in apparent molecular weight. The NH2-terminal sequence of the heavy chain of Factor X has been published by Fujikawa et al. (3, 22), Try-Ala-Ile, as was that of Xa, Ile-Val-Gly-Gly-. Hence, we determined partial NH2-terminal sequences of the appropriate heavy chains in order to deduce whether the molecular weight changes were due to NH2- or COOH-terminal cleavages. Owing to the small amount of I1 available, its heavy chain was isolated by gel electrophoresis. No NH2-terminal acid was identified by the dansyl technique.
Following two turns of Edman degradation, the following sequence was found (\(-\)-Ala-Ile-). Since Try cannot be identified as its dansyl derivative, this sequence would be expected if the NH$_2$-terminal amino acids of 1$_1$ were the same as in Factor X. Thus, we tentatively conclude that the X \(\rightarrow\) I$_1$ conversion involves cleavage of a peptide from the COOH-terminal end of the heavy chain of Factor X.

The heavy chains of \(\alpha\)-X$_a$ and \(\beta\)-X$_a$ were carboxymethylated (25) and separated from their light chains by gel filtration according to the method of Jackson (26). The NH$_2$-terminal amino acids of both heavy chains were found to be Ile-Val.

Thus, the conversion of \(\alpha\)-X$_a$ to \(\beta\)-X$_a$ appears to involve the loss of a COOH-terminal peptide from \(\alpha\)-X$_a$, but this cannot be considered definitive until the COOH-terminal sequences have been determined.

Only trace amounts of I$_2$ were obtained from gel electrophoresis of the preparation of I$_1$ and, therefore, only a single NH$_2$-terminal amino acid was determined, Thr. On the basis of this preliminary identification it appears as if I$_1$ \(\rightarrow\) I$_2$ involves the cleavage of an NH$_2$-terminal peptide, probably a fragment of the complete activation peptide.

The apparent molecular weights of the heavy chains of the different species determined by sodium dodecyl sulfate gel electrophoresis were as follows: Factor X, 46,400; I$_1$, 41,900; I$_3$, 32,000; \(\alpha\)-X$_a$, 34,300; \(\beta\)-X$_a$, 29,600.

**DISCUSSION**

**Activation Pathways of Factor X**—In the present work we have shown that there are two alternative pathways leading to the formation of X$_a$. In one, initiated by either Russell’s viper venom or TF-VII, an activation peptide is cleaved from the NH$_2$-terminal region of the zymogen leading to the direct formation of \(\alpha\)-X$_a$. Fujikawa et al. (3) have isolated a single activation peptide produced by the action of the venom. As the apparent molecular weight of \(\alpha\)-X$_a$ produced by TF-VII is the same as that produced by the venom, we assume that the peptide bond is hydrolyzed by both enzymes. Definitive proof of this, however, must await isolation and analysis of the peptide (or peptides) released by TF-VII. An additional step in this pathway is the autocatalytic conversion of \(\alpha\)-X$_a$ to \(\beta\)-X$_a$. Both \(\alpha\)-X$_a$ and \(\beta\)-X$_a$ have the NH$_2$-terminal sequence Ile-Val, suggesting that the conversion of \(\alpha\)-X$_a$ to \(\beta\)-X$_a$ involves the cleavage of a COOH-terminal peptide from \(\alpha\)-X$_a$. This reaction is markedly accelerated by phospholipid, but since activation by Russell’s viper venom are not usually performed in the presence of lipids, no \(\beta\)-X$_a$ is formed. When TF-VII is used, however, lipids are present as the lipid component of tissue factor and the conversion of \(\alpha\)-X$_a$ to \(\beta\)-X$_a$ occurs rapidly. Further, we have shown that the TF-VII cannot catalyze this reaction when DIP-\(\alpha\)-X$_a$ is used as a substrate.

An alternative activation pathway is initiated by X$_a$ in which the zymogen is converted to I$_1$. I$_1$ has a lower apparent molecular weight than Factor X (by about 4500) but has the same NH$_2$-terminal sequence, \(\sim\)-Ala-Ile-. We therefore conclude that a peptide is cleaved from the COOH-terminal end of the Factor X heavy chain. As judged by biosay and by the lack of incorporation of $^{35}$PDPF, this intermediate lacks catalytically active activity. I$_1$ is converted directly to \(\beta\)-X$_a$ by either the venom or TF-VII (Fig. 9). We presume this occurs by cleavage of the activation peptide from the NH$_2$-terminal end of the molecule. Direct evidence for this assertion can be obtained determining the NH$_2$- and COOH-terminal sequences of \(\beta\)-X$_a$ formed via each of these pathways. These studies are in progress.

X$_a$ can also convert I$_1$ to \(\beta\)-X$_a$, but the pathway is not certain. During the activation of I$_1$ by X$_a$ (Fig. 6A), a new band appears, I$_2$. I$_2$ also lacks enzymic activity inasmuch as it fails to incorporate $^{35}$PDPF (Fig. 6D). As I$_2$ disappears while \(\beta\)-X$_a$ is accumulating, it is likely that I$_2$ is converted to \(\beta\)-X$_a$. Proof of this, however, requires the isolation of I$_2$ and direct demonstration of its conversion to \(\beta\)-X$_a$. As we have been unable to isolate I$_2$, this reaction must be considered tentative. Alternatively, of course, I$_2$ could be directly converted to \(\beta\)-X$_a$ by the action of X$_a$, as it is by Russell’s viper venom and TF-VII (Fig. 9).

**Structural Model of Factor X, X$_a$, and Intermediates**—On the basis of this evidence we propose the following structural model for the derivatives of Factor X (Fig. 12). (a) All derivatives contain the unmodified light chain of Factor X. (b) Each derivative also contains the following regions of the molecule, as indicated in Fig. 12: X, A–R; I$_1$, A–D; I$_3$, B–D; \(\alpha\)-X$_a$, C–E; \(\beta\)-X$_a$, C–D. The observation that Factor X and I$_1$ have the same NH$_2$-terminal sequence, and that \(\alpha\)-X$_a$ and \(\beta\)-X$_a$ have the same NH$_2$-terminal sequence, is good evidence in support of the proposal that the conversions of Factor X to I$_1$, and \(\alpha\)-X$_a$ to \(\beta\)-X$_a$, are a result of the cleavage of COOH-terminal peptides. Further, we propose for the following reasons that these peptides are identical: (a) \(\beta\)-X$_a$ produced by the three possible pathways is of identical apparent molecular weight (29,600, heavy chain), and (b) the differences in the apparent molecular weight between Factor X and I$_1$, and between \(\alpha\)-X$_a$ and \(\beta\)-X$_a$, are the same within the limits of error, 4,500 and 4,700, respectively. Final proof of these structures, however, awaits the determination of COOH-terminal sequences.

Radelife and Barton (1) isolated X$_a$ which was generated by Russell’s viper venom, TF-VII, trypsin, and other techniques, and found that all forms were of identical molecular weight and had the same NH$_2$- and COOH-terminal residues. In each instance, however, X$_a$ was purified by chromatography; we believe that during this procedure any \(\alpha\)-X$_a$ would be converted to \(\beta\)-X$_a$ by autocatalysis. Other forms of X$_a$ have been reported. Aronson and Mustafa (27) found a species of human X$_a$ which activated prothrombin, but was not accelerated by Ca$^{2+}$, Factor V, and phospholipid. A similar species was prepared by Milstone et al. (28) by digesting bovine X$_a$ with chymotrypsin. These forms of the enzyme are clearly different from \(\alpha\)-X$_a$ and \(\beta\)-X$_a$ because the latter require these co-factors for full activity. The three forms of X$_a$ described by Seegers et al. (20) have different NH$_2$-terminal amino acids from those we found and therefore do not seem to correspond to either \(\alpha\)-X$_a$ or \(\beta\)-X$_a$. Bajaj and Mann (24) recently showed that trypsin activation of Factor X resulted in an 8-fold higher yield of X$_a$ than obtained with the venom. Whether this is due to the formation of a different species of X$_a$
is unknown. In a previous study (1), we noticed that $X_a$ activity seemed to appear more rapidly than the corresponding bands on gel electrophoresis. On that basis we presumed $\alpha$-$X_a$ to be more active than $\beta$-$X_a$. However, by bioassay of the isolated forms it is now clear they have equivalent coagulant activity.

**Kinetics of Activation**—In a synergogen activation in which an alternative pathway is initiated by the product (in this case $X_a$) the alternative pathway will occur to a greater extent at lower initial rates of activation. This is illustrated in Fig. 1, in which $I_1$ is seen only in the slower activation (Panel B). This can be explained by the fact that in rapid activations, Factor X is completely converted to $X_a$; thus no Factor X remains to form $I_1$. Conversely, in slow activations, $X_a$ formed initially catalyzes the reaction in which residual Factor X is converted to $I_1$. During many studies of the activation of factor X by TF-VII, we have repeatedly observed the yield of $X_a$ to be related to the rate of activation, i.e. the concentration of activator. These low yields were paralleled by the accumulation of $I_1$, which appeared to be very slowly activated. It therefore occurred to us that the low yields may be a result of $I_1$ being refractory to activation by TF-VII. However, isolation of $I_1$ enabled us to show that it is in fact converted by TF-VII at the same rate as Factor X (Fig. 8).

We therefore considered other means of control whereby yield may be a function of rate: (a) inactivation of TF-VII by $X_a$; (b) inhibition of TF-VII by peptides released during the reaction, and (c) inactivation of $X_a$. Only the first of these possibilities was studied, and it was found that at a concentration of Factor X generated in the early stages of activation, $X_a$ rapidly inactivates TF-VII (Fig. 11). This rate of inactivation could account for the low yields of $X_a$ and accumulation of $I_1$. The second mechanism will be tested when we can prepare sufficient quantities of the peptides. The third possibility has not yet been studied in detail, but preliminary results suggest that $X_a$ undergoes some degradation on prolonged incubation at 37°C.

The tissue factor pathway proceeds via the sequential activation of Factor X and prothrombin. The sequence is initiated by Factor VII after it has formed a complex with tissue factor. The reaction in which residual Factor X is converted to $I_1$ yields may be a result of $I_1$ being refractory to activation by Factor X, and propose that it is at least partially responsible for the incomplete activation of Factor X that we have observed at low levels of activator. Furthermore, in view of the fact that in all the reactions of the tissue factor pathway the enzymatically active product can rise to a higher level than that of the activator, we consider that this type of control may be of general importance in the control of coagulation.

**Note Added in Proof**—We have recently found the COOH terminus of the heavy chain of $\alpha$-$X_a$ to be Leu and that of $\beta$-$X_a$ to be Arg, thus confirming the loss of a COOH-terminal peptide during the conversion of $\alpha$-$X_a$ to $\beta$-$X_a$.

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**REFERENCES**

The Mechanism of Activation of Factor X: KINETIC CONTROL OF ALTERNATIVE PATHWAYS LEADING TO THE FORMATION OF ACTIVATED FACTOR X
Jolyon Jesty, Allen K. Spencer and Yale Nemerson


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