The Mechanism of Activation of Bovine Factor X (Stuart Factor) by Intrinsic and Extrinsic Pathways†

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ABSTRACT: Bovine factor X (Stuart factor) is a coagulation protein (mol wt 55,000) present in plasma in a precursor form. It is a glycoprotein composed of two polypeptide chains held together by a disulfide bond(s). During the coagulation process, factor X is converted to an enzyme, factor X_a, by either factors IX_a and VIII or tissue factor and factor VII. Factor X is also activated by other enzymes such as trypsin or a protease from Russell's viper venom. The present investigations indicate that formation of factor X_a by each of these four catalysts involves the cleavage of a single specific arginyl-isoleucine peptide bond in the heavy chain of the precursor protein. This results in the formation of an activation peptide (a glycopeptide of mol wt 11,000) and factor $X_{a\alpha}$ (a glycoprotein of mol wt 44,000). The new amino-terminal sequence in the heavy chain of factor $X_{a\alpha}$ is Ile-Val-Gly-Gly- when formed by each of the above catalysts. No change was detected in the light chain during the activation reaction. In a second, slower step, factor $X_{a\alpha}$ is degraded by hydrolysis of a peptide bond(s) in the carboxyl-terminal region of the heavy chain, releasing another glycopeptide fragment(s). This also removes the remaining carbohydrate present in factor $X_{a\alpha}$ and gives rise to factor $X_{a\beta}$ (mol wt 40,000). Factor X can also be converted to factor X_β (mol wt 51,000) by factor IX_a and factor VIII or tissue factor and factor VII or trypsin by an initial cleavage of the glycopeptide fragment(s) from the carboxyl-terminal region of the heavy chain. The peptide bond cleaved in this reaction appears to be the same as that involved in the conversion of factor $X_{a\alpha}$ to factor $X_{a\beta}$. Factor X_β is a partially degraded protein which can also be converted to an activated enzyme by cleavage of a specific arginyl-isoleucine bond in the amino-terminal region of the heavy chain. This reaction leads to the formation of the Ile-Val-Gly-Gly- sequence in the heavy chain of the activated enzyme. This enzyme appears to be identical with factor $X_{a\beta}$ formed by degradation of factor $X_{a\alpha}$.

Factor X (Stuart factor)[1] is a plasma glycoprotein which participates in the middle phase of blood coagulation (Davie and Kirby, 1973). In recent years, the bovine factor X preparation has been extensively purified and characterized in a number of different laboratories (Esnouf and Williams, 1962; Jackson and Hanahan, 1968; Fujikawa *et al.*, 1972a; Jackson, 1972; Jesty and Esnouf, 1973; Bajaj and Mann, 1973). The molecular weight of the bovine preparation is 55,000 and it is composed of a heavy and a light chain held together by a disulfide bond(s). A recent report suggests that factor X may also exist as a single polypeptide chain (Mattock and Esnouf, 1973). This preparation, however, has not been well characterized.

Under physiological conditions, factor X is activated by factor IX_a and factor VIII (intrinsic pathway) (Lundblad and Davie, 1964; Biggs and Macfarlane, 1965; Schiffman *et al.*, 1966; Hougie *et al.*, 1967; Barton, 1967) and by tissue factor and factor VII (extrinsic pathway) (Flynn and Coon, 1953; Hjort, 1957; Hougie, 1959; Straub and Duckert, 1961; Deutsch *et al.*, 1964; Williams and Norris, 1966; Nemerson and Pitlick, 1970; Osterud *et al.*, 1972; Jesty and Nemerson, 1974). Both of these pathways require phospholipid and calcium ions. Factor X is also activated by other enzymes such as trypsin (Ferguson *et al.*, 1960; Pechet and Alexander, 1960; Papahadjopoulos *et al.*, 1964; Yin, 1964; Rimon *et al.*, 1966; Bajaj and Mann, 1973), a protease from Russell's viper venom (Macfarlane, 1961; Esnouf and Williams, 1962; Papahadjopoulos *et al.*, 1964; Fujikawa *et al.*, 1972b; Jesty and Esnouf, 1973), cathepsin C (Purcell and Barnhart, 1963), and papain (Alexander *et al.*, 1962).

Fujikawa *et al.* (1972b) have shown that the protease from Russell's viper venom activates factor X by cleavage of a single specific peptide bond in the amino-terminal region of the heavy chain. This gives rise to an activation peptide (a glycopeptide of mol wt 11,000) and factor X_a (a glycoprotein of mol wt 44,000). Jesty and Esnouf (1973) have also studied the activation of factor X by the Russell's viper venom protease and reported a molecular weight of 40,000–42,000 for their factor X_a preparation. They suggested, however, that the venom activation of factor X may involve cleavage of both the heavy and light chains of the zymogen. Bajaj and Mann (1973) reported that factor X_a formed in the presence of trypsin has a molecular weight of 52,000 and this is due to the cleavage of a small peptide of molecular weight 4000 from the heavy chain. This fragment was not isolated and characterized. Jesty and Nemerson (1974) investigated the activation of factor X by tissue factor and factor VII and concluded that more than one peptide bond is cleaved when factor X is converted to factor X_a by this mechanism. Radcliffe and Barton (1972, 1973), however, found that the various activation mechanisms of factor X yield essentially the same molecular form of factor X_a. Furthermore, the factor X_a which they characterized had no carbohydrate moiety.

In the present article, we report our investigations concerning the molecular events which occur during the activation of factor X by factor IX_a and factor VIII or tissue fac-

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[1] The nomenclature for various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

tor and factor VII or trypsin. These results were compared with the well-defined chemical reaction catalyzed by the protease from Russell's viper venom (Fujikawa *et al.*, 1972b; Jesty and Esnouf, 1973). The data indicate that the activation mechanism for factor X is essentially identical in all four cases and is due to the cleavage of a specific arginyl-isoleucine bond in the amino-terminal portion of the heavy chain of factor X.

Materials

Carboxypeptidase A and B and bovine trypsin were products of Worthington Biochemical Co., Freehold, N.J. Prior to use, the carboxypeptidases were treated with diisopropyl phosphofluoridate by the method of Fraenkel-Conrat *et al.* (1955), while trypsin was treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone according to Carpenter (1967). Russell's viper venom was purchased from the Ross Allen Reptile Institute, Inc., Silver Springs, Fla., and partially purified by gel filtration as described by Schiffman *et al.* (1969). Thromboplastin (Bacto) was purchased from Difco Laboratories, Detroit, Mich. Dithiothreitol (A grade) was purchased from Calibiochem, Los Angeles, Calif. 4-Vinylpyridine was obtained from J. T. Baker Co., Phillipsburg, N.J. Sephadex G-50 and G-100 were products of Pharmacia Fine Chemicals, Piscataway, N.J. Benzamidine-HCl was purchased from Aldrich Chemical Co., Milwaukee, Wis. Acrylamide was obtained from Matheson Coleman and Bell, Norwood, Ohio. Guanidine hydrochloride (extreme purity) was purchased from Heiko, Inc., Delaware Water Gap, Pa. *o*-Nitrophenylsulfonyl chloride, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, and soybean trypsin inhibitor were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were the same as described in a previous paper (Fujikawa *et al.*, 1972a) or commercial preparations of the highest quality available.

Methods

Protein concentration was determined by measuring the absorption at 280 nm employing an $\epsilon_{280}^{1\%}$ of 11.5, 14.3, 7.0, and 14.4 for factor X, factor IX_a, factor VIII, and trypsin, respectively. An $\epsilon_{280}^{1\%}$ of 10.0 was assumed for the protease from Russell's viper venom and factor VII.

Amino acid analysis and the preparation of samples were carried out by the methods of Moore and Stein (1963) and Spackman *et al.* (1958) employing a Spinco Model 120 amino acid analyzer. Samples were hydrolyzed in 6 N HCl at 110° for 24, 48, 72, and 96 hr in evacuated tubes. Values for serine and threonine were calculated by extrapolating to zero-time hydrolysis. Tryptophan was determined by the colorimetric method of Scoffone *et al.* (1968) and half-cystine was determined as *S*-pyridylethylcysteine by the method of Friedman *et al.* (1970). Isoleucine and valine were calculated from the 96-hr hydrolysis sample. Neuraminic acid, neutral sugars, and hexosamine were determined as previously described (Fujikawa *et al.*, 1972a).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (1969) and 8.5% gels were used throughout these experiments. Samples were run for 6 hr with 8 mA/tube and gels were stained for protein with Coomassie Brilliant Blue.

Sedimentation equilibrium measurements on the *S*-pyridylethyl derivative of the heavy chain of factor $X_{a\beta}$ were performed at three different concentrations (0.8, 0.4, and 0.25 mg/ml) in 6 M guanidine-HCl as previously described (Fujikawa *et al.*, 1972a). Sedimentation equilibrium experiments were carried out at 21° at a rotor speed of 32,000 rpm. A partial specific volume of $\bar{\nu}$ = 0.725 ml/g was determined by amino acid analysis (Cohn and Edsall, 1943; Longsworth, 1953). The $\bar{\nu}$ for the *S*-pyridylethyl protein was assumed to be identical with that of the native chain.

Automated Edman degradations were performed in a Beckman Sequencer Model 890A as described by Hermodson *et al.* (1972). Carboxyl-terminal amino acids were determined by the method of Fraenkel-Conrat *et al.* (1955) employing pancreatic carboxypeptidase A and B.

Heparin–agarose was prepared by the method of Fujikawa *et al.* (1973) and benzamidine–agarose was prepared by the method of Schmer (1972).

Factor X and factor X_a were assayed by the method of Bachmann *et al.* (1958) except crude Russell's viper venom was deleted from the assay mixture for factor X_a activity. It should be emphasized that factor X is converted to factor X_a in about 5% yield in the Bachmann assay.

Factor VII was assayed as follows. Barium sulfate–aluminum hydroxide adsorbed plasma was prepared as described previously (Fujikawa *et al.*, 1973). The plasma was then added to an equal volume of saline containing 0.1 mg/ml of factor X and 1.0 mg/ml of prothrombin. The prothrombin which was free of factor VII was purified concurrently with factor VII (Fujikawa *et al.*, 1974a). In the assay of factor VII, 0.2 ml of this solution was incubated with 0.1 ml of thromboplastin and 0.1 ml of test sample for 1 min at 37°. Subsequently, 0.1 ml of 0.025 M $CaCl_2$ was added and the clotting time determined in duplicate. The activity was calculated from a calibration curve where the log of factor VII concentration was plotted against the log of the clotting time.

Factor IX_a and factor VIII were assayed as previously described (Fujikawa *et al.*, 1973; Legaz *et al.*, 1973).

Preparation of Various Coagulant Proteins. Bovine factor IX_a was prepared by the method of Fujikawa *et al.* (1974b). Bovine factor VIII was purified by the method of Schmer *et al.* (1972) as modified by Legaz *et al.* (1974). Bovine factor X was isolated by methods previously described (Fujikawa *et al.*, 1972a). Only factor X_1 was used in the present experiments. The *S*-pyridylethyl heavy and light chains and the activation peptide formed in the presence of the protease from Russell's viper venom were also prepared as previously described (Fujikawa *et al.*, 1972b).

Factor VII was initially purified with factor IX up to the DEAE-Sephadex column step according to the method of Fujikawa *et al.* (1973). The two factors were partially separated by DEAE column chromatography where factor VII eluted with prothrombin just prior to factor IX. The fraction rich in factor VII was dialyzed overnight against 0.05 M imidazole buffer (pH 6.0) and 1 M $CaCl_2$ was added to a final concentration of 0.005 M. The solution was then applied to a heparin–agarose column (3 × 80 cm) which was previously equilibrated with 0.05 M sodium acetate buffer (pH 5.7), containing 0.001 M benzamidine-HCl and 0.005 M $CaCl_2$. The column was washed with 500 ml of the same buffer and subsequently washed with this buffer containing 0.2 M NaCl. Factor VII was eluted with a linear gradient prepared from 1.75 l. of 0.2 M NaCl and 0.5 M NaCl, each containing 0.05 M sodium acetate buffer (pH 5.7), 0.001 M benzamidine-HCl, and 0.005 M $CaCl_2$. The active fraction eluted between 2.5 and 3.5 l. of effluent just after prothrombin. Factor VII was further purified by a repetition of the heparin–agarose procedure. The factor VII was dialyzed against 0.05 M imidazole buffer (pH 6.5), contain-

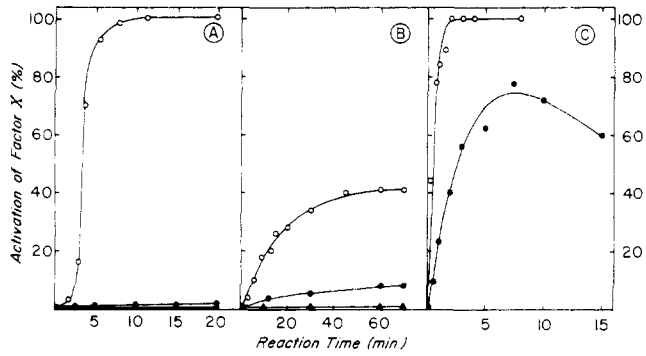

FIGURE 1: Time curves for the activation of factor X by four different enzymatic pathways. (A) Activation of factor X with factor IX_a and factor VIII. The activation mixture contained 25 μl of factor VIII (0.1 mg/ml in 0.05 M Tris-HCl (pH 8.0), containing 0.15 M NaCl), 25 μl of factor IX_a (0.03 mg/ml in 0.05 M Tris-HCl (pH 8.0)), 50 μl of 0.1 M $CaCl_2$, 100 μl of crude soybean phospholipid (0.04% Centrolex-P in 0.01 M Tris-HCl (pH 7.4), containing 0.15 M NaCl), and 200 μl of 0.05 M Tris-HCl buffer (pH 8.0). After a preincubation of the mixture for 5 min at 37°, the activation reaction was initiated by the addition of 200 μl of factor X (1.4 mg/ml). Aliquots were taken at various times, diluted 1000- to 10,000-fold in 0.05 M Tris-HCl (pH 8.0), and assayed for factor X_a as described under Methods: (○) factors IX_a and VIII; (●) factor VIII alone; (▲) factor IX_a alone. (B) Activation of factor X with tissue factor and factor VII. The activation mixture contained 250 μl of thromboplastin, 50 μl of factor VII solution (0.05 mg/ml), 25 μl of 0.1 M $CaCl_2$, 25 μl of water, and 50 μl of 0.5 M Tris-HCl buffer (pH 8.0). The reaction was initiated after a 5-min preincubation by the addition of 100 μl of factor X (1.1 mg of protein/ml in 0.025 M Tris-HCl buffer (pH 7.2), containing 0.2 M NaCl). Incubation was carried out at 37° and 50-μl aliquots of the reaction mixture were removed at various time intervals and the reaction was stopped with 10 μl of 0.1 M EDTA. Factor X_a activity was then assayed as described in A: (○) factor VII–thromboplastin; (●) thromboplastin alone; (▲) factor VII alone. (C) Activation of factor X with the protease from Russell's viper venom or with trypsin. The activation mixture contained 1 ml of factor X (1.0 mg of protein/ml in 0.025 M Tris-HCl buffer (pH 7.2), and 0.2 M NaCl), 0.4 ml of 0.5 M Tris-HCl buffer (pH 8.0), 0.5 ml of saline and 0.1 ml of 0.1 M $CaCl_2$. The reaction was initiated by the addition of 10 μl (10 μg) of the purified Russell's viper venom protease. Incubation was carried out at 37° and 100-μl aliquots were removed at various time intervals and the reaction stopped with 10 μl of 0.1 M EDTA. Factor X_a was assayed as described in A. The activation mixture with trypsin contained 0.67 ml of factor X (1.5 mg in 0.025 M Tris-HCl buffer (pH 7.2), and 0.2 M NaCl), 0.4 ml of 0.5 M Tris-HCl buffer (pH 8.0), in a final volume of 2.0 ml. The reaction was initiated by the addition of 10 μl (10 μg) of trypsin. The mixture was incubated at 37° and 100-μl aliquots were removed at appropriate time intervals and placed in a test tube containing 2 μg of soybean trypsin inhibitor: (○) activation with the protease from Russell's viper venom; (●) activation with trypsin.

ing 0.001 M benzamidine-HCl. This was then applied to a benzamidine–agarose column (2.0 × 20 cm) (Schmer, 1972; Jesty and Nemerson, 1974) equilibrated with the same buffer. After washing with 3.0 l. of the same buffer containing 0.35 M NaCl which eluted prothrombin, factor VII was eluted with a linear gradient prepared from 600 ml of imidazole buffer (pH 6.5), 0.001 M benzamidine-HCl, 0.35 M NaCl, and 600 ml of the buffer with 2.0 M NaCl. Factor VII eluted in the first part of the gradient.

Tissue thromboplastin was prepared by extraction from thromboplastin (1 g) with 28.0 ml of saline for 10 min at 48°. The suspension was then filtered through cheesecloth and the filtrate was centrifuged at 30,000 rpm for 30 min in a Beckman Model L ultracentrifuge using a number 40 rotor. The precipitate was resuspended in 20 ml of saline and the suspension was recentrifuged. This procedure was repeated five times. Finally, the tissue factor particles were resuspended in 5 ml of 0.15 M NaCl. Aliquots were stored at −20° until needed for the activation of factor X or for factor VII assay.

Results

Activation of Bovine Factor X. Time curves for the activation of factor X by four different enzymatic pathways are shown in Figure 1. Panel A shows the curve for factor X activation by factor IX_a and factor VIII (open circles). A distinct lag phase of 2–3 min is observed and the reaction is essentially complete within 8–10 min. With factor VIII alone at the same concentration, no factor X activation occurs (solid triangles). Incubation of factor X with factor IX_a results in the activation of approximately 1% of the factor X (solid circles). This result was similar to that observed previously by Thompson (1971). The final yield of factor X_a formed in the presence of factor IX_a and factor VIII was essentially identical with that obtained by the purified protease from Russell's viper venom. The weight ratio of factor X to factor IX_a in these experiments was 350:1. The molar ratio of factor IX_a to factor VIII was 8:1 employing a molecular weight of 45,000 for factor IX_a (Fujikawa *et al.*, 1974b) and 1.1×10^6 for factor VIII (Schmer *et al.*, 1972).

Panel B (Figure 1) shows the time curve for factor X activation by tissue factor and factor VII (open circles). In these experiments, the weight ratio of substrate to partially purified enzyme was 90:1 and the final yield of factor X_a ranged from 40 to 50% of that observed with the purified protease from Russell's viper venom. Higher concentrations of tissue factor and factor VII gave a higher yield of factor X_a, but these levels of tissue factor and factor VII interfere with the sodium dodecyl sulfate gel electrophoresis patterns as presented below. Some activation of factor X occurred in the presence of only tissue factor (solid circles), while little or no activation occurred in the presence of only factor VII (solid triangles).

The activation of factor X by trypsin and the purified protease from Russell's viper venom is shown in panel C (Figure 1). In each of these experiments, the weight ratio of substrate to enzyme was 100:1. The final yield of factor X_a by trypsin activation (solid circles) ranged from 70 to 80% of that obtained by the purified protease from Russell's viper venom (open circles). Inactivation of factor X_a by trypsin was also apparent after 8–10 min of incubation. The inactivation of factor X_a by trypsin has also been noted by others (Papahadjopoulos *et al.*, 1964; Bajaj and Mann, 1973). Crude viper venom (not shown) was a relatively poor activator of factor X, presumably due in part to the large amount of protease inhibitor present in the crude venom (Takahashi *et al.*, 1972).

The molecular changes in factor X during the activation reaction were then examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In these experiments, aliquots were removed at various times from an activation mixture corresponding to each of those shown in Figure 1 and analyzed by gel electrophoresis (Figure 2). Panel A shows the activation of factor X by factor IX_a and factor VIII. At zero time, a major protein band corresponding to factor X was observed. After 3–4 min of incubation, however, a new major faster moving band appeared and this band coincided with the appearance of factor X_a activity (see Figure 1). Also, this band has the same electrophoretic migration as factor X_a formed by the purified protease from Russell's viper venom (gel on the far right, panel A, Figure 2). This protein, which has enzymatic activity, has been referred to as factor X_{aa} (see panel B for the gel patterns for

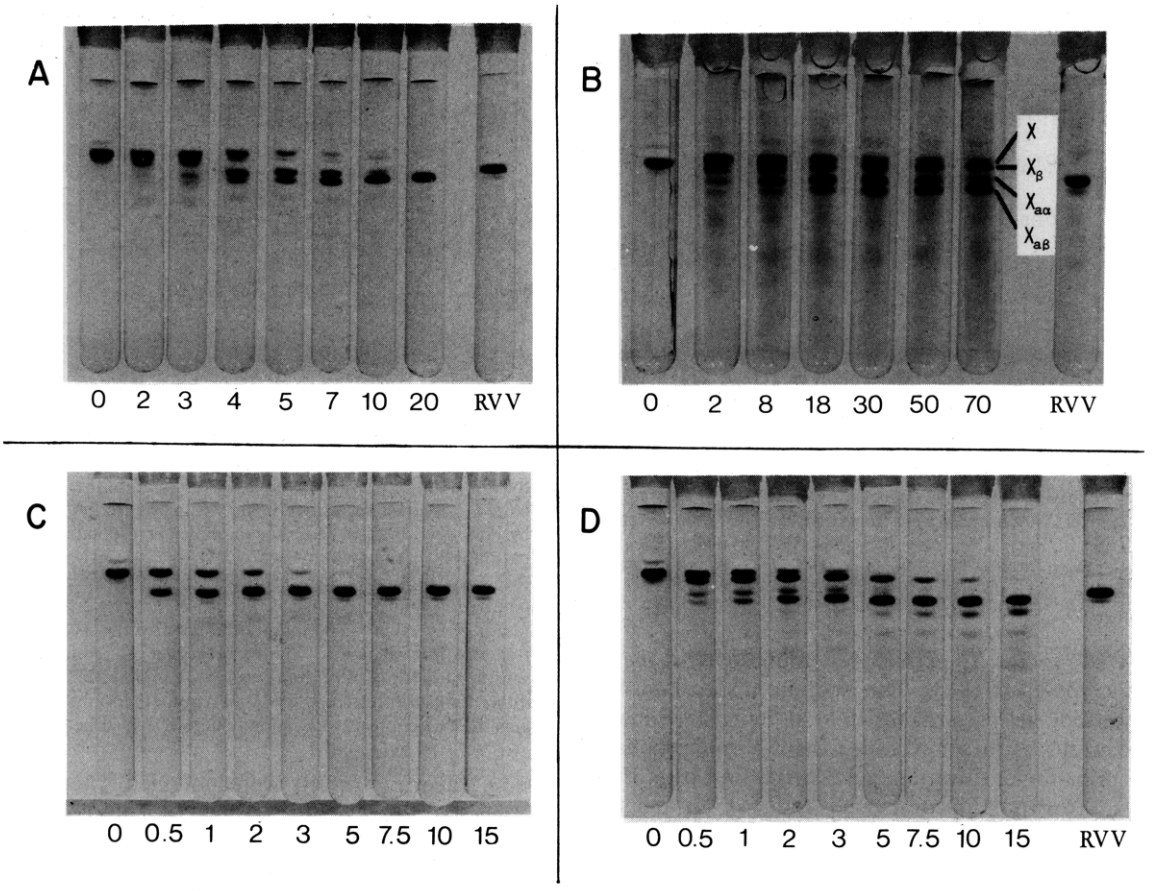

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel patterns of factor X activated by four different enzymatic pathways. Samples from each of the four activation mixtures shown in Figure 1 (12–20 μg of protein were incubated with 2% sodium dodecyl sulfate, applied to the gels, and run at 8 mA/tube for 6 hr): (A) activation of factor X with factor IX_a and factor VIII; (B) activation of factor X with tissue factor and factor VII; (C) activation of factor X with the protease from Russell's viper venom; (D) activation of factor X with trypsin. A 15-min activation mixture of factor X with the protease from Russell's viper venom is shown in A, B, and D for comparison.

factors X, X_β, $X_{a\alpha}$, and $X_{a\beta}$). After 4–5 min of incubation with factor IX_a and factor VIII, factor $X_{a\alpha}$ is slowly converted to a slightly faster moving component, referred to as factor $X_{a\beta}$. Since the total factor X_a activity is unchanged (as shown in panel A, Figure 1), factor $X_{a\alpha}$ and factor $X_{a\beta}$ each have comparable coagulant activity. Five to ten per cent of factor X was initially converted to another component, referred to as factor X_β. This protein, which migrated slightly faster than factor X, appeared after the first few minutes of incubation. The migration of this protein, however, was slower than factor $X_{a\alpha}$ or factor $X_{a\beta}$. Furthermore, factor X_β was slowly converted to factor $X_{a\beta}$ and was completely absent in the 20-min incubation sample.

A similar polyacrylamide gel pattern was observed with factor X activation catalyzed by tissue factor and factor VII (panel B, Figure 2). Factor $X_{a\alpha}$ was rapidly formed and was slowly converted to factor $X_{a\beta}$. Twenty to thirty per cent of the factor X was also converted to factor X_β. Under the conditions of these experiments, substantial amounts of factor X and factor X_β were present after 70 min of incubation.

The gel patterns for factor X activation by Russell's viper venom protease and trypsin are shown in panels C and D, respectively (Figure 2). The formation of factor $X_{a\alpha}$ was nearly quantitative in the presence of low concentrations of the purified protease from Russell's viper venom. With trypsin, factor $X_{a\alpha}$, factor $X_{a\beta}$, and factor X_β are formed, and the gel pattern after 2 or 3 min of incubation is very similar to the final gel patterns observed for tissue factor and factor VII (70-min sample, panel B, Figure 2). With trypsin, however, the reaction continues leading to the complete conversion of factor $X_{a\alpha}$ to factor $X_{a\beta}$. Furthermore, there is a complete disappearance of factor X and factor X_β after 15 min of incubation yielding mainly factor $X_{a\beta}$. Some loss of factor X_a activity also occurs after 15 min (panel C, Figure 1) and this loss of activity is accompanied by several new protein bands which migrate faster than factor $X_{a\beta}$.

These results suggest that the conversion of factor X to factor $X_{a\alpha}$ by factor IX_a and factor VIII or tissue factor and factor VII or trypsin proceeds by the same mechanism as that involved in the activation by the protease from Russell's viper venom. In each case, a new protein is formed which corresponds to factor $X_{a\alpha}$ on gels, and the appearance of this protein parallels the appearance of factor X_a activity. Factor$X_{a\alpha}$ is also converted to factor $X_{a\beta}$ by factor IX_a and factor VIII or tissue factor and factor VII or trypsin. This reaction, however, is very slow in the presence of low concentrations of the protease from Russell's viper venom.

Conversion of Factor X_β to Factor $X_{a\beta}$. In the activation of factor X by factor IX_a and factor VIII or tissue factor and factor VII or trypsin, 5–30% of the precursor molecule was converted to factor X_β (Figure 2). To further identify and characterize this protein, factor X was partially activated with trypsin (2-min incubation) and the reaction mixture was then subjected to gel filtration on Sephadex G-100 (Figure 3). Three major protein peaks were observed. Coagulant activity was found in the first and second peaks (solid

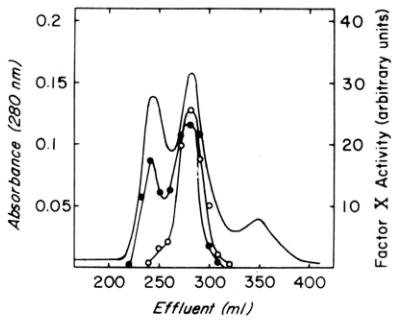

FIGURE 3: Separation of factor X_β from a partial activation mixture catalyzed by trypsin. Factor X (10.5 mg) was activated at 37° for 2 min by 0.1 mg of trypsin in 5 ml of 0.025 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl. The reaction was terminated by the addition of 0.2 mg of soybean trypsin inhibitor and the reaction mixture was then applied to a 2.5 × 90 cm Sephadex G-100 column. Protein was eluted with 0.025 M Tris-HCl (pH 7.2) containing 2×10^{-3} M benzamidine and 10^{-4} M EDTA. Samples were collected in 5 ml/tube. Factor X and factor X_a activity was assayed as described under Methods. A basal absorption at 280 nm due to benzamidine was subtracted from all tubes: (●) factor X; (○) factor X_a.

circles) while factor X_a activity was found only in the second peak (open circles). The sodium dodecyl sulfate–polyacrylamide gel patterns for these proteins are shown in Figure 4. Gel 1 is factor X; gel 2 is the 2-min incubation mixture prior to gel filtration; gel 3 is the first peak shown in Figure 3 (this protein corresponds to factor X_β and small amounts of intact factor X); and gel 4 is the second peak shown in Figure 3 (this protein corresponds to factor $X_{a\beta}$).

When factor X_β was incubated with the protease from Russell's viper venom, factor X_a activity was rapidly formed. The exclusive formation of factor $X_{a\beta}$ in these experiments was demonstrated by sodium dodecyl sulfate gel electrophoresis (gel 5, Figure 4). Similar results were obtained with factor IX_a and factor VIII. In these experiments, the weight ratio of factor X_β to factor IX_a and factor VIII was 20:1 and the molar ratio of factor IX_a to factor VIII was 13:1. In the presence of factor IX_a and factor VIII, factor X_β was converted to factor $X_{a\beta}$ in less than 5

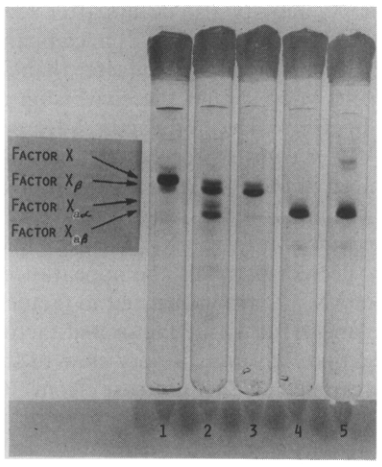

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern for factor X_β and factor $X_{a\beta}$: (gel 1) factor X standard; (gel 2) a partial activation mixture catalyzed by trypsin (same as the 2-min sample shown in Figure 2D); (gel 3) first peak shown in Figure 3 (tube No. 47 corresponding to 235 ml of effluent); (gel 4) second peak shown in Figure 3; (gel 5) factor X_β (50-μl sample from tube No. 47) activated with 1 μg of Russell's viper venom protease. Each sample (12–15 μg of protein) was incubated with 2% sodium dodecyl sulfate and electrophoresis was carried out at 8 mA/tube for 6 hr.

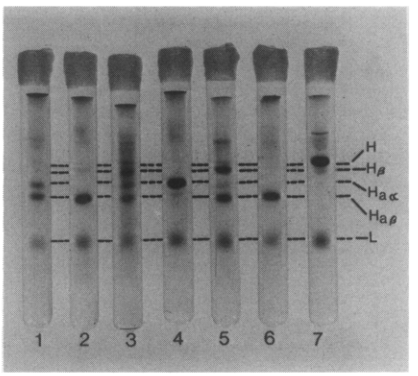

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel pattern for the reduced protein formed during the activation of factor X by four different activation pathways: (gels 1 and 2) activation of factor X by factor IX_a and factor VIII (samples correspond to the 7- and 20-min incubation mixtures, respectively, shown in Figure 2A); (gel 3) activation by factor VII and tissue factor (sample corresponds to the 70-min incubation mixture shown in Figure 2B); (gel 4) activation of factor X by the Russell's viper venom protease (sample corresponds to the 15-min incubation mixture shown in Figure 2C); (gels 5 and 6) activation of factor X by trypsin (samples correspond to the 3- and 10-min incubation mixtures shown in Figure 2D); (gel 7) reduced factor X. Each sample (12–15 μg of protein) was incubated at 37° for 4 hr with 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol before applying to the gels. Electrophoresis was carried out at 8 mA/tube for 6 hr.

min. Identical results were obtained when factor X_β was converted to factor $X_{a\beta}$ with trypsin employing a substrate-to-enzyme ratio of 100:1.

These results suggest that factor IX_a and factor VIII or tissue factor and factor VII or trypsin partially degrade factor X by minor proteolysis leading to the formation of factor X_β. This protein, however, can be converted directly to an activated form (factor $X_{a\beta}$) which has the same sodium dodecyl sulfate gel electrophoresis pattern as factor $X_{a\beta}$ formed from factor $X_{a\alpha}$.

Molecular Changes in the Heavy Chain of Factor X during the Activation Reaction. The gel patterns for the reduced proteins formed by the activation of factor X by the various activation pathways are shown in Figure 5. Gels 1 and 2 correspond to 7- and 20-min samples, respectively, from the factor IX_a and factor VIII activation mixture (panel A, Figure 2); gel 3 corresponds to a 70-min sample from the tissue factor and factor VII activation mixture (panel B, Figure 2); gel 4 corresponds to a 15-min sample from the Russell's viper venom activation mixture (panel C, Figure 2); gels 5 and 6 correspond to 3- and 10-min samples from the trypsin activation mixture (panel C, Figure 2), respectively; and gel 7 is reduced factor X.

A fast-moving band, labeled L, was present in all four activation reactions. This protein has the same migration as the light chain of factor X (gel 7) or factor X_a formed by the protease from Russell's viper venom (gel 4). Previous studies from our laboratory have shown that this protein isolated from either factor X or factor X_a formed in the presence of the protease from Russell's viper venom has a molecular weight of 17,000 and contains an amino-terminal sequence of Ala-Asn-Ser-Phe- (Fujikawa *et al.*, 1972b). Thus, little or no change has occurred in the light chain of factor X during its activation by factor IX_a and factor VIII or tissue factor and factor VII or trypsin, as well as with the protease from Russell's viper venom.

The two protein bands, labeled $H_{a\alpha}$ and $H_{a\beta}$, arise from the heavy chain of the activated enzyme. The $H_{a\alpha}$ chain which is formed during the activation of factor X by the

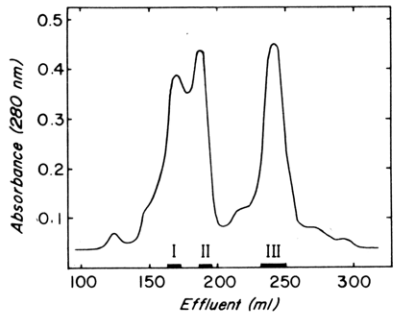

FIGURE 6: Gel filtration pattern for the heavy and light chains of factor X partially activated with trypsin. Factor X (21 mg) was partially activated by incubation with 0.2 mg of trypsin at 37° for 2 min in a final volume of 20 ml of 0.025 M Tris-HCl buffer (pH 8.0). The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 5%. Protein was collected by centrifugation and *S*-pyridylethyl derivatives were prepared and subjected to gel filtration on a column (2.5 × 90 cm) of Sephadex G-100 with 9% formic acid in 2 M urea. The fractions of peak I, peak II, and peak III shown by the heavy bars were combined and desalted by filtration on a Sephadex G-25 column (2.5 × 47 cm) with 9% formic acid followed by lyophilization. The yields of peaks I, II, and III were 4.5, 4.0 and 3.5 mg, respectively.

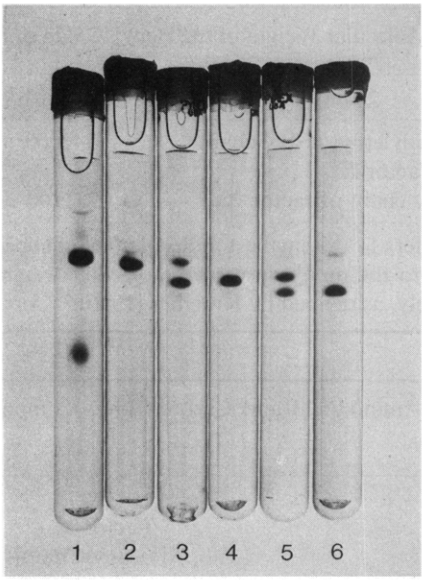

FIGURE 7: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the various factor X heavy chains isolated by gel filtration as shown in Figure 6: (gel 1) reduced factor X; (gel 2) peak I shown in Figure 6 corresponding to the *S*-pyridylethyl heavy chain of factor X_β; (gel 3) a mixture of the two proteins shown in gels 2 and 4; (gel 4) *S*-pyridylethyl heavy chain of factor $X_{a\alpha}$ formed in the presence of the protease from Russell's viper venom; (gel 5) a mixture of the proteins shown in gels 4 and 6; (gel 6) peak II shown in Figure 6 corresponding to the *S*-pyridylethyl heavy chain of factor $X_{a\beta}$. Samples (12–20 μg) were applied to the gels and electrophoresis was carried out at 8 mA/tube for 6 hr.

protease from Russell's viper venom (gel 4) has also been characterized previously in our laboratory (Fujikawa *et al.*, 1972b). This protein has a molecular weight of 27,000 and contains an amino-terminal sequence of Ile-Val-Gly-Gly-. It is formed by the cleavage of a glycopeptide from the amino-terminal portion of the precursor heavy chain which has a molecular weight of 38,000. This $H_{a\alpha}$ chain is also present in each of the factor X activation mixtures from factor IX_a and factor VIII (gel 1) or tissue factor and factor VII (gel 3) or trypsin (gel 5). These data clearly demonstrate that the activation of factor X by each of the four pathways is due to the cleavage of a fragment(s) from the heavy chain of the precursor and the site of cleavage is probably the same in all four reactions. On longer incubation, the $H_{a\alpha}$ chain is converted to $H_{a\beta}$ chain with factor IX_a and factor VIII (gel 2) or tissue factor and factor VII (not shown) or trypsin (gel 6). The $H_{a\beta}$ chain migrated a little faster than the $H_{a\alpha}$ chain which suggests that it has a lower molecular weight.

The slow-moving band, labeled H_β, was present primarily in the activation mixtures from tissue factor and factor VII (gel 3) or trypsin (gel 5). This band migrated slightly faster than the heavy chain in the precursor protein (labeled H). Furthermore, this H_β chain is present in factor X_β. This was shown by gel electrophoresis following reduction of the isolated protein. These data indicate that factor X_β arises from minor proteolysis of the heavy chain of factor X.

Isolation and Further Characterization of the Light and Heavy Chains of Factor $X_{a\alpha}$, Factor $X_{a\beta}$, and Factor X_β. In previous experiments, we have described the separation of the heavy and light chains of factor X and factor X_a by gel filtration following the reduction and alkylation of the proteins (Fujikawa *et al.*, 1972a). Similar experiments were performed with each of the reaction mixtures for the activation of factor X by factor IX_a and factor VIII or tissue factor and factor VII or trypsin, as well as the protease from Russell's viper venom. In each of these experiments, a partially or fully activated factor X activation mixture was treated with cold trichloroacetic acid and the precipitate was separated from the supernatant fraction by centrifugation. The precipitated protein was then reduced with dithiothreitol, alkylated with 4-vinylpyridine, and subjected to gel filtration on Sephadex G-100. The gel filtration pattern for a 5-min reaction mixture of factor X activated by trypsin is shown in Figure 6. This sample contained primarily the heavy and light chains of factor X and factor X_a. Chromatography of this preparation yielded three major protein peaks. Peaks I and II contained the heavy chains of factor X_β and factor $X_{a\beta}$, respectively, and peak III contained the light chain of factor X. This was shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In these experiments, the fractions shown by the bars in Figure 6 were desalted and lyophilized and the proteins were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 7). Gel 1 is a control sample of reduced factor X with its characteristic light (fast-moving band) and heavy (slow-moving band) chains. Gel 2 corresponds to peak I of Figure 6. This protein migrated slightly faster than the heavy chain of factor X and corresponds to the H_β chain previously described. Its molecular weight was estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to be 34,000, or about 4000 less than the heavy chain of the precursor molecule. The amino-terminal sequence of this polypeptide was determined in a Beckman Sequencer and found to be Trp-Ala-Ile-His-. These experiments prove that this polypeptide arises from the heavy chain of the precursor molecule. Furthermore, the corresponding reduction in its molecular weight is due to the removal of a peptide(s) from the carboxyl-terminal region of the polypeptide chain. Gel 4 is the heavy chain of factor $X_{a\alpha}$ formed in the presence of the purified protease from Russell's viper venom. This protein has a molecular weight of 27,000 and has an amino-terminal sequence of Ile-Val-Gly-Gly- (Fujikawa *et al.*, 1972b). Gel 3 is a mixture of the two proteins shown in gels 2 and 4.

Gel 6 is the protein present in peak II of Figure 6. This

TABLE I: Molecular Weight of the Heavy Chain of Factor $X_{a\beta}$ by Sedimentation Equilibrium.

Sample	M_1[a]	M_n	M_w	M_z
S-Pyridylethyl heavy chain of factor $X_{a\beta}$	24,000 ± 400	24,000 ± 400	24,500 ± 650	25,300 ± 800
Heavy chain of factor $X_{a\beta}$[b]	23,100 ± 400			

[a] M_1 refers to the smallest molecular weight species calculated by the methods described by Teller *et al.* (1969). M_n, M_w, and M_z refer to the number average molecular weight, the weight average molecular weight, and the z-average molecular weight, respectively, as defined by Kraemer (1940). [b] Corrected for 8.4 *S*-pyridylethyl residues.

TABLE II: Amino Acid and Carbohydrate Composition of Factor $X_{a\alpha}$, Factor $X_{a\beta}$, and the Heavy Chains of Factor $X_{a\alpha}$ and Factor $X_{a\beta}$.

Components	Factor $X_{a\alpha}$[a] [Heavy Chain] (mol/27,000)	Factor $X_{a\beta}$ [Heavy Chain] (mol/23,000)	Factor $X_{a\alpha}$ [Heavy plus Light Chains] (mol/44,000)	Factor $X_{a\beta}$ [Heavy plus Light Chains] (mol/40,000)	Factor X_a[b] (mol/40,000)
Amino acid					
Lysine	13.5	15.3	20.1	21.9	21.1
Histidine	5.9	5.9	8.4	8.4	8.1
Arginine	14.4	14.0	22.7	22.3	22.0
Aspartic acid	17.6	18.4	32.9	33.7	30.3
Threonine	19.3	18.3	26.3	25.3	22.3
Serine	12.2	10.0	24.4	22.2	24.1
Glutamic acid	24.9	24.2	57.7	57.0	52.9
Proline	11.9	3.5	14.6	6.2	9.1
Glycine	21.7	21.4	38.4	38.1	34.3
Alanine	17.7	13.6	26.2	22.1	22.2
Half-cystine	8.4	7.9[c]	24.8	24.3	20.2
Valine	19.9	16.6	26.3	23.0	21.5
Methionine	5.3	4.8	5.3	4.8	5.1
Isoleucine	8.1	7.6	10.5	10.0	9.2
Leucine	15.1	13.5	23.2	21.6	21.1
Tyrosine	6.2	4.4	9.4	7.6	8.4
Phenylalanine	12.5	10.4	20.6	18.5	18.4
Tryptophan	5.2	4.8[d]	5.6	6.2	7.9
Carbohydrate					
Hexose	2.9	0	4.2	1.3	0
Hexosamine	1.7	0	2.0	0.3	0
Neuraminic acid	0.4	0	0.6	0.2	0
Protein (%)	96.6	100	97.1	99.2	100
Carbohydrate (%)	3.4	0	2.9	0.8	0

[a] Taken from the data of Fujikawa *et al.* (1972a). [b] Modified from the data of Radcliffe and Barton (1973). Their data were presented in residues/50,000 g of factor X_a. A summation of the total amino acids listed in their table, however, yields a molecular weight of approximately 43,000. Thus, the data shown in this column were adjusted from 43,000 to 40,000 rather than from 50,000 to 40,000. [c] Determined as *S*-pyridylethylcysteine by the method of Friedman *et al.* (1970). [d] Determined by the colorimetric method of Scoffone *et al.* (1968).

polypeptide migrated slightly faster than the heavy chain of factor $X_{a\alpha}$. Gel 5 is a mixture of the two proteins shown in gels 4 and 6. The amino-terminal sequence of the polypeptide shown in gel 6 was found to be Ile-Val-Gly-Gly- and its molecular weight was 23,000 ± 400 as determined by sedimentation equilibrium (Table I). These data indicate that peak II contains the heavy chain of factor $X_{a\beta}$. Furthermore, this chain is formed by cleavage of a fragment(s) from the carboxyl-terminal region of the heavy chain of factor $X_{a\alpha}$ and this fragment(s) would have a molecular weight of about 4000.

The amino acid and carbohydrate composition for the heavy chain of factor $X_{a\beta}$ is shown in column 2, Table II. The amino acid and carbohydrate composition is also shown in column 1 for the heavy chain of factor $X_{a\alpha}$. A small reduction in a number of amino acids is apparent in comparing the compositions of the two heavy chains. A striking difference is noted, however, in proline and the carbohydrate content. The heavy chain of factor $X_{a\alpha}$ contains 3.4% carbohydrate, while the heavy chain of factor $X_{a\beta}$ is free of carbohydrate. Thus, the fragment(s) removed from the carboxyl-terminal region of the heavy chain during the conver-

sion of factor $X_{a\alpha}$ to factor $X_{a\beta}$ is a glycopeptide(s).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the protein in peak III of Figure 6 indicated that it has the same migration as the light chain of factor X (gels not shown). Its amino-terminal sequence was found to be Ala-Asn-Ser-Phe- which is the same sequence as that found in the light chain of the precursor molecule. These experiments provide further evidence to support the conclusion that little, if any, change occurs in the light chain of factor X during the activation of factor X by trypsin.

A similar analysis was then made on the heavy and light chains of factors X_{β}, $X_{a\alpha}$, and $X_{a\beta}$ isolated from an activation reaction catalyzed by factor IX_a and factor VIII or tissue factor and factor VII. The various heavy and light chains were isolated by gel filtration following reduction of a factor X activation mixture as described above. The heavy chain from factor $X_{a\beta}$ isolated from the reaction mixture catalyzed by factor IX_a and factor VIII had the same migration in sodium dodecyl sulfate–polyacrylamide gel electrophoresis as that found for the heavy chain of factor $X_{a\beta}$ formed in the presence of trypsin (gel 6, Figure 5). Also, the amino-terminal sequence of this chain was found to be Ile-Val-Gly-Gly-. Following activation of factor X by tissue factor and factor VII, the heavy chain isolated by gel filtration was a mixture of heavy chains from factor $X_{a\alpha}$ and factor $X_{a\beta}$ and corresponded to gel 5, Figure 5. The amino-terminal sequence of this mixture was also Ile-Val-Gly-Gly-indicating that each peptide has the same amino-terminal sequence. These data demonstrate that factor X is converted to factor $X_{a\alpha}$ by cleavage of a single peptide bond in the amino-terminal region of the heavy chain, giving rise to a new amino-terminal sequence of Ile-Val-Gly-Gly- in the active enzyme. Furthermore, the same bond is cleaved whether the activation reaction is catalyzed by factor IX_a and factor VIII or tissue factor and factor VII or trypsin or the protease from Russell's viper venom.

Effect of Carboxypeptidase A and B on the Factor X Activation Peptide. In order to further characterize the peptide bond split during the activation reaction, the factor X activation peptide (mol wt 11,000) was treated with carboxypeptidase A or carboxypeptidase B to identify the carboxyl-terminal amino acid. Amino acids released by these enzymes were then analyzed by the amino acid analyzer. Arginine (0.52 mol/mol of activation peptide), valine (0.96 mol), and glutamine and/or serine and/or asparagine (0.29 mol) were found after incubation with carboxypeptidase B for 60 min at 37°. Only traces of amino acids were released by carboxypeptidase A treatment under essentially identical conditions. Since carboxypeptidase B is specific for carboxyl-terminal basic amino acids, the liberation of valine in the first experiment appears to be due to trace contamination by carboxypeptidase A. These results indicate that the activation peptide contains a carboxyl-terminal arginine residue which is probably adjacent to two valine residues. Since the heavy chain of factor X_a formed by the various activation reactions has an Ile-Val-Gly-Gly- sequence at the amino-terminal end, it can be concluded that a specific arginyl-isoleucine peptide bond in the heavy chain of factor X is split during the activation reaction by factor IX_a and factor VIII or tissue factor and factor VII or trypsin or the protease from Russell's viper venom.

Discussion

The present experiments indicate that the activation of bovine factor X by factor IX_a and factor VIII or tissue factor and factor VII or trypsin or the protease from Russell's viper venom is due to the cleavage of a specific arginyl-isoleucine bond which is located in the amino-terminal region of the heavy chain. This reaction is illustrated in Figure 8 which shows the cleavage of this peptide bond leading to the formation of factor $X_{a\alpha}$. In this reaction, the molecular weight of the precursor molecule is reduced from 55,000 to 44,000 with the liberation of an activation peptide of molecular weight 11,000. No change has been detected in the light chain of factor X during the activation reaction by these four different catalysts. These results indicate that factor X activated by either the intrinsic or extrinsic pathway in blood coagulation is converted to an active enzyme by the same mechanism, and the formation of the active enzyme from the two-chain precursor molecule involves the cleavage of only a single peptide bond. This mechanism is consistent with the well-established specificity of trypsin which hydrolyzes peptide bonds involving arginine or lysine residues (Walsh, 1970). These results also indicate that factor IX_a and factor VIII, tissue factor and factor VII, as well as the protease from Russell's viper venom possess endopeptidase activity directed toward basic amino acids. The protease from Russell's viper venom was originally reported to contain esterase activity toward basic amino acids (Williams and Esnouf, 1962). More recent experiments, however, have demonstrated the separation of the arginine esterase activity from the coagulant activity (Jackson *et al.*, 1971). The present results indicate, however, that this protease is capable of hydrolyzing arginine-containing peptide bonds present in intact proteins such as factor X.

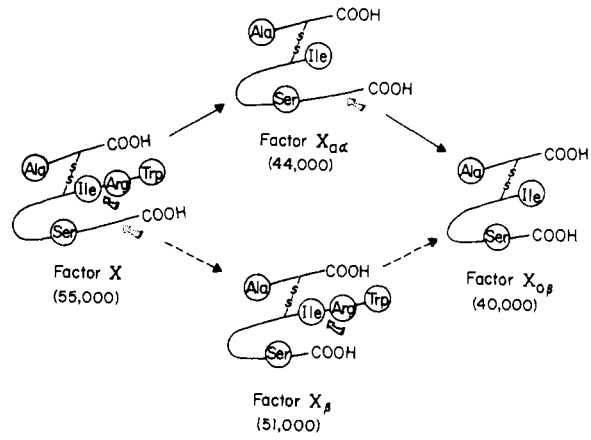

FIGURE 8: The mechanism of activation for bovine factor X.

The carboxyl-terminal arginine in the activation peptide was identified by treatment of the glycopeptide with carboxypeptidase B. This result is consistent with the preliminary data on the carboxyl-terminal sequence of the activation peptide isolated from bovine factor X_1. This has been shown to be -Ser-Gln-Val-Val-Arg by Dr. K. Titani.[2]

In a second slower step, degradation of factor $X_{a\alpha}$ occurs by the splitting of a small glycopeptide fragment(s) (mol wt 4000) from the carboxyl-terminal end of the heavy chain (Figure 8). This gives rise to factor $X_{a\beta}$, a protein of molecular weight 40,000. This protein is free of carbohydrate. The rate of this reaction was found to be very slow in the presence of low concentrations of the protease from Russell's viper venom. Thus, the major product formed by this activation mechanism is factor $X_{a\alpha}$. In the presence of factor IX_a and factor VIII or tissue factor and factor VII or

[2] K. Titani, personal communication.

trypsin, factor $X_{a\alpha}$ is converted more readily to factor $X_{a\beta}$. Consequently, the major product isolated from these activation reactions is factor $X_{a\beta}$. The glycopeptide(s) which is split from the carboxyl-terminal end of the heavy chain during the second reaction has not been identified. The peptide bond(s) which is split in this reaction, however, may also involve an arginine residue since this amino acid has been identified at position 16 (or 17) and position 23 (or 24) from the carboxyl-terminal end of the heavy chain.[2] Furthermore, these peptide bonds containing arginine have been shown to be very susceptible to hydrolysis by trypsin.[2] Cleavage of the peptide bond containing arginine at position 23 (or 24) would liberate a peptide fragment with a molecular weight of about 2400. The heavy chain of factor $X_{a\alpha}$ contains 3.4% carbohydrate which is also liberated during the conversion of factor $X_{a\alpha}$ to factor $X_{a\beta}$. This is equivalent to about 900 g of carbohydrate/mol of heavy chain (Table II). Thus, a glycopeptide fragment split from the carboxyl-terminal region of the heavy chain at position 23 (or 24) would reduce the molecular weight of factor $X_{a\alpha}$ by approximately 3300. This value would be in good agreement with the difference in molecular weight between the heavy chains of factor $X_{a\alpha}$ and factor $X_{a\beta}$ as determined by sedimentation equilibrium (Table I).

In a much slower reaction, factor X is converted to factor X_β by factor IX_a and factor VIII or tissue factor and factor VII or trypsin by the initial cleavage of a glycopeptide(s) from the carboxyl-terminal region of the heavy chain (Figure 8). This partially degraded factor X has no enzymatic activity. It is, however, converted to factor $X_{a\beta}$ by all four catalysts by cleavage of the same specific arginyl-isoleucine bond in the amino-terminal region of the heavy chain. This reaction results in the formation of the same Ile-Val-Gly Gly- sequence in the heavy chain of the activated enzyme.

Factor $X_{a\beta}$ appears to be identical with factor X_a isolated and characterized by Radcliffe and Barton (1972, 1973). This is indicated by the data in Table II which show the amino acid and carbohydrate composition of factor $X_{a\beta}$ and similar data obtained by Radcliffe and Barton for their factor X_a. There is good agreement in the amino acid composition between these two different preparations of factor X_a. Furthermore, factor $X_{a\beta}$ is essentially free of carbohydrate as noted previously for the factor X_a prepared by Radcliffe and Barton.

Bajaj and Mann (1973) have studied the activation of factor X by insolubilized trypsin. They concluded that factor X_a has a molecular weight of 52,000 and was formed by the cleavage of a peptide of molecular weight 4000 from the heavy chain. Our data suggest that they had identified factor X_β and not factor X_a. They also reported that the maximum yield of factor X_a is eight times higher with trypsin than with crude Russell's viper venom. In experiments employing highly purified protease from Russell's viper venom, the yield of factor X_a is essentially the same by both catalysts (panel C, Figure 1). Similar results have also been published previously by Papahadjopoulos *et al.* (1964).

Jesty and Nemerson (1974) have studied factor X activation by highly purified preparations of tissue factor and factor VII. They observed essentially the same sodium dodecyl sulfate gel electrophoresis pattern for factor X activation as that shown in panel B, Figure 2. They concluded from the appearance of several bands that factor X activation involves the cleavage of more than a single peptide bond. From the present experiments, it is clear that factor X can be converted to factor X_β and subsequently activated by conversion to factor $X_{a\beta}$. This pathway involving the cleavage of more than one peptide bond, however, appears to be a minor pathway for the activation of factor X.

Factor X is readily activated by factor IX_a and factor VIII. In the absence of thrombin, however, a typical lag phase was consistently observed prior to the appearance of factor X_a activity. In the presence of thrombin-modified factor VIII, however, the activation of factor X occurs without any detectable lag phase.[3]

Mattock and Esnouf (1973) have suggested that factor X may also exist as a single polypeptide chain in plasma. This proposal is consistent with the concept that factor X is synthesized as a single polypeptide chain which could be split into two chains *in vivo* or during the isolation procedure (Fujikawa *et al.*, 1974a). Starting with a single chain, it appears probable that the activation of factor X will require two steps: the first being the cleavage of the single chain into a two-chain structure, followed by an activation step resulting in the cleavage of the activation peptide from the amino-terminal region of the heavy chain. This mechanism would be essentially identical with that recently observed for the activation of bovine factor IX. This coagulation factor is readily isolated from bovine plasma as a single chain glycoprotein and it is activated by factor XI_a in a two-step reaction (Fujikawa *et al.*, 1974b).

With factor X_a and factor IX_a, the part of the precursor molecule which becomes the light chain remains attached to the heavy chain in the activated enzyme. In the activation of bovine prothrombin, however, the amino-terminal portion of the precursor molecule is cleaved from the protein (Heldebrant and Mann, 1973; Esmon *et al.*, 1974). In this case, the light chain or A chain of thrombin is derived from an internal portion of prothrombin. In all three enzymes, however, the active site or reactive site is located in the heavy chain of the molecule.

Acknowledgments

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