Bovine Factor X_1 (Stuart Factor). Mechanism of Activation by a Protein from Russell's Viper Venom†

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ABSTRACT: Bovine factor X_1 (Stuart factor) is a plasma glycoprotein (mol wt 55,000) composed of a heavy chain (mol wt 38,000) and a light chain (mol wt 17,000). During its conversion to activated factor X_1 by a protein present in Russell's viper venom, a specific peptide bond is cleaved resulting in the release of a glycopeptide (mol wt 11,000) from the amino-terminal end of the heavy chain. Liberation of the glycopeptide or activation peptide decreases the molecular weight of the precursor molecule from 55,000 to 44,000 and results in the formation of a new amino terminal sequence of Ile-Val-Gly-Gly- in the heavy chain of factor X_{1a}. No change in the amino-terminal end of the light chain in factor X_1 was observed during the activation reaction. Once factor X_1 is activated, it is inhibited by relatively high concentrations of diisopropyl phosphofluoridate. With [^{32}P]diisopropyl phosphofluoridate, it was shown that 0.78 mol of [^{32}P]diisopropyl phosphate was incorporated into the heavy chain per mol of factor X_{1a}. Factor X_{1a} is also inhibited by phenylmethylsulfonyl fluoride, but other inhibitors such as *N*-tosyl-L-phenylalanylchloromethane and *N*-tosyl-L-lysylchloromethane have no effect on the activity of the enzyme. These results suggest that bovine factor X_1 is converted to a serine protease by hydrolysis of a specific peptide bond in the precursor molecule resulting in the liberation of an activation peptide.

A number of different physiological and nonphysiological mechanisms have been described for the activation of factor X (Stuart factor),[1] a protein which participates in the middle phases of blood coagulation (Davie *et al.*, 1969). The physiological mechanism involves two different pathways called the extrinsic and intrinsic systems. In the extrinsic system, factor X is activated in the presence of tissue factor and factor VII (Flynn and Coon, 1953; Hjort, 1957; Hougie, 1959; Straub and Duckert, 1961; Deutsch *et al.*, 1964; Nemerson and Spaet, 1964; Williams, 1964, 1966; Nemerson, 1966; Williams and Norris, 1966; Nemerson and Pitlick, 1970). Highly purified tissue factor preparations have peptidase activity, suggesting that tissue factor participates in blood coagulation by the splitting of peptide bonds in its substrate (Pitlick *et al.*, 1971).

In the intrinsic system, factor X is activated in the presence of factor IX_a (activated Christmas factor), factor VIII (antihemophilic factor), calcium ions, and phospholipid (Bergsagel and Hougie, 1956; Hougie *et al.*, 1957; Spaet and Cintron, 1963; Lundblad and Davie, 1964). Kinetic data suggested that the activation of factor X involved activated factor VIII (Lundblad and Davie, 1965; Biggs and Macfarlane, 1965), but more recent data indicate that factor X is activated by a complex of factors IX_a, VIII, phospholipid, and calcium ions (Hougie *et al.*, 1967; Osterud and Rapaport, 1970).

Several nonphysiological mechanisms for the activation of factor X have also been described. These include a protein present in Russell s viper venom (Macfarlane, 1961; Esnouf and Williams, 1962; Papahadjopoulos *et al.*, 1964), trypsin (Ferguson *et al.*, 1960; Pechet and Alexander, 1960; Papahadjopoulos *et al.*, 1964; Yin, 1964), cathepsin C (Purcell and Barnhart, 1963), and papain (Alexander *et al.*, 1962).

The mechanism of factor X activation by these various pathways has not been described in detail. It is clear, however, from the work of Papahadjopoulos *et al.* (1964) that a reduction in molecular weight and a change in electrophoretic properties of factor X occur during its activation by either Russell's viper venom or trypsin. Once factor X is activated by one of the various mechanisms described above, it participates in the activation of prothrombin in the presence of factor V (proaccelerin), phospholipid, and calcium ions (Papahadjopoulos and Hanahan, 1964; Milstone, 1964; Denson, 1967; Barton *et al.*, 1967; Esnouf and Jobin, 1967; Jobin and Esnouf, 1967; Ferguson *et al.*, 1967).

The present communication describes some of the molecular events associated with the activation of bovine factor X_1 (Fujikawa *et al.*, 1972) by a protein present in Russell's viper venom. The data indicate that the activation reaction involves the splitting of a glycopeptide from the amino-terminal end of the heavy chain in factor X_1 giving rise to factor $X_{1a(RVV)}$, a serine protease.

Materials

Bovine factor X_1 was purified to a homogeneous state, as described in the preceding paper (Fujikawa *et al.*, 1972). The venom enzyme which activates factor X was purified by gel filtration on a Sephadex G-100 column from crude venom of *Vipera russelli* (Ross Allen Reptile Institute, Inc., Silver Springs, Fla.) by the method of Schiffman *et al.* (1969). [^{32}P]-DFP[2] was purchased from Amersham/Searle Co., Des Plaines, Ill. *N*-α-*p*-Tosyl-L-lysylchloromethane and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co., St. Louis, Mo. *N*-Tosyl-L-phenylalanylchloromethane was a product of Cyclo Chemical Co., Los Angeles, Calif. Dithiothreitol (A grade) was purchased from Calbiochem,

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[1] The nomenclature for various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959). Factor X_1 which has been activated by Russell's viper venom has been called factor $X_{1a(RVV)}$.

[2] Abbreviations used are: DFP, diisopropyl phosphofluoridate; DIP, diisopropyl phosphate; RVV, Russell's viper venom.

Los Angeles, Calif. 4-Vinylpyridine was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Sephadex G-50 was a product of Pharmacia Fine Chemicals, Piscataway, N. J. All other chemicals were the same as described in the preceding paper (Fujikawa *et al.*, 1972) or commercial preparations of the highest quality available.

Methods

Polyacrylamide disc gel electrophoresis was performed at pH 9.4 according to the method of Davis (1964), and sodium dodecyl sulfate polyacrylamide electrophoresis was done by the method of Weber and Osborn (1969). Molecular weights were determined by the high-speed technique of Yphantis (1964), as described in the preceding paper (Fujikawa *et al.*, 1972).

For amino acid analyses, samples were prepared by the method of Moore and Stein (1963) and analyzed for amino acids according to the method of 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. The values for serine and threonine were determined by extrapolation to zero-time hydrolysis. Isoleucine and valine values were calculated from the 96-hr hydrolysis. Tryptophan was estimated by the method of Bencze and Schmid (1957), and half-cystine was determined as pyridylethylcysteine by the method of Friedman *et al.* (1970).

Neutral sugar was determined by the phenol–sulfuric acid method of Dubois *et al.* (1956), amino sugar by the method of Elson and Morgan as described by Gardell (1957), and neuraminic acid by the thiobarbituric acid method of Warren (1959), as described in the preceding paper (Fujikawa *et al.*, 1972).

Reduction and pyridylethylation of factor X_1 or factor $X_{1a(RVV)}$ were performed by a minor modification of the method of Friedman *et al.* (1970). Reduction and carboxymethylation of factor $X_{1a(RVV)}$ were done by the method of Crestfield *et al.* (1963).

Amino-terminal sequence was determined by automated Edman degradations using a Beckman Sequencer Model 890A. The mode of operation of the instrument and the method of sequenator analysis are adaptions (Hermodson *et al.*, 1972) of the technique of Edman and Begg (1967).

For the isolation of the activation peptide at neutral pH, a factor X_1 activation mixture was reduced and carboxymethylated followed by gel filtration on Sephadex G-25 to remove salt. The sample was then lyophilized and dissolved in 5 ml of 0.025 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl. The activation peptide and light chain of factor $X_{1a(RVV)}$ were soluble in this buffer. Only part of the heavy chain, however, was soluble under these conditions. The activation peptide was separated from the light chain and the heavy chain by gel filtration on a column (2.5 × 90 cm) of Sephadex G-100 using 0.025 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl. This preparation of activation peptide was employed only for amino-terminal analysis.

For the assay of factor $X_{1a(RVV)}$ activity, 10 μl of the activation mixture was added to 1.0 ml of Michaelis buffer containing 1×10^{-2} M EDTA. The sample was further diluted with Michaelis buffer to a final dilution of 25,000- to 100,000-fold depending upon the degree of activation. An aliquot (0.1 ml) of the final dilution was incubated at 37° for 30 sec with 0.1 ml of phospholipid solution and 0.1 ml of factor X deficient plasma. A 0.1-ml solution of 0.025 M $CaCl_2$ was then added to the incubation mixture and the clotting time was determined. The per cent activation was determined from a calibration curve prepared from the fully activated sample. By this method, 0.5 ng of factor $X_{1a(RVV)}$ gives a clotting time of 25–30 sec.

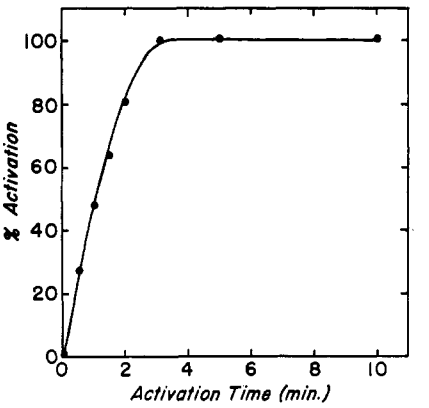

FIGURE 1: Time curve for the activation of factor X_1 with a protein from Russell's viper venom. The activation mixture contained 0.3 ml of factor X_1 (0.312 mg of protein in 0.025 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl), 0.3 ml of 0.025 M $CaCl_2$, and 0.3 ml of water. The reaction was initiated by the addition of 3 μl (3.75 μg) of the purified Russell's viper venom protein. Incubation was carried out at 37° and 10-μl aliquots of the reaction mixture were removed at various time intervals, and factor X_{1a} activity was then assayed as described in Methods.

Results

Activation of Bovine Factor X_1. A time curve for the activation of factor X_1 by a protein in Russell's viper venom is shown in Figure 1. With an enzyme-to-substrate ratio of 1:100 (weight basis), the activation reaction goes to completion in about 5 min. The factor $X_{1a(RVV)}$ which is formed is extremely active in initiating blood coagulation since 1–2 ng/ml of the enzyme in the final assay reaction mixture will form a clot in about 30 sec.

During the activation reaction, there is a molecular change in factor X_1, as shown by polyacrylamide gel electrophoresis experiments (Figure 2). These experiments were carried out under conditions essentially the same as those described in Figure 1. At zero time, a single protein band for factor X_1 is observed. During the first minute of activation, a new major fast-moving component, factor $X_{1a(RVV)}$, is observed with a corresponding decrease in the concentration of the original factor X_1. After an incubation of 3–4 min, the activation reaction is nearly complete and, at this time, all of the original factor X_1 has disappeared. A minor second fast-moving band also appears and this protein migrates slightly ahead of the major new band. Both of the fast-moving bands have factor X_a activity. This was shown by splitting the gels in half longitudinally and staining one of the gels for protein and assaying the corresponding protein bands in the other half of the gel for factor X_a activity. The relative amounts of the two forms of factor X_a varied from one preparation of factor X_1 to another, but, generally, the minor component was less than 20% of the major component. Whether the fast-moving minor component was formed from the major component has not been investigated. These experiments indicate that some modification in factor X_1 has occurred during the activation reaction by Russell's viper venom, and this modification has given rise to a major and a minor fast-moving protein, each of which possesses factor X_a activity.

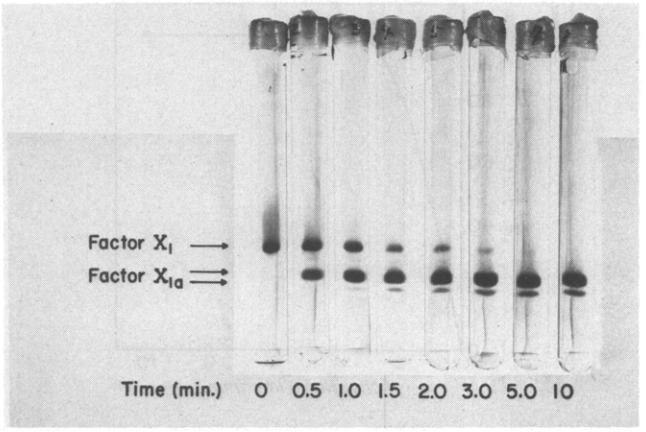

FIGURE 2: Polyacrylamide disc gel electrophoresis pattern of samples removed from the incubation mixture containing factor X_1 and Russell's viper venom. The activation of factor X_1 was carried out essentially as described in Figure 1. Aliquots (50 μl) of the reaction mixture containing 15 μg of protein were removed at various times, and the reaction was terminated by the addition of 10 μl of 0.1 M EDTA. The samples were then applied to the gel columns and electrophoresis was carried out at pH 9.4 as described in Methods. The protein was stained with Coomassie Brilliant Blue for 4 hr prior to destaining.

In previous experiments, we have shown that factor X_1 is composed of a heavy and a light chain (Fujikawa *et al.*, 1972). Following reduction of the protein with 2-mercaptoethanol, the heavy and light chains are readily separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Similar experiments which were carried out on factor X_1 before and after activation with the protein from Russell's viper venom are

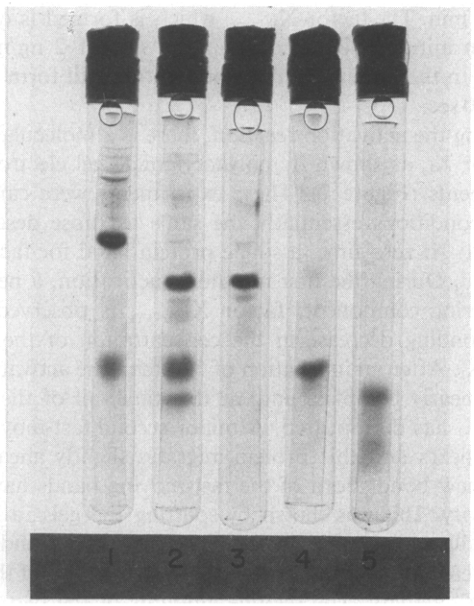

FIGURE 3: Sodium dodecyl sulfate polyacrylamide disc gel electrophoresis of reduced factor X_1 before and after activation with Russell's viper venom and various isolated components of the reaction mixture. Sodium dodecyl sulfate gel electrophoresis was carried out in 8.5% gels, as described in Methods. Gel 1 is 10 μg of factor X_1, and gel 2 is 10 μg of the activation mixture containing factor $X_{1a(RVV)}$. The proteins shown in gels 3, 4, and 5 were isolated by gel filtration as shown in Figure 4. Gel 3 is 6 μg of the heavy chain of factor $X_{1a(RVV)}$, gel 4 is 75 μg of the light chain of factor $X_{1a(RVV)}$, and gel 5 is 20 μg of the activation peptide.

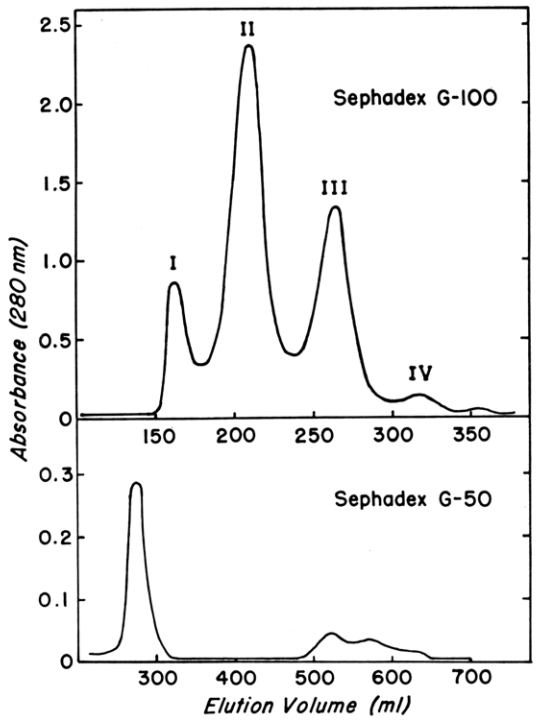

FIGURE 4: Gel filtration patterns of the reaction mixture from factor X_1 and Russell's viper venom. The top figure is the gel filtration pattern on a Sephadex G-100 column of the acid-precipitable protein, and the lower figure is the gel filtration pattern on a Sephadex G-50 column of the acid-soluble protein. The activation mixture contained 94.4 mg of factor X_1, 0.94 mg of purified Russell's viper venom protein, 4.0 ml of 0.1 M $CaCl_2$, and 16.0 ml of 0.1 M Tris-HCl buffer (pH 8.0), in a final volume of 80 ml. The incubation was carried out at 37° for 5 min, and the reaction was stopped by the addition of cold trichloroacetic acid (final concentration of 10%). The precipitate and supernatant were then separated by centrifugation. The precipitated protein was reduced and pyridylethylated as described in Methods and applied to a Sephadex G-100 column (2.0 × 70 cm) which was previously equilibrated with 9% formic acid in 3 M urea. The protein was eluted with the same formic acid–urea buffer. The supernatant was extracted four times with ether to remove trichloroacetic acid and then lyophilized. It was then dissolved in 5 ml of 9% formic acid and applied to a Sephadex G-50 column (2.0 × 100 cm) and eluted with 9% formic acid.

shown in Figure 3. Gel number one corresponds to a zero-time sample of the activation mixture which was reduced with 2-mercaptoethanol. The usual heavy and light chains of factor X_1 are obtained in which the light chain or fast-moving band is clearly separated from the slow-moving heavy chain. The second gel is a protein sample taken from the 5-min activation reaction and reduced with 2-mercaptoethanol. Three distinct protein bands are obtained. The slow-moving band originates from the heavy chain of factor X_1. It has been reduced in size, however, during the activation reaction resulting in the formation of a new faster moving band. Little or no change has occurred in the second band which corresponds to the original light chain of factor X_1. A third very fast-moving band is also obtained which moves ahead of the light chain. These experiments, shown in gels one and two, indicate that the reduction in molecular weight which has occurred in factor X_1 during its activation by Russell's viper venom is due to the hydrolysis of a polypeptide fragment from the heavy chain of the precursor protein.

To further characterize the activation reaction, an aliquot of the 5-min activation mixture was treated with cold trichloroacetic acid and centrifuged, and the precipitate was sep-

TABLE I: Molecular Weight of Factor X_{1a} Heavy Chain and Factor X_1 Activation Peptide by Sedimentation Equilibrium.

Mol Wt	$X_{1a(H)}$	$X_{1(AP)}$	$X_{1(H+AP)}$	$X_{1(H)}$
M_1[a]	27,000 ± 1250	10,800 ± 400[b]	37,800	38,000[c]
M_n	30,200 ± 1400	10,830 ± 520		
M_w	33,700 ± 1400	10,730 ± 540		
M_z	40,500 ± 1100	11,600 ± 650		

[a] M_1 refers to the smallest molecular weight species calculated by the methods described by Teller *et al.* (1969). The M_n, M_w, and M_z values shown above are molecular weight averages calculated throughout the whole cell in experiments employing three different protein concentrations in 6 M guanidine hydrochloride, as described in Methods. [b] Calculated on the basis of 25% carbohydrate (Table III). [c] Fujikawa *et al.* (1972).

arated from the supernatant fraction. The precipitated protein was then reduced, alkylated with 4-vinylpyridine, and subjected to gel filtration on Sephadex G-100 (top diagram, Figure 4). Three major and one minor ultraviolet absorbing peaks were obtained. The first peak contained little or no protein and was discarded. The second peak showed one protein band on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and this band corresponds to the heavy chain in factor $X_{1a(RVV)}$ (gel 3, Figure 3). The third peak also yields one band on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and this band corresponds to the light chain in factor $X_{1a(RVV)}$ (gel 4, Figure 3). The fourth peak corresponds to the fast-moving protein band seen in gel 2 (Figure 3). The amount of protein in this peak was small, however, and in some experiments, this minor peak was absent.

The supernatant was also examined for polypeptide material. In these experiments, the trichloroacetic acid was removed by ether extraction, and the remaining solution was lyophilized and subjected to gel filtration on Sephadex G-50 (bottom diagram, Figure 4). One major ultraviolet-absorbing, ninhydrin-positive peak was found. This peak is a small protein or polypeptide which is not readily detected by polyacrylamide gel electrophoresis since it stains poorly with Coomassie Blue or Aniline Blue Black. When the gels are stained for 24 hr, however, a major protein band running ahead of the light chain was observed (gel 5, Figure 3). This protein band corresponds to the fast-moving band seen in gel 2 (Figure 3) and was called the activation peptide. With some preparations, no fast-moving contaminants were observed while other preparations contained variable amounts of the fast-moving contaminants. It appears probable that some degradation of the activation peptide occurs during the acid precipitation step, which may involve the loss of some of the carbohydrate, as discussed below.

Table I shows the molecular weight data for the activation peptide and the heavy chain from factor $X_{1a(RVV)}$, as determined by sedimentation equilibrium. These proteins were isolated by gel filtration and dissolved in 6 M guanidine hydrochloride. The heavy chain was extensively dialyzed against guanidine hydrochloride prior to the sedimentation equilibrium studies, and its minimal molecular weight was found to be 27,000. Considerable aggregation of the heavy chain was occurring, however, during the sedimentation studies. The molecular weight of the activation peptide was found to be 10,800, and no evidence of heterogeneity was observed for this protein. A summation of equal amounts of the heavy chain of factor $X_{1a(RVV)}$ and the activation peptide is 37,800, and this value is in good agreement with the 38,000 molecular weight which was determined for the heavy chain of the precursor protein.

TABLE II: Amino Acid and Carbohydrate Compositions of the Heavy Chain of Factor $X_{1a(RVV)}$ and the Activation Peptide.[a]

Components	Heavy Chain (Mol/27,000)	Activation Peptide (Mol/10,800)	Heavy Chain + Act. Peptide (Mol/37,800)	Heavy Chain for Factor X_1[b] (Mol/38,000)
Amino acid				
Lysine	13.7	0.4	14.1	15.0
Histidine	5.9	2.0	7.9	7.0
Arginine	14.4	4.2	18.6	17.6
Aspartic acid	17.6	10.0	27.6	27.4
Threonine	19.3	3.6	22.9	21.9
Serine	12.2	7.1	19.3	18.6
Glutamic acid	24.9	9.4	34.3	36.3
Proline	11.9	4.5	16.4	17.1
Glycine	21.7	6.4	28.1	27.7
Alanine	17.7	6.3	24.0	22.8
Half-cystine[c]	8.4	0.6	9.0	9.2
Valine	19.9	5.1	25.0	23.3
Methionine	5.3	0	5.3	4.6
Isoleucine	8.1	2.4	10.5	9.9
Leucine	15.1	7.2	22.3	22.8
Tyrosine	6.2	2.5	8.7	6.2
Phenylalanine	12.5	0.7	13.2	10.4
Tryptophan[d]	5.2	2.0	7.2	7.2
Carbohydrate				
Hexose	2.9 (1.7%)	5.5 (8.3%)	8.4	8.1
Hexosamine[e]	1.7 (1.3%)	4.0 (7.6%)	5.7	6.3
Neuraminic acid[f]	0.4 (0.4%)	3.2 (8.9%)	3.6	5.3
Protein (%)	96.6	75.2		
Carbohydrate (%)	3.4	24.8		

[a] The heavy chain and activation peptide were isolated by acid precipitation of the reaction mixture followed by gel filtration, as described in Figure 4. [b] The amino acid composition for the heavy chain of the precursor (factor X_1) is taken from Fujikawa *et al.* (1972). [c] Determined as pyridylethylcysteine by the method of Friedman *et al.* (1970). [d] Determined by the spectrophotometric assay. [e] Hexosamine was calculated as residues of *N*-acetylhexosamine. [f] Neuraminic acid was calculated as residues of *N*-acetylneuraminic acid.

The amino acid and carbohydrate compositions for the heavy chain of factor $X_{1a(RVV)}$ and the activation peptide are shown in Table II. A summation of the amino acids for equimolar amounts of the two proteins is also included. These values are in reasonably good agreement with the amino acid composition of the heavy chain from the precursor protein (Fujikawa *et al.*, 1972). Some disagreement is evident, however, in the case of phenylalanine and tyrosine, and the

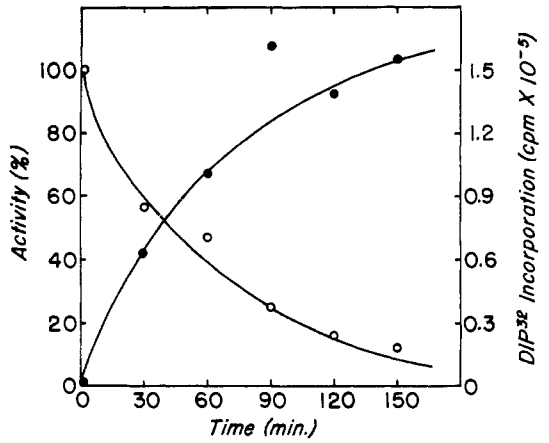

FIGURE 5: Inactivation of factor $X_{1a(RVV)}$ by DFP and the incorporation of [^{32}P]DIP from [^{32}P]DFP into the enzyme. The activation of factor X_1 was carried out for 5 min under the conditions described in Figure 4, and 8 ml of 0.1 M EDTA was then added to terminate the reaction. Factor $X_{1a(RVV)}$ was then incubated with 5×10^{-3} M DFP (465 μmol of [^{32}P]DFP; total counts 7.8×10^8 cpm), and 10-μl aliquots of the reaction mixture were removed at various intervals and added to 1.0 ml of Michaelis buffer. Factor $X_{1a(RVV)}$ activity was assayed as described in Methods from one portion of the sample (10 μl), and another portion (50 μl) was placed on Whatman No. 3MM filter paper (2.2 cm), washed in cold 5% trichloroacetic acid four times with stirring. The radioactivity bound to the filter was then determined in a Packard Tri-Carb liquid scintillation counter after the addition of 10 ml of Omnifluor.

reasons for these discrepancies are not known. It is also clear from these experiments that the activation peptide is a glycopolypeptide containing approximately 25% carbohydrate. The heavy chain of factor $X_{1a(RVV)}$ contains only 3.4% carbohydrate. These values are a little low, however, since the recovery of the carbohydrate found in the activation peptide plus the heavy chain of factor $X_{1a(RVV)}$ is approximately 82% of that present in the heavy chain of factor X_1. Thus, some loss of carbohydrate, which was primarily neuraminic acid, has occurred during the isolation of the activation peptide and the heavy chain of factor $X_{1a(RVV)}$ in the presence of trichloroacetic acid. It is clear, however, that a major portion of the carbohydrate present originally in factor X_1 is split from the precursor molecule during the activation reaction by the protein from Russell's viper venom.

Amino-Terminal Analysis. Amino-terminal sequences of the activation peptide and the heavy chain and light chain of factor $X_{1a(RVV)}$ were then carried out on a Beckman Sequencer. In these experiments, the activation peptide was isolated at neutral pH from the activation reaction, as described in Methods. It was found to contain the amino-terminal sequence of Trp-Ala-Ile-His-. This is the same sequence as that found in the heavy chain of the precursor molecule (Fujikawa *et al.*, 1972). The amino-terminal sequence of Trp-Ala-Ile-His- was observed only in preparations of the activation peptide which were isolated at neutral pH. With preparations isolated in 10% trichloroacetic acid, no amino-terminal residue was identified by the method of Edman and Begg (1967). Furthermore, no amino acids were identified in these preparations after turns 2, 3, and 4 in the sequenator.

The results for the amino-terminal analysis of the heavy chain of factor $X_{1a(RVV)}$ are shown in Table III. In these experiments, an amino-terminal sequence of Ile-Val-Gly-Gly- was found for the heavy chain. These data indicate that the activation of factor X_1 by the protein in Russell's viper venom results in the formation of a new amino-terminal isoleucine residue in the heavy chain of factor $X_{1a(RVV)}$. These results also show that the activation peptide is split from the amino-terminal end rather than the carboxyl-terminal end of the heavy chain of factor X_1.

TABLE III: Amino-Terminal Sequence of the Heavy Chain of Factor $X_{1a(RVV)}$ and Several Other Plasma and Pancreatic Proteolytic Enzymes.[a]

Enzyme	Amino-Terminal Sequence			
Bovine factor X_{1a}[b]	*Ile*	*Val*	*Gly*	*Gly*
Bovine thrombin[c]	*Ile*	*Val*	Glu	*Gly*
Bovine plasmin[d]	*Ile*	*Val*	*Gly*	*Gly*
Human plasmin[e]	Val	*Val*	*Gly*	*Gly*
Bovine trypsin[f]	*Ile*	*Val*	*Gly*	*Gly*
Bovine chymotrypsin A[g]	*Ile*	*Val*	Asn	*Gly*

[a] The underscored amino acids are those which are found in the same position for more than one protein. [b] Heavy chain of factor $X_{1a(RVV)}$. [c] B chain of thrombin (Magnusson, 1971). [d] Light chain of bovine plasmin (Nagasawa and Suzuki, 1970). [e] B chain of human plasmin (Robbins *et al.*, 1972). [f] Walsh and Neurath (1964). [g] B chain of α-chymotrypsin (Hartley, 1964).

The light chain of factor $X_{1a(RVV)}$ was shown to contain the amino terminal sequence of Ala-Asn-Ser-Phe-. This sequence is the same as that found in the light chain of the precursor molecule (Fujikawa *et al.*, 1972). These data provide further evidence that little or no change has occurred in the light chain of factor X_1 during the activation reaction by the protein present in Russell's viper venom.

Inhibition of Factor $X_{1a(RVV)}$. Jackson and Hanahan (1968) reported that factor X_a is inhibited by 0.01 M DFP, resulting in the loss of both clotting and esterase activity of the enzyme. Similar results with high concentrations of DFP were reported by Leveson and Esnouf (1968) who also noted the binding of the inhibitor to a serine residue in the enzyme. Lower concentrations of DFP have little or no effect on factor X_a (Zucker-Franklin and Spaet, 1963; Lundblad and Davie, 1965; Marciniak and Seegers, 1966; Aronson and Ménaché, 1968). The inhibition of factor $X_{1a(RVV)}$ by high concentrations of DFP was also observed in the present experiments. In these studies, factor $X_{1a(RVV)}$ was inhibited approximately 90% by 5.0×10^{-3} M DFP after incubation for 1 hr at 37°, and the inhibition was greater than 95% after 2 hr. In these experiments, carried out in 0.04 M Tris-HCl buffer (pH 8.0), the molar ratio of enzyme to inhibitor was 1:250. Under essentially the same conditions, 2.5×10^{-3} M phenylmethylsulfonyl fluoride inhibited factor $X_{1a(RVV)}$ 52% after 1 hr, and this inhibition increased to about 63% after 2 hr. *N*-Tosyl-L-phenylalanylchloromethane and *N*-tosyl-L-lysylchloromethane at 2.5×10^{-3} M had little or no effect on factor $X_{1a(RVV)}$ after incubation for 2 hr at 37°.

The inhibition of factor $X_{1a(RVV)}$ by DFP involves the covalent binding of the diisopropylphosphoryl group to the heavy chain of the enzyme. This was shown by employing [^{32}P]DFP which becomes covalently bound to the enzyme, and this binding occurs in parallel with the loss of biological activity of the enzyme (Figure 5). At maximal inhibition of factor $X_{1a(RVV)}$, 0.78 mol of ^{32}P was incorporated per mol of factor $X_{1a(RVV)}$ employing an enzyme-to-inhibitor ratio of

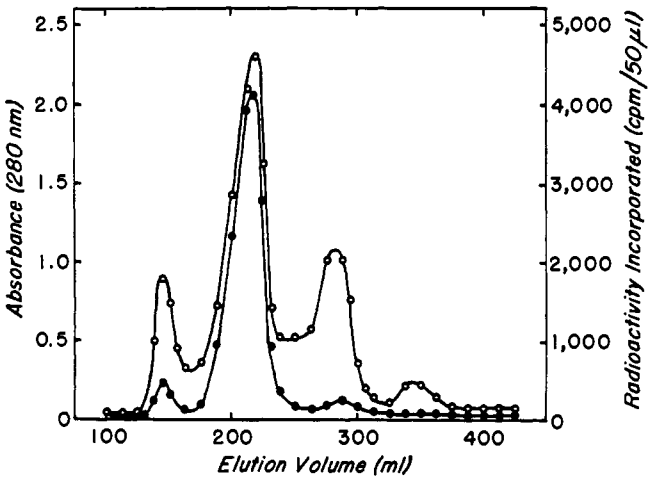

FIGURE 6: Gel filtration of reduced [^{32}P]diisopropylphosphoryl factor $X_{1a(RVV)}$ on a Sephadex G-100 column. Factor X_1 was activated as described in Figure 4 except that 86.7 mg of factor X_1 was employed. Factor $X_{1a(RVV)}$ was labeled with 907 µmol of [^{32}P]DFP (total counts 2.5×10^8 cpm) as described in Figure 5. The protein precipitated by trichloroacetic acid was reduced and alkylated as described in Methods and applied to a Sephadex G-100 column (2.0×90 cm) which was previously equilibrated with 9% formic acid in 3 M urea. Elution was carried out with a formic acid–urea solution, and samples were assayed for protein and radioactivity. (●) Radioactivity; (○) absorbance.

1:264. The incorporation of ^{32}P occurs in the heavy chain of factor $X_{1a(RVV)}$, and this was shown by reducing the ^{32}P-labeled factor $X_{1a(RVV)}$ with 0.1 M dithiothreitol followed by pyridylethylation and gel filtration on Sephadex G-100 (Figure 6). Four major peaks were observed. The first peak contained little or no protein, as previously observed (Figure 3). The second peak is the heavy chain of factor $X_{1a(RVV)}$, and it contains about 95% of the total radioactivity. These experiments indicate that the binding site for the diisopropylphosphoryl group in factor $X_{1a(RVV)}$ is present in the heavy chain of the enzyme.

Discussion

The present experiments suggest that the hydrolysis of a specific peptide bond in the heavy chain of factor X_1 by a protein present in Russell's viper venom is responsible for the conversion of this inactive plasma protein to a serine esterase. Final proof of the conclusion, however, will require experiments which directly correlate the release of the activation peptide with the appearance of factor $X_{1a(RVV)}$ activity. Figure 7 summarizes our present information regarding the structure of bovine factor X_1 and the location of the peptide bond split during the activation reaction by Russell's viper venom. At the present time, there is no evidence that the hydrolysis of other peptide bonds in factor X_1 is required for the activation reaction. Hydrolysis of other peptide bonds in factor X_1 or $X_{1a(RVV)}$ does occur, however, giving rise to the formation of minor amounts of another factor $X_{1a(RVV)}$ molecule. This was apparent in experiments in which a minor fast-moving protein was observed by disc gel electrophoresis, and this protein also contained factor X_{1a} activity. Preliminary data indicate that the minor fast-moving protein results from an additional peptide of approximately 3000 molecular weight being hydrolyzed from the carboxyl-terminal end of the heavy chain.[3]

[3] K. Fujikawa and E. W. Davie, unpublished results.

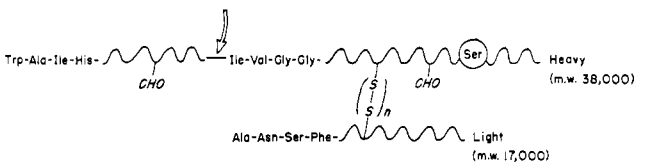

FIGURE 7: Structure of factor X_1 showing the peptide bond split during activation by a protein from Russell's viper venom.

The activation of factor X by trypsin also involves the removal of the same activation peptide split by Russell's viper venom. This results in the formation of the same Ile-Val-Gly-Gly- amino-terminal sequence in the heavy chain. With trypsin, however, the hydrolysis of a small fragment with an approximate molecular weight of 3000 from the carboxyl-terminal end of the heavy chain occurs faster than the hydrolysis of the activation peptide. Thus, factor X_{1a} formed by trypsin activation is smaller in molecular weight than factor $X_{1a(RVV)}$.[3] It appears probable that the activation of factor X in the intrinsic and extrinsic pathways will also lead to the formation of the Ile-Val-Gly-Gly- sequence in the heavy chain of factor X_a. This suggests that the complex of factors VIII and IX_a as well as tissue factor and factor VII participate in blood coagulation as proteolytic enzymes.

Thus far, the carboxyl-terminal residue in the activation peptide has not been established. Preliminary evidence suggests that it is arginine, and this is consistent with the known specificity of trypsin. Additional experiments will be required, however, to confirm these findings.

The present studies were carried out primarily with factor X_1. Preliminary experiments with factor X_2, however, indicate that the mechanism of activation for this protein by the protein present in Russell's viper venom is essentially the same as that for factor X_1. This was indicated by experiments carried out with factor X_2 which were similar to those shown in Figures 2 and 3. After activation of factor X_2, a new fast-moving band was observed by polyacrylamide disc gel electrophoresis. After reduction of an aliquot of the reaction mixture with 2-mercaptoethanol, three bands were obtained which were essentially identical to those shown in gel 2 (Figure 3). Thus, it was concluded that the mechanism of activation of factor X_2 by Russell's viper venom is probably identical with the mechanism of activation of factor X_1.

The activation mechanism for factor X_1 is very similar to the activation reaction for other proteolytic enzymes involving the formation of a new amino-terminal polypeptide chain with similar amino acids (Table III). The same sequence of Ile-Val-Gly-Gly- occurs in the amino-terminal end of bovine trypsin following the activation of trypsinogen by trypsin (Walsh and Neurath, 1964). In this case, the hydrolysis of an activation peptide also occurs from the amino-terminal end of the precursor protein (Davie and Neurath, 1955). These same four amino-terminal acids are also found in the amino-terminal end of bovine thrombin, bovine chymotrypsin, and bovine plasmin. Thus, the mechanism of activation and the homology of factor X_1 with various other serine esterases are quite apparent. The homology of these enzymes is dealt with in considerable detail in the following manuscript (Titani *et al.*, 1972).

The factor X_1 activation peptide was found to contain an amino-terminal tryptophan. This residue was found only in the activation peptide preparation isolated at neutral pH. Preparations isolated under acidic conditions contained no detectable N-terminal amino acid, and it appears probable that *N*-formylkynurenine derivatives were formed under these

conditions which interfered with the amino-terminal analysis (Spande *et al.*, 1970). The activation peptide also contains approximately 25% carbohydrate which represents a major portion of the carbohydrate originally present in the protein. The removal of a large carbohydrate component containing neuraminic acid from the precursor protein may be of importance *in vivo* since most plasma proteins lacking neuraminic acid are readily cleared by the liver (Morell *et al.*, 1971). Thus, factor X_a when formed *in vivo* is probably neutralized by plasma inhibitors shortly after its activation and participation in blood coagulation (Yin *et al.*, 1971), and this protein with a reduced carbohydrate level probably is cleared rapidly by the liver. Further experiments are required, however, to test this hypothesis.

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Bovine Factor X_{1a} (Activated Stuart Factor). Evidence of Homology with Mammalian Serine Proteases†

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ABSTRACT: Amino acid sequences of peptides containing the amino-terminal and active-site regions of the heavy chain of bovine factor X_{1a} (activated Stuart factor) have been determined. The amino-terminal sequence is *Ile-Val-Gly-Gly*-Arg-Asp-*Cys*-Ala-Glu-Gly-Glu-Cys-*Pro-Trp-Gln*-Ala-Leu-*Leu*-Val-Asn-Glu-Glu-Asn-Glu-Gly-*Phe-Cys-Gly-Gly*-Thr-Ile-Leu-*Asn*-Glu-Phe-Tyr-*Val*-. The sequence which includes the reactive serine of the active site (capitalized) is *Phe-Cys-Ala-Gly-Tyr*-Asp-Thr-Gln-Pro-Glu-*Asp*-Ala-*Cys-Gln-Gly-Asp-SER-Gly-Gly-Pro*-His-*Val*-Thr-Arg-. The amino acids which are in italics are identical with those found in the amino-terminal region and active-site region of bovine trypsin, indicating considerable homology between the two proteins. These sequences are also very similar to the corresponding sequences of other proteases including bovine thrombin, bovine chymotrypsin, and human plasmin. It is concluded that bovine factor X_{1a} is a typical serine protease which has evolved from an ancestor common to other plasma and pancreatic proteolytic enzymes.

Factor X (Stuart factor)[1] is a protein which participates in the middle phase of blood coagulation (Davie *et al.*, 1969). The purified protein can be separated into two chromatographic fractions (factors X_1 and X_2) having similar chemical and biological properties (Jackson and Hanahan, 1968; Fujikawa *et al.*, 1972a).

An enzyme in Russell's viper venom activates factors X_1 and X_2 by hydrolyzing a peptide bond in the amino-terminal region of the heavy chain of the precursor molecule (Fujikawa *et al.*, 1972b). The activated protein (factor X_{1a} or X_{2a}) is a proteolytic enzyme which converts prothrombin to thrombin. It also hydrolyzes various synthetic esters such as *p*-toluenesulfonyl-L-arginine methyl ester and benzoyl-L-arginine ethyl ester (Esnouf and Williams, 1962; Milstone, 1964; Jackson and Hanahan, 1968; Aronson and Ménaché, 1968; Adams and Elmore, 1971). Like certain proteolytic enzymes, activated factor X is inhibited by soybean trypsin inhibitor (Breckenridge and Ratnoff, 1964; Milstone, 1964; Lundblad and Davie, 1965; Jackson and Hanahan, 1968) and by diisopropyl phosphorofluoridate (DFP)[2] although relatively high concentrations of this organic phosphate are required (Jackson and Hanahan, 1968; Leveson and Esnouf, 1968; Fujikawa *et al.*, 1972b).

This communication describes experiments which show that factor X_{1a} contains amino acid sequences which are homologous with sequences found in other proteolytic enzymes, specifically in bovine trypsin (Walsh and Neurath, 1964), chymotrypsin A (Hartley, 1964), elastase (Hartley and Shotton, 1971), and thrombin (Magnusson, 1971). These homologous sequences are found in the amino-terminal segment of the heavy chain of factor X_{1a} and in the region of the serine residue which reacts with DFP.

Experimental Procedure

Materials

Bovine factor X_1 and the enzyme from Russell's viper venom which activates factor X were prepared as previously described (Fujikawa *et al.*, 1972a,b). DFP was obtained from Pierce Chemical Co. and [^{32}P]DFP from Amersham/Searle Co. Sephadex products were obtained from Pharmacia Fine Chemicals and the anion-exchange resin, AG-1X2 (200–400 mesh), from Bio-Rad Laboratories. Porcine trypsin, a product of Novo Industri A/S, was purified on an affinity column as described by Robinson *et al.* (1971). Reagents used for the sequenator (sequenal grade) were from Pierce Chemical Co.

Methods

Activation of Factor X_1. The conversion of factor X_1 to the active form (factor X_{1a}) by Russell's viper venom and its subsequent inactivation with DFP or [^{32}P]DFP were carried out as previously described (Fujikawa *et al.*, 1972b).

Reduction, pyridylethylation, and separation of the heavy and light chains of factors X_1, X_{1a}, and DIP factor X_{1a} were also carried out as previously described (Fujikawa *et al.*, 1972b).

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

[2] Abbreviations used are: DFP, diisopropyl phosphorofluoridate; DIP, diisopropylphosphoryl.