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Activation of Human Factor X (Stuart Factor) by a Protease from Russell's Viper Venom†

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ABSTRACT: Human Factor X (Stuart factor) is a plasma protein (mol wt 59 000) that participates in the middle phase of blood coagulation. It is composed of a heavy and a light chain held together by a disulfide bond(s). Factor X is readily converted to an enzyme, factor X_a, by a protease from Russell's viper venom. In this reaction, a specific arginyl-isoleucine bond is cleaved in the amino-terminal region of the heavy chain giving rise to an activation peptide and factor X_a. This results in the formation of an Ile-Val-Gly-Gly-Gln-Glu-Cys-Lys-Asp-Gly-Glu-Cys-Pro-Thr-Gln-Ala-Leu- sequence in the heavy chain of the enzyme. No change was observed in the light chain of factor X during the activation reaction. The heavy chain of human factor X_a also contains the active site sequence of Phe-Cys-Ala-Gly-Tyr-Asp-Thr-Lys-Gln-Glu-Asp-Ala-Cys-Gln-Gly-Asp-SER-Gly-Gly-Pro-His-Val-Thr-Arg-Phe-. The amino-terminal and active site sequences of the heavy chain are homologous with the corresponding amino-terminal and active site sequences of bovine factor X_a and a number of other plasma serine proteases. Human factor X_a was rapidly inhibited by antithrombin III and formed a stable one-to-one molar complex with the inhibitor. These data indicate that the mechanism of activation and inhibition of human factor X are essentially identical with that previously described for bovine factor X.

Factor X (Stuart factor)[1] is one of four known coagulation factors present in plasma that requires vitamin K for its biosynthesis. During the coagulation process, factor X is converted to an enzyme, factor X_a, which in turn converts prothrombin to thrombin [see Davie and Fujikawa (1975) and Suttie and Jackson (1977) for reviews].

In recent years, human factor X has been purified and characterized in several different laboratories (Aronson et al., 1969; Rosenberg et al., 1975; Kosow, 1976; Vician and Tishkoff, 1976; Di Scipio et al., 1977). It is a glycoprotein composed of a heavy and a light chain held together by a disulfide bond(s). In the present article, we describe the mechanism of activation of human factor X by a protease from Russell's viper venom. In this reaction, factor X is converted to factor X_a by the cleavage of a specific arginyl-isoleucine bond in the amino-terminal portion of the heavy chain of the precursor molecule. This mechanism of activation is essentially identical with that previously observed for bovine factor X when it is activated by (a) the protease from Russell's viper venom, (b) trypsin, (c) factor IX_a and factor VIII, or (d) factor VII and tissue factor (Fujikawa et al., 1972a, 1974, 1975; Jesty and Esnouf, 1973; Jesty and Nemerson, 1974).

Experimental Section

Materials

Human factor X was purified to homogeneity by a slight modification of the method of Di Scipio et al. (1977). In the present experiments, a single 0–40% ammonium sulfate precipitation was substituted for the sequential 0–10% and 10–40% ammonium sulfate precipitation. This preparation does not contain two forms of factor X similar to bovine factor X_1 and factor X_2 (Fujikawa et al., 1972b). The protease from Russell's viper venom that activates factor X (RVV-X)[2] was purified from crude *Vipera russeli* venom by the method of Schiffman et al. (1969) or Kisiel et al. (1976).

Barbital, urethane, imidazole (grade I), morpholinoethanesulfonic acid (Mes), *N*-acetylneuraminic acid, Coomassie brilliant blue R, galactosamine, dithiothreitol, aldolase, bovine serum albumin, bovine carbonic anhydrase, and myoglobin were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium sulfate, sodium arsenate, and 4-vinylpyridine were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. The 4-vinylpyridine was vacuum distilled before use. Periodic acid was a product of Frederick Smith Chemical Co., Columbus, Ohio. 2-Thiobarbituric acid, 2-mercaptoethanol, *N,N*-methylenebisacrylamide, iodoacetic acid, and *N,N,N,N'*-tetraethylenediamine were purchased from Eastman Kodak Co., Rochester, N.Y. Agarose was a product of

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

[2] Abbreviations used: RVV-X, protease from Russell's viper venom that activates factor X; DFP, diisopropyl phosphorofluoridate; Tris, tris(hydroxymethyl)aminomethane; Mes, morpholinoethanesulfonic acid.

Marine Colloids, Rockland, Maine. Sodium lauryl sulfate was purchased from BDH Chemicals Ltd., Poole, England. Acrylamide was obtained from Bio-Rad Laboratories, Richmond, Calif. Cyclohexanone was a product of Aldrich Chemical Co., Milwaukee, Wis. Lithium heparin (181 U/mg) from porcine intestinal mucosa was obtained from Riker Laboratories, Inc., Northridge, Calif. Sephadex G-100, Sephadex G-75 superfine, and Sephadex G-25 coarse were products of Pharmacia Fine Chemicals, Piscataway, N.J. Constant boiling HCl and cyanogen bromide were obtained from Pierce Chemical Co., Rockford, Ill. Phosphorylase *b* was kindly provided by Dr. E. Fischer, and human antithrombin III was kindly provided by Dr. K. Kurachi (Kurachi et al., 1976a,b). Centrolex P was purchased from Central Soya, Chicago, Ill., and carboxypeptidases A and B were products of Worthington Biochemicals Co., Freehold, N.J. Carboxypeptidases A and B were pretreated with 10^{-3} M diisopropyl phosphorofluoridate (DFP) before use. Normal human plasma was obtained from the Puget Sound Blood Center, Seattle, Wash.; it had been stored for 4 months at −20 °C before use in the present studies. Human plasma from factor X deficient patients (GK-1001, GK-1002) was purchased from George King Biomedicals, Salem, N.H. All other materials were of the highest quality available.

Methods

Protein concentration was determined by absorption employing an extinction coefficient of $E_{280}^{1\%} = 11.6$ for human factor X and assuming the same value for human factor X_a (Di Scipio et al., 1977); $E_{280}^{1\%} = 5.7$ was employed for human antithrombin III (Kurachi et al., 1976a); and $E_{280}^{1\%} = 10$ was assumed for the protease from Russell's viper venom (Kisiel et al., 1976).

Amino acid analyses and preparation of samples were carried out by the methods of Moore and Stein (1963) and Spackman et al. (1958) employing a Durrum Model D500 amino acid analyzer. Samples were hydrolyzed in constant boiling 6 N HCl at 110 °C for 24, 48, 72, and 96 h in evacuated tubes. The values of threonine and serine were determined by extrapolation to zero hydrolysis time. Isoleucine and valine were calculated from the 96-h hydrolysis time. Tryptophan was estimated by the method of Bencze and Schmid (1957). Half-cystine was determined as cysteic acid by the method of Hirs (1967) or as *S*-carboxymethylcysteine by the method of Crestfield et al. (1963).

N-Acetylneuraminic acid was determined by the thiobarbituric acid method of Warren (1959) using *N*-acetylneuraminic acid as a standard. Neutral sugar was determined by the phenol–sulfuric acid method of Dubois et al. (1956) using a 1:1 mixture of mannose and galactose as a standard. For the determination of hexosamine, samples were hydrolyzed in 2 N HCl for 24 h at 110 °C in evacuated tubes and analyzed by the method of Elson and Morgan as described by Gardell (1957) using galactosamine as a standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (1969) as modified by Kisiel et al. (1976). Samples were run on either 7.2 or 8.5% gels made up in 0.1 M Tris-H_3PO_4 buffer (pH 7.0) containing 0.2% sodium dodecyl sulfate. The gels were run for 3.5 to 4 h at 4.2 mA/gel and stained for protein with a 0.2% solution of Coomassie brilliant blue R. Molecular weights were estimated by interpolation from a linear semi-logarithmic plot of apparent molecular weight vs. migration distance using the following protein standards: phosphorylase *b* (97 000), bovine serum albumin (68 000), aldolase (40 000), bovine carbonic anhydrase (29 000), and myoglobin (17 000).

The heavy and light chains of factor X were prepared as previously described (Di Scipio et al., 1977), and the *S*-pyridylethyl and *S*-carboxymethyl derivatives were made according to Friedman et al. (1970) and Crestfield et al. (1963), respectively. The heavy and light chains of factor $X_{a\alpha}$ were isolated as follows: factor X (15 mg at 1 mg/mL) was incubated with RVV-X (0.07 mg) for 25 min as described below, and the reaction terminated by the addition of 1 mL of 0.2 M EDTA. The protein was desalted on a Sephadex G-25 column (2 × 40 cm) previously equilibrated with 10% formic acid, and the protein fraction was pooled and lyophilized. The protein was then dissolved in 1 mL of 10% formic acid and applied to a Sephadex G-75 column (1.6 × 84 cm) which had been previously equilibrated with 10% formic acid. The flow rate was 1 mL/10 min. The peak containing the activation peptide and the peak containing factor $X_{a\alpha}$ were pooled separately and lyophilized. Factor $X_{a\alpha}$ was then reduced and carboxymethylated (or pyridylethylated) and the heavy and light chains were separated on a Sephadex G-100 column (1.6 × 85 cm) which had been previously equilibrated with 10% formic acid. The flow rate was 3 mL/10 min. The heavy and light chains were then pooled separately and lyophilized.

Cyanogen bromide digestion of the heavy chain of factor X was performed by dissolving 20 mg of salt-free *S*-pyridylethyl factor X heavy chain in 2.0 mL of 70% formic acid, and 40 mg of cyanogen bromide was added. The reaction was allowed to proceed for 24 h at 4 °C. After lyophilization, the digest was fractionated by gel filtration on Sephadex G-50 superfine (1.6 × 85 cm column) in 10% formic acid. The flow rate was 0.12 mL/min.

Amino-terminal sequence analyses were performed with a Beckman sequenator Model 890C. The mode of operation of the instrument and the methods of sequenator analysis are adaptations (Hermodson et al., 1972) of the technique of Edman and Begg (1967). Phenylithiohydantoin amino acids were identified and quantitated by high-pressure liquid chromatography (Bridgen et al., 1976) or by gas chromatography after silylation. Phenylthiohydantoin amino acids (Cys, Arg, and His) that remained in the water layer were identified by spot tests and not quantitated. Also, in the present experiments, it was not possible to quantitate Ser, Thr, Lys, or Gln. For amino-terminal analyses, 1 mg of the activation peptide, 2 mg of the *S*-carboxymethyl light chain of factor $X_{a\alpha}$, 2.5 mg of the *S*-pyridylethyl cyanogen bromide peptide containing the active site of factor X, and 7 mg of the *S*-pyridylethyl heavy chain of factor $X_{a\alpha}$ were used. The analyses of the activation peptide and the light chain of factor X were performed only once since these data confirmed earlier experiments (Di Scipio et al., 1977). The analyses of the active site region and the heavy chain of factor X were carried out in duplicate. In the quantitation of amino-terminal residues, protein concentration was determined by amino acid analysis after hydrolysis of the sample in 6 N HCl. Norleucine was employed as an internal standard to calculate protein recovery.

Carboxyl-terminal analysis was carried out by a modification of the procedure of Fraenkel-Conrat et al. (1955). Pancreatic carboxypeptidase A and B were each pretreated with 1×10^{-3} M DFP before use. The activation peptide (approximately 1 mg) was dissolved in 0.5 mL of 0.01 M sodium phosphate buffer (pH 8.0) containing 1.0% sodium dodecyl sulfate. The sample was then boiled for 10 min and diluted by the addition of 0.5 mL of 0.01 M sodium phosphate buffer (pH 8.0). Half of the sample was incubated for 16 h at 37 °C with carboxypeptidase A (10 μg) and the other half with carboxypeptidase B (7.2 μg) for 16 h at 37 °C. The liberated amino acids were then analyzed in the amino acid analyzer. The

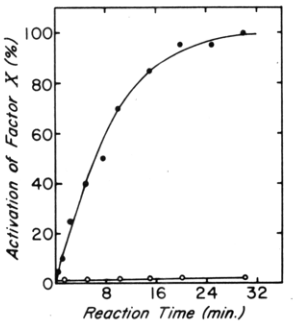

FIGURE 1: Time curve for the activation of factor X with a protease from Russell's viper venom. The reaction mixture (0.5 mL) contained 1 mg/mL of factor X in 0.05 M Tris-HCl, pH 7.8, 0.15 M NaCl, and 5 mM $CaCl_2$. The reaction was initiated by the addition of 2 μL (0.75 mg/mL) of the purified Russell's viper venom protein. Incubation was carried out at 37 °C; aliquots (10 μL) were removed at various times and diluted, and factor X_a was assayed as described in Methods. (●—●) Complete reaction mixture; (○—○) reaction mixture in the absence of $CaCl_2$.

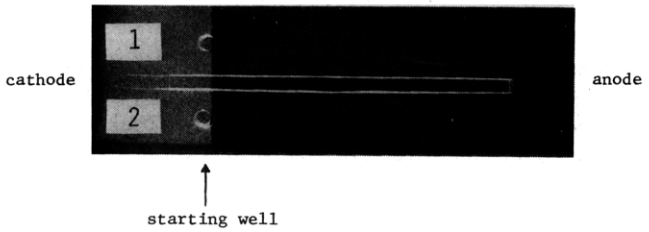

FIGURE 2: Immunoelectrophoresis of human factor X and human factor $X_{a\alpha}$. Factor X (5 μL containing 7 μg of protein) was placed in well number 1 and factor $X_{a\alpha}$ (5 μL containing 4 μg of protein) was placed in well number 2. Electrophoresis was carried out in 0.05 M sodium barbital buffer, pH 8.6, for 50 min at 4 °C at 150 V. Following electrophoresis, 50 μL of rabbit antibody solution (1.5 mg/mL) against human factor X was placed in the center trough. After 24 h, precipitin lines appeared and the slides were then photographed.

amount of peptide (mol wt approximately 14 000) was quantitated by amino acid analysis of a 24-h hydrolysate employing norleucine as an internal standard.

Antibodies to human factor X were prepared as previously described (Fujikawa et al., 1972b).

Immunoelectrophoresis was performed on glass slides (25 × 75 cm) with 1.0% agarose in 0.05 M sodium barbital buffer (pH 8.6) containing 0.01% sodium azide according to the method of Scheidegger (1955). Samples (4–7 μg) were subjected to electrophoresis for 50 min at 150 V at 4 °C and 50 μL of antibody solution (1.5 mg/mL) was added to the trough and allowed to diffuse through the agarose gel overnight.

Activation of Human Factor X by RVV-X. Human factor X (0.5–1.0 mg/mL) in 0.05 M Tris-HCl buffer (pH 7.8) containing 0.15 M NaCl and 5 mM $CaCl_2$ was activated by the addition of RVV-X to give a final enzyme-to-substrate weight ratio of 1:100 to 1:300. The reaction was terminated by the addition of ethylenediaminetetraacetic acid to a final concentration of 10 mM. For coagulant assays, aliquots (10 μL) were withdrawn at various times from the reaction mixture and diluted 100–25 000-fold with Michaelis buffer. An aliquot (0.1 mL) of the diluted sample was then incubated at 37 °C for 1 min with 0.1 mL of 0.2% Centrolex P suspension and 0.1 mL of normal human plasma in a siliconized glass tube. The clotting time was determined after the addition of 0.1 mL of 0.033 M $CaCl_2$. The percent activation was determined from a calibration curve from the fully activated sample.

Inhibition of Human Factor $X_{a\beta}$ by Antithrombin III. The inhibition of human factor $X_{a\beta}$ was carried out by incubating human antithrombin III (0.7 mg) with factor $X_{a\beta}$ (0.3 mg) in 1 mL of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl in the presence or absence of heparin (20 units). Samples were incubated at 37 °C and 10-μL aliquots were withdrawn at various times, diluted with ice-cold Michaelis buffer, and assayed immediately for coagulant activity as previously described.

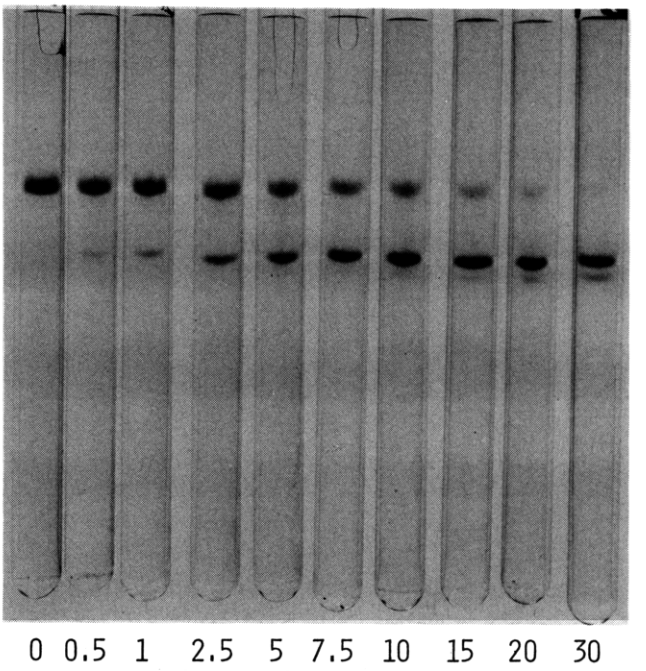

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of human factor X activated with the protease from Russell's viper venom. Aliquots (containing approximately 10 μg of protein) were removed at various times from an incubation mixture corresponding to that shown in Figure 1 and subjected to electrophoresis on 7.2% polyacrylamide gels as described in Methods. The numbers under the gels refer to the incubation time in minutes. The anode was at the bottom of the gels.

Results

Activation of Human Factor X. A time course for the activation of human factor X by RVV-X is shown in Figure 1. The activation was performed with an enzyme-to-substrate ratio of 1:300 at pH 7.8 in the presence of 5 mM $CaCl_2$ (solid circles). Little, if any, activation of factor X occurred in the absence of calcium (open circles). Factor X_a at a final concentration of 30 ng/mL in the coagulant assay (see Methods) gave a clotting time of about 24 s.

A difference in the electrophoretic properties of factor X and factor X_a was apparent when aliquots of the reaction mixture were examined by immunoelectrophoresis (Figure 2). Factor X (sample 1) had a faster electrophoretic mobility than factor X_a (sample 2) when examined in 0.05 M barbital buffer (pH 8.6). Furthermore, both proteins formed single sharp precipitin lines.

Further evidence of a change in the structure of factor X during the activation reaction was shown in experiments employing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 3). In these experiments, aliquots were removed at various times from an activation mixture corresponding to that shown in Figure 1 and analyzed by gel electrophoresis. At zero time, a single protein band (apparent mol wt 66 000) was observed for factor X. During the first few minutes of incubation, a new faster moving band (apparent mol wt 51 000) was observed, and the appearance of this band

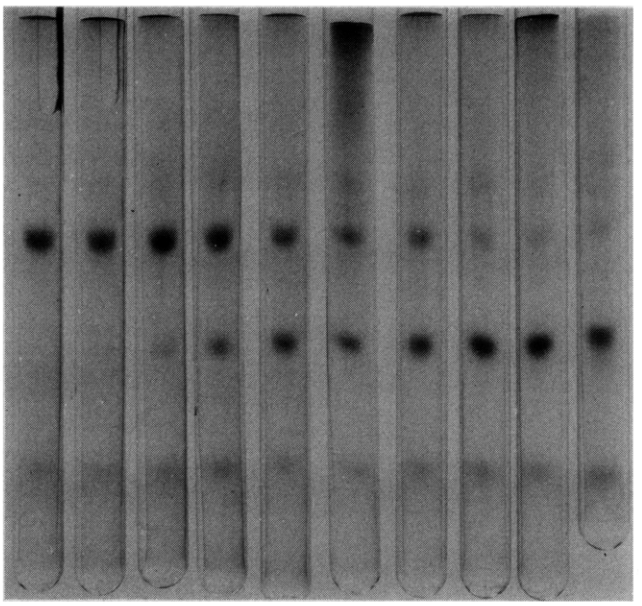

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of the samples shown in Figure 3 after reduction. Aliquots containing approximately 15 μg of protein were removed from the reaction mixture at various times, reduced with 2-mercaptoethanol, and subjected to electrophoresis on 8.5% gels as described in Methods. The numbers under the gels refer to the incubation time in minutes. The anode was at the bottom of the gels.

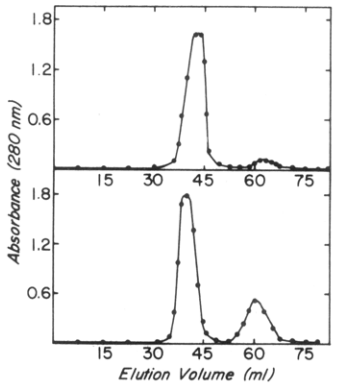

FIGURE 5: Separation of factor $X_{a\alpha}$, the activation peptide, and the heavy and light chains of factor $X_{a\alpha}$. Human factor X (15 mg) was activated at 37 °C for 25 min with 0.1 mg of RVV-X as described in Methods, and factor $X_{a\alpha}$ and the activation peptide were separated on a Sephadex G-75 column (2.5 × 84 cm) in 10% formic acid (upper panel). The flow rate was 0.10 mL/min. Factor $X_{a\alpha}$ was then reduced, carboxymethylated, and fractionated on a Sephadex G-100 column (1.6 × 85 cm) in 10% formic acid (lower panel). The flow rate was 0.3 mL/min.

occurred in parallel with the formation of factor X_a coagulant activity. The resulting molecule was called factor $X_{a\alpha}$ employing the nomenclature previously used for bovine factor X (Fujikawa et al., 1974). After prolonged incubation (15–30 min), small amounts of a second, fast-moving band (apparent mol wt 47 000) were observed. This protein, which was called factor $X_{a\beta}$, was formed by incubating human factor X with RVV-X for 30 min at 37 °C and then allowing the reaction to continue for an additional 10 h at 4 °C. These experiments provide additional evidence to indicate that a fragment(s) (approximate mol wt 15 000) has been split from factor X giving rise to factor $X_{a\alpha}$ with a lower molecular weight and lower electrophoretic mobility. These experiments also suggest that the fragment(s) split from factor X during the activation reaction stain very poorly with Coomassie brilliant blue since they were not observed in the present experiments. Upon prolonged incubation, factor $X_{a\alpha}$ was converted to factor $X_{a\beta}$ by minor degradation.

Since human factor X is composed of a heavy and a light chain, it was of interest to examine the activation mixture by gel electrophoresis after reduction to determine which chain(s) was modified during the activation reaction (Figure 4). The zero-time sample showed two bands that correspond to the heavy chain (apparent mol wt 49 000) and the light chain (apparent mol wt 17 000) of factor X. Within the first few minutes of activation, a new band appeared (apparent mol wt 32 000), and the appearance of this new band occurred in parallel with the disappearance of the heavy chain of factor X. No change was noted in the light chain (fast-moving chain) during the activation reaction. These data provide further evidence for a change in the molecular weight of factor X during the activation reaction, and this change occurs in the heavy chain of the molecule.

Isolation of the Heavy and Light Chains of Human Factor $X_{a\alpha}$ and the Activation Peptide. Factor X (15 mg) was activated by RVV-X and the reaction mixture was fractionated on a Sephadex G-75 column in the presence of 10% formic acid (Figure 5, upper panel). Two major peaks were obtained. The first peak contained factor $X_{a\alpha}$ and the second peak contained a polypeptide identified as the activation peptide (see below). The peak containing factor $X_{a\alpha}$ was then reduced, subjected to carboxymethylation (or pyridylethylation), and the heavy and light chains were separated on a Sephadex G-100 column (Figure 5, lower panel). The heavy chain of factor $X_{a\alpha}$ appeared in the first peak and the light chain appeared in the second peak. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the heavy and light chains of factor X and factor $X_{a\alpha}$ and the activation peptide gave single sharp bands (Figure 6). The heavy and light chains of factor X are shown in gels 1 and 2, respectively, and the heavy and light chains of factor $X_{a\alpha}$ are shown in gels 3 and 4, respectively. The activation peptide (apparent mol wt 21 000) is shown in gel 5.

Composition of the Heavy and Light Chains of Factor X and Factor $X_{a\alpha}$ and the Activation Peptide. The amino acid and carbohydrate compositions of the two chains of factor X and factor $X_{a\alpha}$ and the activation peptide are shown in Table I. The molecular weights employed for these calculations were as follows: heavy chain of factor X, 42 100; light chain of factor X, 16 900; heavy chain of factor $X_{a\alpha}$, 29 100; and the activation peptide, 14 000 (see Discussion). A summation of the amino acid and carbohydrate compositions of the heavy and light chains of factor X (mol wt 59 000) is shown in column 3. These data are in good agreement with those previously published for the intact protein (Di Scipio et al., 1977). A summation of the amino acid and carbohydrate compositions of the heavy and light chains of factor $X_{a\alpha}$ (mol wt 46 000) is shown in column 5, and a summation of factor $X_{a\alpha}$ (column 5) and the activation peptide (column 6) is shown in column 7. There is fairly good agreement between the data shown in column 7 and column 3 (heavy and light chains of factor X), except for aspartic acid, serine, and glutamic acid. Human factor X contains approximately 15% carbohydrate (Di Scipio et al., 1977), and essentially all of the carbohydrate is located in the heavy chain of factor X. Furthermore, the major portion of the carbohydrate of factor X is released during the activation reaction. This results in the formation of an activation peptide containing nearly 40% carbohydrate.

Amino-Terminal Analysis. In previous studies from our laboratory, it was shown that the amino-terminal sequences of the light and heavy chains of human factor X were Ala-

TABLE I: Amino Acid and Carbohydrate Compositions of Human Factor X.[a]

Component	Factor X			Factor $X_{a\alpha}$			$X_{a\alpha}$ heavy + $X_{a\alpha}$ light + Act. peptide
	Heavy	Light	Heavy + light	Heavy	Heavy + light	Act. peptide	
Amino acid							
Lys	21.1	11.6	32.7	17.4	29.0	4.0	33.0
His	7.0	4.0	11.0	5.5	9.5	0.9	10.4
Arg	16.3	5.7	22.0	12.5	18.2	3.3	21.5
Asp	29.7	16.8	46.5	22.1	38.9	11.5	50.4
Thr	23.5	10.0	33.5	17.0	27.0	7.2	34.2
Ser	16.8	7.7	24.5	13.1	20.8	6.4	27.2
Glu	37.1	25.8	62.9	32.2	58.0	9.9	67.9
Pro	14.5	5.0	19.5	10.7	15.7	5.8	21.5
Gly	26.5	13.7	40.2	21.6	35.3	5.4	40.7
Ala	21.7	6.8	28.5	15.0	21.8	7.5	29.3
½-cystine	10.4	14.0	24.4	8.1	22.1	2.1	24.2
Val	17.7	3.7	21.4	13.1	16.8	3.0	19.8
Met	4.7	1.5	6.2	5.1	6.6	0.2	6.8
Ile	13.9	2.1	16.0	10.2	12.3	2.6	14.9
Leu	20.2	9.2	29.4	15.3	24.5	5.8	30.3
Tyr	7.2	4.3	11.5	5.8	10.1	1.1	10.2
Phe	11.6	6.1	17.7	10.0	16.1	2.2	18.3
Trp	6.0	1.9	7.9	5.5	7.4	0.6	8.0
Mol wt[b] (protein)	34000	16800	50800	27000	43800	8500	52300
Carbohydrate[c]							
Hexose	13.7	0.4	14.1	3.4	3.6	8.9	12.7
Hexosamine	12.8	0	12.8	3.2	3.2	8.4	11.6
Neuraminic acid	9.6	0	9.6	2.6	2.6	7.0	9.6
Carbohydrate (%)	19.2	0.4	13.7	7.3	4.3	39.1	12.7
Protein (%)	80.8	99.6	86.3	92.7	95.7	60.9	87.3
Mol wt (glycoprotein)	42100	16900	59000	29100	46000	14000	60000

[a] Expressed in residues per molecule of glycoprotein. [b] Molecular weights were rounded off to the nearest 100. [c] Carbohydrate was expressed as *N*-acetylhexosamine and *N*-acetylneuraminic acid.

Asn-Ser-Phe-Leu- and Ser-Val-Ala-Gln-Ala-, respectively (Di Scipio et al., 1977). Accordingly, it was of interest to examine the amino-terminal sequences of the heavy and light chains of factor $X_{a\alpha}$ and the activation peptide to further identify the origin of the activation peptide and what bond(s) was cleaved during the activation reaction. Alanine was the only amino-terminal residue identified in the light chain of human factor $X_{a\alpha}$. The first five residues and the equivalents per 16 900 g of protein were as follows: Ala (0.9), Asn (0.6), Ser (not quantitated), Phe (0.7), Leu (0.7). These data are consistent with the sodium dodecyl sulfate–polyacrylamide gel electrophoresis experiments which indicated that little, if any, change occurred in the light chain of factor X during the activation reaction.

Isoleucine was identified as the amino-terminal residue in the heavy chain of factor $X_{a\alpha}$ and no other amino acids were detected. The sequence of the first 17 residues and the equivalents per 29 100 g of protein were as follows: Ile (0.7), Val (0.8), Gly (0.8), Gly (0.8), Gln (not quantitated), Glu (0.7), Cys (not quantitated), Lys (not quantitated), Asp (0.5), Gly (0.5), Glu (0.5), Cys (not quantitated), Pro (0.6), Thr (not quantitated), Gln (not quantitated), Ala (0.4), Leu (0.6). The repetitive yields for the degradations were greater than 96%. The amino acids that are underlined are identical with those found in the heavy chain of bovine factor $X_{a\alpha}$ (Titani et al., 1975). These data indicate that the activation peptide originated from the amino-terminal end of the heavy chain of factor X during the activation reaction. Further evidence for this conclusion was obtained by an amino-terminal analysis of the activation peptide. Serine was identified as the amino-terminal residue of this polypeptide and no other amino acids were detected. The first 16 residues in this fragment were Ser-Val-Ala-Gln-Ala-Thr-Ser- ? -Ser-Gly-Glu-Ala-Pro-Asp-Thr-Ile. The residues in this polypeptide were not quantitated. This sequence, however, is identical with that previously obtained for the heavy chain of factor X (Di Scipio et al., 1977). As before, no amino acid residue was clearly identified in position 8.

Effect of Carboxypeptidases A and B on the Activation Peptide. To characterize further the peptide bond split during the activation reaction, the activation peptide was treated with carboxypeptidase A or carboxypeptidase B in order to identify the carboxyl-terminal amino acid. Amino acids released by these enzymes were then analyzed by the amino acid analyzer. Arginine (0.96 mol/14 000 g of activation peptide) was found after incubation with carboxypeptidase B for 16 h at 37 °C in 0.05% sodium dodecyl sulfate. No other amino acids were liberated by carboxypeptidase B. Carboxypeptidase A released only traces of amino acids. These results indicate the presence of a carboxyl-terminal arginine on the activation peptide. Furthermore, it can be concluded that a specific arginyl-isoleucine peptide bond in the heavy chain of factor X was hydrolyzed during the activation reaction.

Amino Acid Sequence of the Active Site. In order to determine the amino acid sequence in the active site region of factor X, the *S*-pyridylethyl heavy chain of factor X was

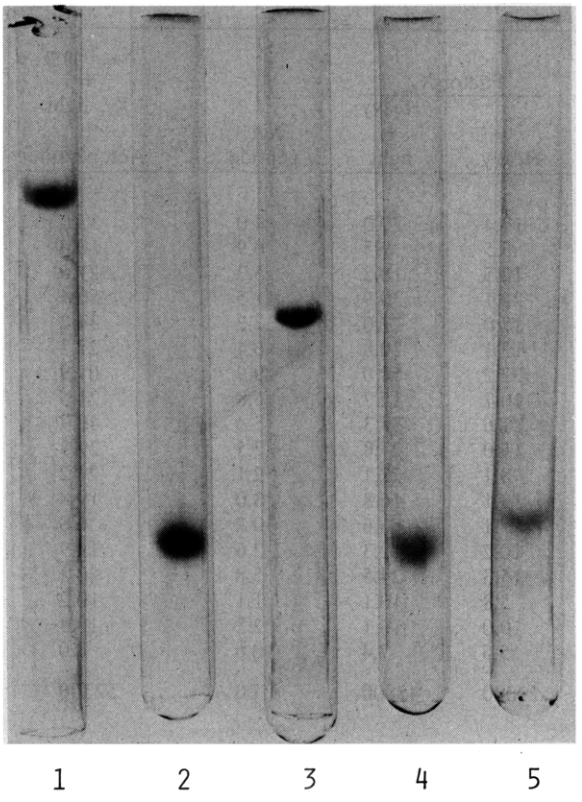

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of the various polypeptides of human factor X and human factor $X_{a\alpha}$. Gel electrophoresis was carried out on 8.5% gels as described in Methods. Gel 1 is 10 μg of the *S*-pyridylethyl heavy chain of factor X. Gel 2 is 10 μg of the *S*-pyridylethyl light chain of factor X. Gel 3 is 7 μg of the *S*-pyridylethyl heavy chain of factor $X_{a\alpha}$. Gel 4 is 6 μg of the *S*-pyridylethyl light chain of factor $X_{a\alpha}$. Gel 5 is 10 μg of the activation peptide. The anode is at the bottom of the gel.

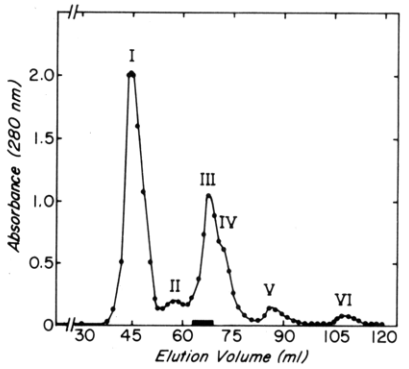

FIGURE 7: Gel filtration of the cyanogen bromide peptides from the *S*-pyridylethyl heavy chain of human factor X. The digest (20 mg) was dissolved in 2 mL of 25% aqueous formic acid and applied to a Sephadex G-50 column (1.6 × 85 cm) which was previously equilibrated with 10% formic acid. The column was eluted with 10% formic acid at a flow rate of 0.13 mL/min, and 1.5-mL fractions were collected. The various peaks were pooled individually and lyophilized. The fractions containing fragment III were combined as indicated by the solid bar.

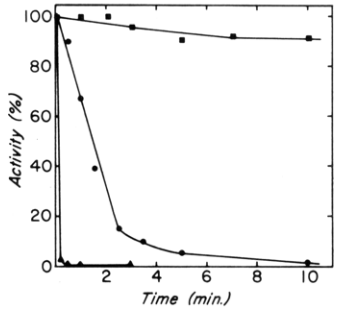

FIGURE 8: Inhibition of human factor $X_{a\beta}$ by antithrombin III in the presence and absence of heparin. The reaction mixture contained 0.3 mg/mL of factor $X_{a\beta}$ and 0.7 mg/mL of antithrombin III in a final volume of 1 mL. Aliquots (10 μL) were removed at various times, diluted with cold Michaelis buffer, and immediately assayed for coagulant activity. (■—■) Factor $X_{a\beta}$ plus heparin (20 U/mL); (●—●) factor $X_{a\beta}$ plus antithrombin III; (▲—▲) factor $X_{a\beta}$ plus antithrombin III and heparin (20 U/mL).

subjected to cyanogen bromide digestion, as described in Methods. The cyanogen bromide fragments were then fractionated on a Sephadex G-50 column (2.5 × 85 cm) in 10% formic acid (Figure 7). Six peaks were observed. These peaks were pooled separately and lyophilized. Fragment III was identified as the polypeptide containing the active site serine residue. It migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and had an approximate molecular weight of 7000. Phenylalanine was identified as the amino-terminal residue of this polypeptide, and no other amino acids were detected. The sequence of the first 25 residues and the equivalents per 7000 g of protein were as follows: Phe (0.8), Cys (not quantitated), Ala (0.8), Gly (0.7), Tyr (0.8), Asp (0.5), Thr (not quantitated), Lys (not quantitated), Gln (not quantitated), Glu (0.6), Asp (0.6), Ala (0.6), Cys (not quantitated), Gln (not quantitated), Gly (0.4), Asp (0.4), SER (not quantitated), Gly (0.3), Gly (0.4), Pro (0.3), His (not quantitated), Val (0.3), Thr (not quantitated), Arg (not quantitated), Phe (0.2). The repetitive yields for the degradations were greater than 95%. The active site serine residue (capital letters) corresponding to serine-195 in chymotrypsin appeared in position 17 of the peptide. The residues that are underlined are identical with the corresponding region in bovine factor X (Titani et al., 1975). These data indicate that human factor X contains an amino acid sequence which is nearly identical with the active site region in bovine factor X.

Inhibition of Factor $X_{a\beta}$ with Antithrombin III. A time curve for the inhibition of human factor $X_{a\beta}$ by human antithrombin III is shown in Figure 8. In these experiments, the molar ratio of factor $X_{a\beta}$ to antithrombin III was approximately 1:2. Within 5 min, essentially all of the factor $X_{a\beta}$ activity was inhibited by antithrombin III in the absence of heparin (solid circles). In the presence of 20 U/mL of heparin, the inhibition was almost instantaneous (solid triangles).

The inhibition of factor $X_{a\beta}$ as a function of antithrombin III concentration is shown in Figure 9. Factor $X_{a\beta}$ and antithrombin III, in the presence and absence of heparin, were incubated for 10 min at 37 °C, and aliquots were diluted and assayed for coagulant activity. In each case, a one-to-one molar ratio of the antithrombin III to factor $X_{a\beta}$ was found to give maximal inhibition. When a mixture of factor $X_{a\beta}$ and antithrombin III, in the presence or absence of heparin, was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, a complex with an apparent molecular weight of 97 000 was observed. These data also indicate that a stable one-to-one molar complex was formed between antithrombin III and factor $X_{a\beta}$. In the presence of 2-mercaptoethanol, a band with an apparent molecular weight of 83 000 was observed. These data suggest that the light chain of factor $X_{a\beta}$ has been removed from the complex by reduction and the new band is due to a complex containing the heavy chain of factor $X_{a\beta}$ and antithrombin III. Substantial degradation of the complex from proteolysis was also noted. This degradation, however, was much less in the presence of heparin.

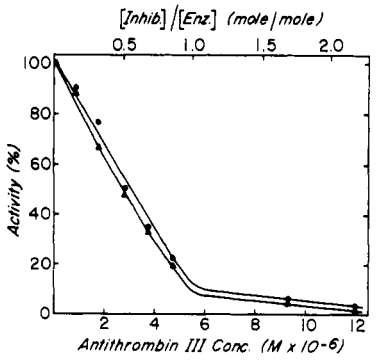

FIGURE 9: Inhibition of human factor $X_{a\beta}$ by increasing concentrations of antithrombin III in the presence and absence of heparin. The reaction mixture contained 6×10^{-6} M of factor $X_{a\beta}$ (0.05 mg in a final volume of 0.2 mL) in 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, and increasing concentrations of antithrombin III as shown. After incubation for 10 min at 37 °C, aliquots (10 μL) were removed, diluted with Michaelis buffer, and assayed for coagulant activity. (●—●) Factor $X_{a\beta}$ plus antithrombin III; (▲—▲) factor $X_{a\beta}$ plus antithrombin III and heparin (20 U/mL).

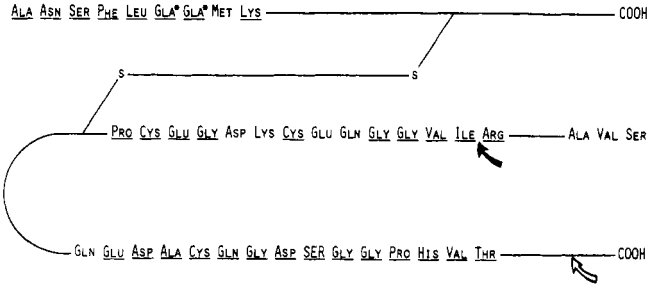

FIGURE 10: Partial structure for human factor X. The solid arrow indicates the site of cleavage in the heavy chain during the activation reaction leading to factor $X_{a\alpha}$. The open arrow indicates the probable site of cleavage in the heavy chain of factor $X_{a\alpha}$ leading to the formation of factor $X_{a\beta}$. Gla* (γ-carboxyglutamic acid) shown in positions 6 and 7 of the light chain is tentative. Residues that are underlined are identical with those present in bovine factor X. The active site serine is shown in capital letters. Taken in part from Di Scipio et al. (1977).

Discussion

The data presented in this manuscript indicate that human factor X is converted to an enzyme by the cleavage of a specific arginyl-isoleucine peptide bond located in the amino-terminal region of the heavy chain of the molecule (solid arrow, Figure 10). This results in the formation of factor $X_{a\alpha}$ with a new amino-terminal isoleucine residue in the heavy chain. A slower degradation reaction (open arrow, Figure 10) gives rise to factor $X_{a\beta}$, a molecule with slightly lower molecular weight but with the same coagulant activity. These reactions are essentially identical with those occurring during the activation of bovine factor X by (a) the protease from Russell's viper venom, (b) trypsin, (c) factor IX_a and factor VII, or (d) factor VII and tissue factor (Fujikawa et al., 1972a, 1974, 1975; Jesty and Esnouf, 1973; Jesty and Nemerson, 1974). In the activation of the bovine molecule, an initial cleavage occurs between Arg-51 and Ile-52 leading to factor $X_{a\alpha}$ followed by a degradation reaction between Arg-290 and Gly-291 giving rise to factor $X_{a\beta}$ (Fujikawa et al., 1975). In each case, a glycopeptide is liberated. The activation peptide from bovine factor X has a molecular weight of 9500 and the degradation peptide has a molecular weight of 2700. The critical event in the activation of bovine factor X by various enzymes such as those in the intrinsic pathway (factor IX_a and factor VIII) or the extrinsic pathway (tissue factor and factor VII), or by trypsin or the protease from Russell's viper venom is the cleavage of the peptide bond between Arg-51 and Ile-52. Thus, it is extremely likely that the same situation exists in the case of the activation of human factor X by various enzymes.

The activation of factor X by the protease from Russell's viper venom has an absolute requirement for calcium ions (Esnouf and Williams, 1962). Recently, Kosow (1976) has reported that calcium ions increase the V_{max} of the activation reaction and exhibit a cooperative effect.

Human factor X contains an active site sequence which is nearly identical with bovine factor X. This sequence is also homologous with many other serine proteases (Kisiel et al., 1977). Thus, it seems probable that the basic mechanism for the proteolytic activation of human factor X will be identical with that for bovine factor X and presumably involves ion pair formation between the newly formed amino-terminal isoleucine residue and the carboxyl group of the aspartic acid adjacent to the active serine (Sigler et al., 1968; Stroud et al., 1975; Titani et al., 1975). These reactions could then lead to the charge relay network which is characteristic of the pancreatic serine proteases (Matthews et al., 1967; Sigler et al., 1968; Blow et al., 1969; Shotton and Watson, 1970). Other small changes in the three-dimensional structure of factor X during the activation reaction have also been noted. Furie and Furie (1976) reported that the activation of bovine factor X by the protease from Russell's viper venom is associated with exposure of tryptophan and tyrosine side chains previously buried within the protein.

The effect of antithrombin III on human factor X_a is also very similar to the bovine system (Yin et al., 1971; Kurachi et al., 1976b). In each case, a one-to-one molar complex is formed between the enzyme and inhibitor, and this complex is stable to heating at 100 °C (pH 7.0) in 0.1% sodium dodecyl sulfate. The nature of the binding sites between the enzyme and inhibitor, however, is not known.

Human factor X (mol wt approximately 60 000) is slightly larger than bovine factor X (mol wt 55 100). This is due to the higher percent of carbohydrate in human factor X (15 vs. 10) and the larger size of the heavy chain of human factor X. Indeed, the first 16 amino-terminal residues in the heavy chain of human factor X show no similarity in sequence to the bovine molecule. This suggests that an additional 20–30 amino acid residues may be present on the amino-terminal portion of the heavy chain of the human protein as compared to the bovine molecule. The apparent molecular weight of the light chain of human factor X and factor $X_{a\alpha}$ as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 17 000. This value is probably very close to the correct value since it corresponds well with the molecular weight of the light chain of bovine factor X as determined by amino acid sequence (Enfield et al., 1975). Furthermore, the light chain of human factor X contains little if any carbohydrate. Thus, the apparent molecular weight of this chain as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis will not be influenced by the binding of decreased levels of detergent (Segrest and Jackson, 1972). Accordingly, the correct molecular weights for the heavy chain of factor X and factor $X_{a\alpha}$ are probably closer to 43 000 and 29 000, respectively, and the molecular weight for human factor $X_{a\alpha}$ is about 46 000. This gives a value of 14 000 for the activation peptide. Accordingly, these values were employed for the amino acid and carbohydrate compositions presented in Table I and are considered more reliable than the apparent molecular weights estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It is clear, however, that some minor adjustments of these values will be necessary when the various molecular weights

are established by more precise techniques such as amino acid sequence analysis.

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