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Activation of Human Factor XI (Plasma Thromboplastin Antecedent) by Factor XII_a (Activated Hageman Factor)†

Kotoku Kurachi and Earl W. Davie*

ABSTRACT: Factor XI (plasma thromboplastin antecedent) is a plasma protein that participates in the early phase of blood coagulation. It was isolated from human plasma by barium citrate adsorption of the vitamin K dependent proteins followed by ammonium sulfate fractionation, heparin-agarose, carboxymethyl-Sephadex, and benzamidine-agarose column chromatography. Factor XI is a glycoprotein containing 5% carbohydrate and has a molecular weight of about 124 000. It is composed of two identical polypeptide chains (mol wt about 60 000), and these two chains are held together by a disulfide bond(s). Its amino-terminal sequence is Gly-Cys-Val-Thr-Gln-Leu-Leu-Lys-Asp-Thr-Gln-Phe-Glu-Gly-Gly-. Factor XI is converted to an enzyme, factor XI_a, by factor XII_a (activated Hageman factor). This reaction readily occurs with an enzyme-to-substrate ratio of 1 to 50. Factor XI_a is composed of a pair of heavy chains (mol wt approximately 35 000) and a pair of light chains (mol wt approximately 25 000). These four chains are held together by disulfide bonds. The amino-terminal sequence of the heavy chains is Gly-Cys-Val-Thr-Gln-Leu-Leu-Lys-Asp-Thr-Gln-Phe-Glu-Gly-Gly-Asp-Ile- which is identical with that found in the precursor molecule. The amino-terminal sequence of the light chains is Ile-Val-Gly-Gly-Thr-Val-Ala- ? -His-Gly-Glu-Trp-Pro-Trp-Gln-Val-. This chain also contains the active site sequence of Ile-Cys-Ala-Gly-Tyr-Arg-Glu-Gly-Gly-Lys-Asp-Ala-Cys-Lys-Gly-Asp-SER-Gly-Gly-Pro-. The active site serine is shown in capital letters. These data indicate that factor XI is converted to factor XI_a by the cleavage of an internal peptide bond in each of the two precursor chains. Human factor XI_a is inhibited by diisopropyl phosphorofluoridate and antithrombin III. The inhibitor in each case is bound to the light chain of factor XI_a which contains the active site serine residue. The stoichiometry of the complex formed between factor XI_a and antithrombin III was shown to be 1 mol of enzyme combined with 2 mol of inhibitor. These data further support the conclusion that factor XI_a is a serine protease containing two active sites per mole of enzyme.

Factor XI (plasma thromboplastin antecedent)[1] is a coagulation protein present in plasma in a precursor form. It participates in the early phase of the coagulation process (Davie and Fujikawa, 1975). Individuals with factor XI deficiency often bleed excessively after injury and minor surgery (Rosenthal et al., 1953). Factor XI deficiency has also been reported in a cow by Kociba et al. (1969).

The isolation and detailed characterization of bovine factor XI has been reported recently from our laboratory (Koide et al., 1977a). It is a glycoprotein (mol wt 124 000) composed of two similar or identical polypeptide chains held together by a disulfide bond(s). It contains an active site serine sequence which is homologous to that of other serine proteases (Koide

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

et al., 1977b). Factor XI has also been substantially purified from human plasma (Ratnoff et al., 1961; Ratnoff and Davie, 1962; Schiffman et al., 1963; Kingdon et al., 1964; Wuepper, 1972; Saito et al., 1973; Schiffman and Lee, 1974; Movat and Ozge-Anwar, 1974; Connellan and Castaldi, 1975). It is also composed of two similar or identical chains, and these two chains are held together by a disulfide bond(s) (Wuepper, 1972).

During the coagulation process, factor XI is converted to a serine protease, factor XI_a. The activation reaction is catalyzed by factor XII_a and is enhanced by high molecular weight kininogen and kaolin (Schiffman and Lee, 1974; Schiffman et al., 1975; Saito et al., 1975; Griffin and Cochrane, 1976). Factor XI is also activated by other serine proteases such as trypsin (Wuepper, 1972; Saito et al., 1973; Kato et al., 1974). A change in the molecular structure of human factor XI during the activation reaction has been reported by Wuepper (1972) and Bouma and Griffin (1976).[2] These investigators observed a cleavage of the subunits of factor XI from about 80 000 daltons to 46 000 and 27 000, and 83 000 daltons to 50 000 and 33 000, respectively. These experiments suggest that factor XI consists of two disulfide-linked subunits, each of which is cleaved upon activation.

In the present study, human factor XI has been purified to homogeneity by a method similar to that previously employed for bovine factor XI (Koide et al., 1977a). With this preparation, it was possible to study in detail the mechanism of activation of factor XI employing a homogeneous preparation of factor XII_a (Fujikawa et al., 1977).

Experimental Section

Materials

Bovine factor XII_a (Fujikawa et al., 1977) was kindly provided by Dr. Kazuo Fujikawa. Human antithrombin III was prepared by a slight modification of the method of Kurachi et al. (1976). Bovine carbonic anhydrase, bovine serum albumin, ovalbumin, dithiothreitol, tosyl-L-arginine methyl ester, ammonium persulfate, rabbit brain cephalin, and Coomassie brilliant blue were obtained from Sigma Chemical Co., St. Louis, Mo. Trypsin (4× crystallized) was purchased from Worthington Biochemical Co., Freehold, N.J. Heparin lithium salt (158 USP units/mg) was a product of Riker Laboratories, Inc., Northridge, Calif. Polybrene (hexadimethrine bromide), benzamidine hydrochloride, and diisopropyl phosphorofluoridate (DFP)[3] were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Tritium-labeled DFP (0.25 mCi/0.051 mg in 0.25 mL) and Aquasol were purchased from New England Nuclear, Boston, Mass. Cyanogen bromide was obtained from Baker Chemical Co., Phillipsburg, N.J. Acrylamide, 2-mercaptoethanol, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Phillipsburg, N.J. CM-Sephadex C-50, Sephadex G-150, Sephadex G-75, and Sepharose 4B were products of Pharmacia Fine Chemicals, Piscataway, N.J. *N*-Benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide hydrochloride (S-2160) was a product of Kabi Diagnostica, Stockholm, Sweden. Kaolin was obtained from Matheson Coleman and Bell, Norwood, Ohio, and sodium dodecyl sulfate was obtained from British Drug House, Poole, England. Phosphorylase *b* and myosin were kindly provided by Drs. Edmond Fischer and Walter Kisiel, respectively, in our department, and bovine factor XI deficient plasma was a generous gift of Dr. G. Kociba of Ohio State University. Human Fitzgerald factor deficient plasma and human Fletcher factor deficient plasma were purchased from George King Biochemicals, Salem, N.H. Human factor XII deficient plasma from a Hageman trait patient in the Seattle area was kindly provided by Dr. Gottfried Schmer in our department. Benzamidine-agarose with an ε-aminocaproic acid spacer was prepared by a modification of the procedure of Schmer (1972) as previously described (Fujikawa et al., 1977). Heparin-agarose was prepared by the cyanogen bromide method of Fujikawa et al. (1977). All other chemicals were commercial preparations of the highest quality available.

Methods

Protein concentrations were determined by absorption at 280 nm employing an $E_{280}^{1\%}$ of 5.7 for human antithrombin III (Kurachi et al., 1976) and 14.2 for bovine factor XII and factor XII_a (Fujikawa et al., 1977). The protein concentrations for factor XI and factor XI_a were also determined by absorption employing an $E_{280}^{1\%}$ of 13.4. This value was determined in the analytical ultracentrifuge for factor XI employing the methods of Babul and Stellwagon (1969) and Richards et al. (1968).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (1969) as modified by Kurachi et al. (1976). Samples were run at 6 mA/tube for 2.5 h employing 6.0 or 7.5% gels. All gels were stained for protein with Coomassie brilliant blue.

Amino acid analyses and the 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 6 N HCl at 110 °C for 24, 48, 72, and 96 h 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-h hydrolysis time, and cystine was determined as cysteic acid by the method of Hirs (1967). Tryptophan was determined by the spectrophotometric procedure of Edelhoch (1967).

Hexosamine was determined after hydrolysis of the sample (200 μg) in 95% glacial acetic acid containing 0.5 N H_2SO_4 for 16 h at 80 °C according to the method of Laine et al. (1974). Inositol was employed as an internal standard. Hexose and *N*-acetylneuraminic acid were determined after hydrolysis of the sample (200 μg) in 1.0 N methanolic hydrochloride for 5 h at 80 °C using inositol as an internal standard according to the method of Esselman et al. (1972). Sugars were identified and quantitated by gas chromatography and mass spectrometry employing a Hewlett-Packard Model 402 gas chromatograph and a Finnigan Model 3300 gas chromatograph-mass spectrometer (Esselman et al., 1972; Lindberg, 1972; Laine et al., 1974).

Automated Edman degradations were performed with a Beckman sequenator Model 890C. The mode of operation of the instrument and the methods of sequenator analysis are adaptations of the technique of Edman and Begg (1967). The specific program employed was the dimethylbenzylamine system of Hermodson et al. (1972). Phenylthiohydantoin (Pth) amino acids were identified and quantitated by high pressure liquid chromatography (Bridgen et al., 1976) or by gas chromatography after silylation. In the quantitation of amino-terminal residues, protein concentration was determined by amino acid analysis after hydrolysis of the sample in 6 N HCl.

[2] After this manuscript was submitted, a full report on human factor XI by B. N. Bouma and J. H. Griffin appeared in print ((1977), *J. Biol. Chem. 252*, 6432).

[3] Abbreviations used: DFP, diisopropyl phosphorofluoridate; Pth, phenylthiohydantoin; CM, carboxymethyl.

The analysis of factor XI was carried out on a sample (2.8 mg) that was reduced and carboxymethylated according to Crestfield et al. (1963). The protein was separated from the salt and excess reagents by gel filtration on a Sephadex G-50 column (2.5 × 30 cm) in 9% formic acid. The sequence analysis of peak 2 (Figure 4) was carried out on 3.8 mg of carboxymethyl protein, and the analysis of peak 3 (Figure 4) was carried out on 2.5 mg of carboxymethyl protein. The analysis of the cyanogen bromide fragment containing the active site was carried out on 0.2 mg of polypeptide.

The activation of factor XI by factor XII_a was carried out as follows: 0.1 mL of factor XI (1 mg/mL in 0.025 M Tris-HCl, pH 7.5, containing 0.15 M NaCl) was added to 0.01 mL of the same buffer with or without 0.05 M $CaCl_2$ and incubated at 37 °C for 1 min. To this incubation mixture was added 2.5 μL of factor XII_a (0.8 mg/mL in 0.02 M Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl) and aliquots (10 μL) were removed and assayed for esterase activity and coagulant activity.

The large scale preparation of factor XI_a was carried out as follows: factor XI (9 mg) in 19 mL of 0.025 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl was added to 0.5 mL of 0.5 M Tris-HCl buffer (pH 7.5) and incubated at 37 °C for 5 min. Factor XII_a (0.3 mg in 0.4 mL of 0.025 M Tris-HCl buffer, pH 7.5) was then added and the reaction mixture was incubated for 3 h. Aliquots were examined for esterase activity and also sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments were performed to ensure that the reaction went to completion. The incubation mixture was then dialyzed extensively against 9% formic acid and lyophilized.

The preparation of factor XI_a radiolabeled with DFP was as follows: factor XI_a (3 mg) was incubated with 0.2 mL of [^{3}H]DFP (0.05 mg containing 198 μCi) in 6 mL of 0.025 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. After incubation for 1 h at room temperature, the sample was dialyzed extensively against 9% formic acid and lyophilized.

The separation of the heavy and light chains of factor XI_a was carried out as follows: radiolabeled factor XI_a (approximately 9 mg) was reduced and carboxymethylated in 2 mL of 0.5 M Tris-HCl buffer (pH 8.6) containing 6 M guanidine hydrochloride and 0.2% ethylenediaminetetraacetic acid by the method of Crestfield et al. (1963). The reaction mixture was then applied directly to a Sephadex G-150 column (1.6 × 100 cm) previously equilibrated with 9% formic acid containing 3 M urea. The polypeptides were then eluted by 9% formic acid containing 3 M urea. Aliquots (10 μL) from each tube were added to 10 mL of Aquasol solution and radioactivity was measured in a Beckman liquid scintillation counter LC100. The two main protein peaks were pooled separately and desalted by passing through a Sephadex G-50 column (2.5 × 30 cm) in 9% formic acid and then each was lyophilized.

Cyanogen bromide digestion was carried out by dissolving 3 mg of the salt-free light chain of radiolabeled factor XI_a and 12 mg of cyanogen bromide in 2 mL of 70% formic acid, and the reaction was allowed to proceed for 24 h at 4 °C. After lyophilization, the digest was fractionated by gel filtration on a Sephadex G-75 superfine column (1.5 × 85 cm). The column, previously equilibrated with 9% formic acid, was eluted with 9% formic acid at a flow rate of 4.0 mL/h.

The inhibition of factor XI_a by antithrombin III was carried out essentially as previously described (Kurachi et al., 1976).

For the coagulant assay of factor XI, the sample was diluted 100- to 500-fold with 0.02 M Tris-HCl buffer (pH 7.4) containing 0.10 M NaCl and 1.0 mg/mL bovine serum albumin. The sample (0.1 mL) was then incubated at 37 °C for 10 min (unless mentioned otherwise) with 0.05 mL of factor XI deficient plasma and 0.1 mL of kaolin suspension (50 mg of kaolin/10 mL of saline). One-tenth milliliter of the stock cephalin solution and 0.1 mL of 0.05 M $CaCl_2$ were then added and the clotting time was determined. Activity was calculated from a calibration curve where the log of factor XI concentration was plotted against the log of the clotting time. This plot was linear from 45 to 150 s when citrated normal human plasma was used as a source of factor XI. One unit of activity was defined as that amount of activity present in 1 mL of normal plasma. For the assay of factor XI_a, the preincubation time for the sample was reduced to 30 s with kaolin or with 0.1 mL saline. For some assays, factor XI_a activity was also measured in the presence and absence of kaolin with a 10-min preincubation period.

The assays for factor XII, prekallikrein, and high molecular weight kininogen with factor XII deficient plasma, Fletcher factor deficient plasma, and Fitzgerald factor deficient plasma were carried out in a similar manner. In these experiments, the sample (0.05 mL) was incubated with 0.05 mL of kaolin and 0.05 mL of the respective deficient plasma for 2 min at room temperature. Cephalin (0.05 mL) and 0.05 mL of 0.025 M $CaCl_2$ were then added and the clotting time was determined.

Factor XI_a was measured for esterase activity at 30 °C as follows: a sample containing 1 to 10 μg of protein was added to 1.0 mL of 1.0 mM tosyl-L-arginine methyl ester in 0.025 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and the solution was mixed immediately. The initial velocity of hydrolysis was followed by the change in absorption at 247 nm with a Beckman-Gilford recording spectrophotometer. One unit of esterase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of ester substrate per min in 1 mL reaction mixture (Walsh, 1970).

The amidase activity of factor XI_a was determined in a similar manner except *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide was employed as substrate. The initial velocity was measured by following the change in absorption at 405 nm. The micromoles of substrate hydrolyzed were calculated using a molar extinction coefficient of $E_{405}^{1M} = 10\,600$ for free *p*-nitroaniline.

Purification of Human Factor XI. All operations were performed at 4 °C and direct contact of the samples with glass was avoided by employing siliconized glassware and plastic containers. Human citrated cryosupernatant (15 L) was mixed with 600 mL of 1 M barium chloride and the mixture was stirred for 30 min at 4 °C (Di Scipio et al., 1977). The barium citrate pellet was removed by centrifugation at 5000*g* for 15 min and the supernatant was made 0.1 mM in ethylenediaminetetraacetic acid. Subsequent steps, including ammonium sulfate fractionation, first heparin-agarose column chromatography, and CM-Sephadex column chromatography, were carried out as previously described for the purification of bovine factor XI by Koide et al. (1977a). Following the CM-Sephadex column chromatography, the solution was concentrated to about 100 mL by ultrafiltration employing an Amicon Diaflow concentrator (PM-30 membrane) and the sample was then dialyzed overnight against 2 L of 0.05 M phosphate buffer (pH 6.6) containing 0.15 M NaCl. The solution was then applied to a second heparin-agarose column as described for the purification of bovine factor XI (Koide et al., 1977a). Following the second heparin-agarose, the sample was dialyzed overnight against 2 L of 0.05 M imidazole hydrochloride buffer (pH 6.3) containing 0.15 M NaCl and then applied to a benzamidine-agarose column (2.5 × 15 cm). This column was previously equilibrated with 0.05 M imidazole buffer (pH 6.3)

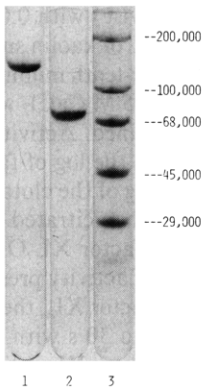

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of human factor XI. Electrophoresis was carried out in 6% polyacrylamide gels at 6 mA/tube for 2.5 h as described in Methods. Gel 1 is 10 μg of factor XI before reduction. Gel 2 is 10 μg of factor XI after reduction. Gel 3 includes the following standards: myosin (mol wt 200 000), phosphorylase *b* (100 000), bovine serum albumin (68 000), ovalbumin (45 000), and carbonic anhydrase (29 000). The anode was at the bottom of the gels.

containing 0.05 M NaCl. The column was washed with 250 mL of 0.05 M imidazole buffer (pH 6.3) containing 0.1 M NaCl and factor XI was eluted with 0.05 M imidazole buffer (pH 6.3) containing 0.1 M NaCl and 0.1 M guanidine hydrochloride. The flow rate was 80 mL/h. Fractions (5 mL) containing factor XI were pooled and concentrated to about 10 mL by ultrafiltration using an Amicon concentrator (PM-30 membrane). Some preparations containing a minor contaminant were further purified by gel filtration. In these experiments, the sample (10 mL) was applied to a Sephadex G-150 column (2.5 × 100 cm) previously equilibrated with 0.025 M Tris-HCl buffer (pH 7.0) containing 0.15 M NaCl. Factor XI appeared in the second peak when elution was carried out with 0.025 M Tris-HCl buffer (pH 7.0) containing 0.15 M NaCl. Fractions containing factor XI were then pooled and concentrated to 0.5–1.0 mg/mL and stored at −70 °C.

Results

Preparation of Human Factor XI. The isolation procedure employed for human factor XI involved the removal of the vitamin K dependent proteins by barium citrate adsorption, followed by ammonium sulfate fractionation, heparin–agarose column chromatography, CM-Sephadex chromatography, a second heparin–agarose column chromatography, and benzamidine–agarose column chromatography (details described in Methods). This procedure gave an overall purification of about 35 000–40 000-fold with a recovery of 20–25%. By this method, it was possible to isolate 5–8 mg of purified protein from 15 L of human plasma. The specific activity of the final product ranged from 700 to 780 units/mg protein employing bovine factor XI deficient plasma in the regular 10-min preincubation assay as described in Methods. In this assay, 25 ng of factor XI gave a clotting time of 60 s in the presence of kaolin and greater than 300 s in the absence of kaolin. The final preparation contained no detectable factor XII, prekallikrein, or high molecular weight kininogen activity when measured in human factor XII deficient plasma, human prekallikrein deficient plasma, or human Fitzgerald factor deficient plasma.

Characterization of Human Factor XI. A single, sharp protein band was observed for human factor XI when it was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1, gel 1). After reduction with 2-mercaptoethanol, a single, faster moving protein band was obtained (Figure 1, gel 2). The apparent molecular weights for factor XI before and after reduction were estimated at 140 000 and 75 000, respectively.

TABLE I: Amino Acid and Carbohydrate Compositions of Human and Bovine Factor XI.

Component	Human Factor XI (Residues/ 124 000)	Bovine Factor XI[a] (Residues/ 124 000)
Amino acid		
Lys	62.8	55.6
His	28.6	38.9
Arg	33.8	40.9
Asp	69.4	82.9
Thr	82.6	75.7
Ser	93.4	79.9
Glu	106.8	123.8
Pro	52.0	52.6
Gly	70.2	68.7
Ala	49.0	44.1
½-Cystine	36.4[b]	42.3
Val	58.8	46.8
Met	7.2	11.6
Ile	49.6	50.2
Leu	71.8	72.1
Tyr	27.2	29.4
Phe	39.4	41.6
Trp	20.8[c]	20.7
Carbohydrate		
Hexose	4.1 (0.6%)	37.2 (5.4%)
N-Acetylhexosamine	15.3 (2.7%)	26.6 (4.7%)
N-Acetylneuraminic acid	7.3 (1.7%)	4.3 (1.0%)
Protein (%)	95.0	88.9
Carbohydrate (%)	5.0	11.1

[a] From Koide et al. (1977a). [b] Determined as cysteic acid according to Hirs (1967). [c] Determined spectrophotometrically according to Edelhoch (1967).

The amino acid and carbohydrate compositions of human factor XI are shown in Table I. The composition of bovine factor XI is included for comparison. Human factor XI is a glycoprotein composed of 95% protein and 5% carbohydrate. The carbohydrate includes 0.6% hexose, 2.7% *N*-acetylhexosamine, and 1.7% *N*-acetylneuraminic acid. The amino acid composition shows some similarity to that of bovine factor XI, although a marked difference in some of the amino acids is apparent. In these calculations, we have tentatively assumed a molecular weight of 124 000 for human factor XI (see Discussion).

An amino-terminal analysis was then carried out on reduced and carboxymethylated factor XI. Glycine was identified as the amino-terminal residue of this protein. The amino acid sequence of the first 15 residues was Gly-Cys-Val-Thr-Gln-Leu-Leu-Lys-Asp-Thr-Gln-Phe-Glu-Gly-Gly. The yield for residues 1 through 15 was 0.2, 0.4, 0.3, 0.4, 0.4, 0.4, 0.2, 0.4, 0.4, 0.3, 0.3, 0.3, 0.3, 0.2, and 0.2 equiv per 62 000 g of protein, respectively. These data suggest that the two chains in factor XI are probably identical or that one of the chains has a blocked amino-terminal residue.

Activation of Human Factor XI. A time curve for the activation of human factor XI by bovine factor XII_a is shown in Figure 2. The activation reaction was performed with an enzyme-to-substrate ratio of 1 to 50 at pH 7.4. Under these conditions, the reaction went to completion in about 100 min (solid circles). In these experiments, tosyl-L-arginine methyl ester was employed as a substrate for the assay of factor XI_a.

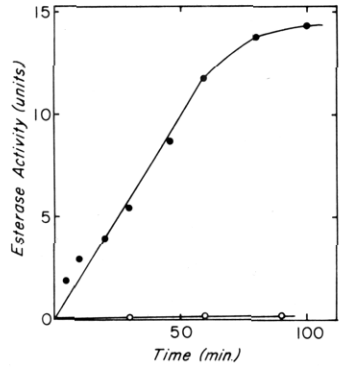

FIGURE 2: Time course for the activation of human factor XI by bovine factor XII_a. The incubation mixtures contained factor XI (0.89 mg/mL) and factor XII_a (0.018 mg/mL) or factor XII (0.018 mg/mL) in 0.025 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. The incubation temperature was 37 °C. At various times, aliquots (10 μL) were removed and directly assayed for esterase activity using tosyl-L-arginine methyl ester as substrate as described in Methods. (●—●) Factor XI plus factor XII_a; (○—○) factor XI plus factor XII.

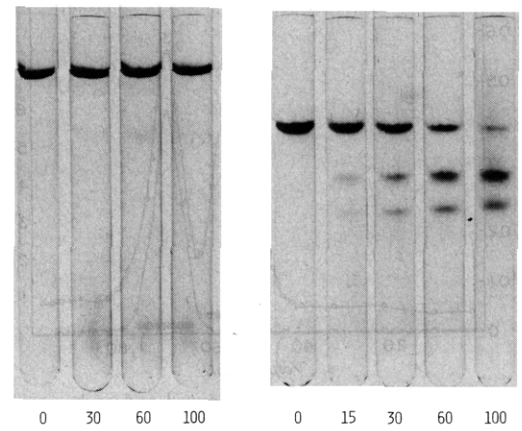

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of human factor XI following activation by bovine factor XII_a. The activation mixture was the same as that described in Figure 2. At various times, aliquots (10 μL) were removed and subjected to electrophoresis employing 6% gels as described in Methods. The left panel shows the gels run in the absence of 2-mercaptoethanol, and the right panel shows the gels run in the presence of 2-mercaptoethanol. Each gel contained about 9 μg of protein. The numbers under each panel refer to the incubation time in minutes. The anode was at the bottom of the gels.

Little, if any, activation of factor XI occurred when factor XII was substituted for factor XII_a (open circles). Also, the activation reaction was not influenced by the presence of $CaCl_2$ at a final concentration of 5 mM.

The specific activity of human factor XI_a in the esterase assay was 18 μmol of tosyl-L-arginine methyl ester hydrolyzed per min per mg of enzyme. The enzyme was assayed at 30 °C with 1.0 mM substrate in 0.025 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl in a final volume of 1.0 mL. Under the same assay conditions, pancreatic trypsin hydrolyzed 165 μmol of substrate per min per mg of protein. Thus, the specific esterase activity of factor XI_a is about 10% of that found for trypsin.

Factor XI_a also has weak amidase activity toward *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide. The specific activity toward this substrate was 1.05 μmol of substrate hydrolyzed per min per mg of enzyme. The substrate concentration was 0.10 mM in 0.025 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. The final volume was 1.0 mL and the assay was carried out at 30 °C.

A marked increase in the coagulant activity was also noted during the activation reaction, and this increase occurred in parallel with the esterase activity. The increase in coagulant activity was observed only when the sample containing factor XI and/or factor XI_a was preincubated with the factor XI deficient plasma and kaolin for 30 s prior to the addition of the phospholipid and calcium ions. Under these conditions, factor XI (200 ng) gave a clotting time of 164 s in bovine factor XI deficient plasma in the presence of kaolin. In the absence of kaolin, the clotting time was greater than 300 s. Factor XI_a (200 ng) gave a clotting time of 84 s in the presence of kaolin and 100 s in the absence of kaolin. Thus, the increase in coagulant activity when factor XI was converted to factor XI_a was about tenfold when measured in the 30-s preincubation assay in the presence of kaolin. These data also indicate that factor XI_a has only about 50% of its coagulant activity expressed when assayed in the absence of kaolin. Also, the 30-s preincubation assay detects only about 10% of the total clotting activity which is measured in the 10-min preincubation assay. In the latter assay, however, no increase in coagulant activity was observed when factor XI was compared with factor XI_a. Under these conditions, the conversion of factor XI to factor XI_a was not one of the rate-limiting reactions leading to fibrin formation.

Evidence for a change in the structure of factor XI during the activation reaction was demonstrated in experiments employing sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In these investigations, aliquots were removed at various times from a reaction mixture corresponding to that shown in Figure 2 and analyzed by gel electrophoresis. No apparent change was noted in samples subjected to gel electrophoresis in the absence of reducing agent (Figure 3, left panel). This suggests that little, if any, change in the molecular weight of factor XI has occurred during its conversion to factor XI_a. A difference in the gel electrophoresis pattern of factor XI during the activation reaction was noted, however, after reduction of the samples (Figure 3, right panel). At zero time, a single protein band (apparent mol wt 75 000) was observed. This band is characteristic of reduced factor XI (Figure 1, gel 2). During the first 30 min of incubation, two new faster moving bands appeared, and the appearance of these heavy and light chains (apparent mol wt 52 000 and 38 000) occurred in parallel with the formation of factor XI_a esterase activity. After 100 min, essentially all of the 75 000 mol wt band was cleaved into heavy and light chains. These data indicate that factor XI is converted to factor XI_a by cleavage of each of the two chains present in the precursor molecule. These data are also consistent with the suggestion that the two chains in the precursor factor XI molecule are identical and each is cleaved at the same peptide bond during the activation reaction.

Isolation and Partial Characterization of the Heavy and Light Chains of Human Factor XI_a. Factor XI_a is readily inhibited by serine protease inhibitors such as diisopropyl phosphorofluoridate, and this inhibitor is bound to a serine residue in the active site of the enzyme (Ratnoff and Davie, 1962; Kingdon et al., 1964). Accordingly, factor XI_a was partially labeled with radioactive DFP (to approximately 30% inhibition) in order to identify the chain containing the active site serine residue. The radiolabeled factor XI_a was then reduced, S-carboxymethylated, and subjected to gel filtration on Sephadex G-150 to separate the heavy and light chains. Two major fractions were obtained (Figure 4, open circles). The first major peak (peak 2) corresponds to the heavy chain of factor XI_a and migrated as a single, sharp band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (left gel, insert, Figure 4). The second major peak (peak 3) also migrated as a single, sharp band on sodium dodecyl sulfate–polyacrylamide

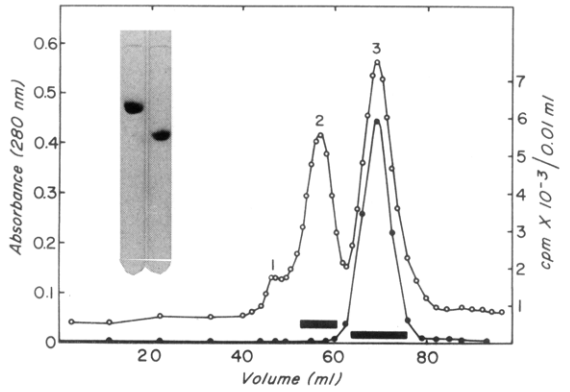

FIGURE 4: Gel filtration pattern of reduced and carboxymethylated human factor XI_a. The radiolabeled sample (3 mg) combined with cold carrier (6 mg) was dissolved in 1.0 mL of 9% formic acid containing 3 M urea and applied to a Sephadex G-150 column (1.6 × 100 cm). The column was eluted with the 9% formic acid–3 M urea, and aliquots were examined for absorbance and radioactivity. Peaks 2 and 3 (shown by the bars) were pooled individually, desalted on a Sephadex G-50 column (1.6 × 20 cm), and lyophilized as described in Methods. (O—O) Absorbance; (●—●) radioactivity. Insert: left gel shows a sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern for peak 2, and the gel on the right is peak 3. Approximately 10 μg of protein was applied to each 7.5% gel.

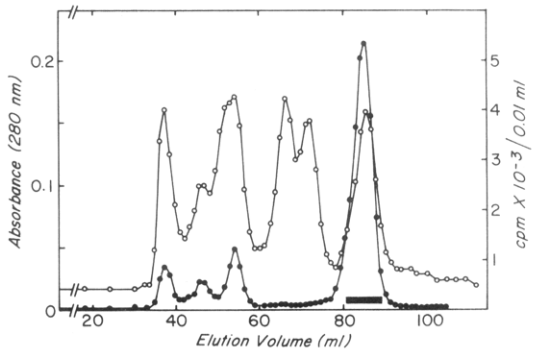

FIGURE 5: Gel filtration pattern of a cyanogen bromide digest of radiolabeled carboxymethylated light chain of human factor XI_a. Approximately 3 mg of the light chain (peak 2, Figure 4) was digested with cyanogen bromide as described in Methods and then applied to a Sephadex G-75 column (1.5 × 85 cm) previously equilibrated with 9% formic acid. The column was eluted with 9% formic acid, and aliquots (10 μL) were removed and measured for radioactivity as described in Methods. The fractions (shown by the bar) were pooled and lyophilized. (O—O) Absorbance; (●—●) radioactivity.

gel electrophoresis (right gel, insert, Figure 4), and this peak corresponds to the light chain of factor XI_a. The light chain also contained all of the radioactivity (solid circles) indicating that it contained the active site serine residue. The minor peak shown in Figure 4 (peak 1) is due to the presence of a small amount of factor XI which had not been converted to factor XI_a.

An amino-terminal analysis was then carried out on the heavy and light chains of factor XI_a in order to establish the origin of these two chains. The amino-terminal residue of the heavy chain was identified as glycine. The amino acid sequence of the first 17 residues was Gly-Cys-Val-Thr-Gln-Leu-Leu-Lys-Asp-Thr-Gln-Phe-Glu-Gly-Gly-Asp-Ile. This is the same sequence as that found in the precursor protein. The yield for residues 1 through 17 was 0.6, 0.4, 0.5, 0.2, 0.3, 0.3, 0.3, 0.4, 0.1, 0.1, 0.2, 0.2, 0.2, 0.1, 0.1, 0.1, and 0.1 equiv per 35 000 g of protein, respectively. These data indicate that the two chains in factor XI give rise to two identical heavy chains in factor XI_a which contain an amino-terminal glycine residue.

The amino-terminal residue of the light chain was identified as isoleucine. The amino acid sequence of the first 16 residues of this chain was Ile-Val-Gly-Gly-Thr-Val-Ala-? -His-Gly-Glu-Trp-Pro-Trp-Gln-Val. The yield for residues 1 through 16 was 0.2, 0.3, 0.2, 0.2, 0.3, 0.2, 0.3, not determined, 0.2, 0.1, 0.2, not quantitated, 0.1, not quantitated, 0.1, and 0.2 equiv per 25 000 g of protein, respectively. No residue was identified in position 8.

The above data indicate that human factor XI_a contains two heavy chains and two light chains held together by disulfide bonds. These experiments also indicate that the heavy chains of factor XI_a originate from the amino-terminal portion of the precursor molecule and the light chains originate from the carboxyl-terminal portion of the precursor molecule following hydrolysis of two internal peptide bonds.

Active Site of Human Factor XI_a. It was then of interest to identify the amino acid sequence in the region of the active site serine in human factor XI_a. Accordingly, the carboxymethyl light chain of human factor XI_a containing the radiolabeled serine residue was digested with cyanogen bromide and the resulting peptides were fractionated on Sephadex G-75. Approximately seven cyanogen bromide fragments were obtained as determined by absorbance at 280 nm (Figure 5, open circles). The last peak contained approximately 85% of the radioactivity (solid circles) and this peak migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (apparent mol wt 6000). An amino-terminal sequence analysis was then carried out on the radiolabeled peptide. The amino-terminal amino acid was identified as isoleucine. The amino acid sequence of the first 20 residues was Ile-Cys-Ala-Gly-Tyr-Arg-Glu-Gly-Gly-Lys-Asp-Ala-Cys-Lys-Gly-Asp-SER-Gly-Gly-Pro. The yield for residues 1 through 20 was 0.6, 0.4, 0.4, 0.4, 0.5, 0.3, 0.2, 0.2, 0.2, 0.1, 0.2, 0.1, 0.1, 0.1, 0.1, 0.1, not quantitated, 0.1, 0.1, and not quantitated when expressed in equivalents per 6000 g of protein, respectively. The active site serine residue, corresponding to serine 195 in chymotrypsin, appeared in position 17 of the peptide and is shown in capital letters. The yield of this residue was poor and was not quantitated. Serine was readily identified, however, in turn number 17 by the presence of a sharp peak of radioactivity. A gradual estrolytic loss of tritium was observed in the first 16 turns, but there was a clear increase in released radioactivity (from 13 000 cpm background to 28 000 cpm) in turn 17, indicating a specific release of Pth-DIP-serine at turn 17. The active site sequence of human factor XI_a is identical with that found in bovine factor XI with the exception that the amino-terminal isoleucine residue in the cyanogen bromide peptide from human factor XI has been replaced by a valine residue in bovine factor XI (Koide et al., 1977b).

The Effect of Antithrombin III on Human Factor XI_a. When factor XI_a was incubated at 37 °C with antithrombin III, a rapid inhibition of esterase activity was observed (Figure 6, solid circles). Furthermore, the rate of inactivation of factor XI_a by antithrombin III was greatly accelerated by the addition of heparin (solid squares). Incubation of factor XI_a with heparin alone had no effect on factor XI_a activity (solid triangles). The effect of increasing concentrations of antithrombin III in the presence of heparin is shown in Figure 7. Complete inhibition of factor XI_a activity was obtained when the molar ratio of inhibitor to enzyme was 2 to 1. In these studies, a molecular weight of 124 000 was employed for human factor XI_a and a molecular weight of 56 000 for human antithrombin III.

The formation of a stable complex between factor XI_a and antithrombin III was also demonstrated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 8). Gels

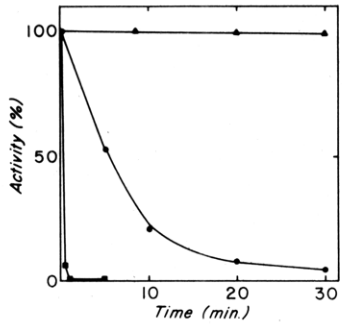

FIGURE 6: Time curve showing the inhibition of human factor XI_a by human antithrombin III. Factor XI_a (3.5×10^{-6} M) was incubated at 37 °C with heparin (1 mg/mL) or antithrombin III (10.7×10^{-6} M) or antithrombin III (10.7×10^{-6} M) plus heparin (1 mg/mL) in 0.025 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. Aliquots (10 μL) were removed at the times shown and assayed for esterase activity as described in Methods. (▲—▲). Factor XI_a plus heparin; (●—●) factor XI_a plus antithrombin III; (■—■) factor XI_a plus antithrombin III plus heparin.

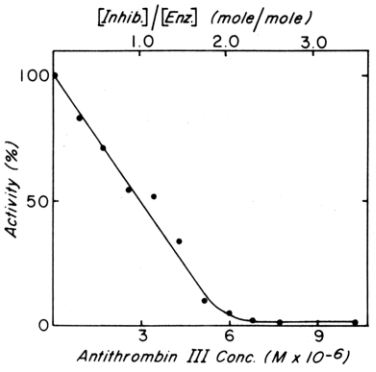

FIGURE 7: Inhibition of human factor XI_a as a function of antithrombin III concentration. The reaction mixture contained 3.1×10^{-6} M factor XI_a and heparin (0.5 mg/mL) in 0.025 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl and increasing concentrations of antithrombin III. After incubation for 5 min at 37 °C, each of the reaction mixtures was assayed for esterase activity.

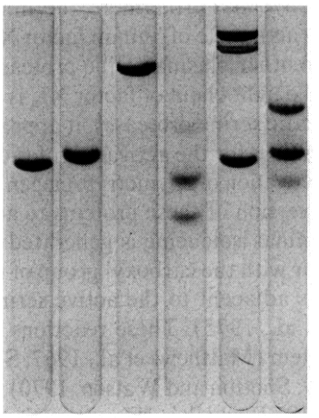

FIGURE 8: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of human factor XI_a incubated with antithrombin III and heparin as described in Figure 7. Gels 1 and 2 contain antithrombin III before and after reduction with 2-mercaptoethanol. Gels 3 and 4 contain factor XI_a before and after reduction with 2-mercaptoethanol. In the experiments shown in gels 5 and 6, factor XI_a was incubated with antithrombin III in the presence of heparin; gel 5 is before reduction; and gel 6 is after reduction. Gels 1 and 2 contained 12 μg of protein; gels 3 and 4 contained 8.6 μg of protein; and gels 5 and 6 contained 12 μg of antithrombin III and 6.0 μg of factor XI_a. The anode was at the bottom of the gels.

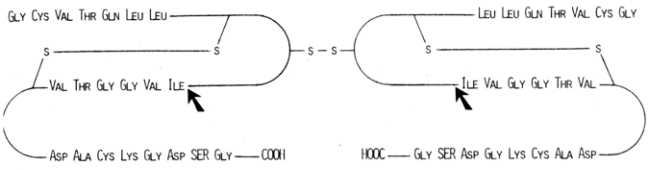

FIGURE 9: Partial structure of human factor XI. The two identical chains are held together by a disulfide bond(s). The active site serine is shown in large caps. The two arrows indicate the site of cleavage in the two identical chains when the protein is converted to factor XI_a by factor XII_a. The resulting four chains are held together by disulfide bonds. The number and exact location of the disulfide bonds are not known.

1 and 2 show antithrombin III before and after reduction with 2-mercaptoethanol, respectively. Gels 3 and 4 show factor XI_a before and after reduction with 2-mercaptoethanol, respectively. Gel 5 shows a reaction mixture containing factor XI_a and antithrombin III (4 molar excess) incubated for 30 min in the presence of heparin. Three protein bands were observed in this gel. The fast-moving band corresponds to residual antithrombin III. The band corresponding to factor XI_a disappeared, but two new slower moving protein bands were present. Each of these two bands contained antithrombin III and factor XI_a. The ratio between these bands varied somewhat from experiment to experiment. This may be due to aggregation of the factor XI_a–antithrombin III complex or the formation of an enzyme–inhibitor complex containing two different amounts of heparin. When the mixture containing the enzyme–inhibitor complex was reduced with 2-mercaptoethanol, three protein bands were observed (gel 6). The fast-moving band corresponds to the heavy chain of factor XI_a. The middle band corresponds to the residual antithrombin III (also seen in gel 5). The slow-moving band corresponds to a complex of antithrombin III and the light chain of factor XI_a. No free light chain of factor XI_a was present in this gel. These data indicate that a very stable complex was formed between factor XI_a and antithrombin III, and this complex involved both of the light chains of factor XI_a which contain the active site serine residue.

Discussion

The present experiments demonstrate that human factor XI is composed of two identical polypeptide chains held together by a disulfide bond(s) (Figure 9). The amino-terminal residue is glycine. Factor XI is converted to factor XI_a by factor XII_a by the cleavage of an internal peptide bond in each of the two identical chains (shown by the two arrows). This gives rise to two heavy chains and two light chains in factor XI_a. Thus, factor XI_a is composed of four polypeptide chains, and these chains are linked by disulfide bonds. The formation of factor XI_a should involve an intermediate containing three polypeptide chains and one active site per mole of enzyme. This intermediate, however, was not isolated. No evidence was obtained for the formation of an activation peptide(s) during the activation reaction.

The specific bond cleaved during the activation reaction has not been identified. It probably involves a basic amino acid such as arginine or lysine since factor XII_a hydrolyzes an Arg-Val bond in factor VII (Kisiel et al., 1977) and has esterase activity toward tosyl-L-arginine methyl ester (Fujikawa et al., 1977) and benzoyl-L-arginine ethyl ester (Schoenmakers et al., 1965; Komiya et al., 1972) and amidase activity toward *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide (Fujikawa et al., 1977).

The new amino-terminal sequence of Ile-Val-Gly-Gly in the light chain of factor XI_a shows considerable homology with the other plasma serine proteases involved in blood coagulation

(Fujikawa et al., 1977). Furthermore, the amino acid sequence surrounding the active site of human factor XI_a is very similar to that found in other plasma serine proteases (Koide et al., 1977b). Since the light chain of factor XI_a is also homologous with the pancreatic serine proteases, it appears probable that the basic mechanism for the activation of factor XI is similar to that for the activation of chymotrypsinogen and trypsinogen. During the conversion of these proteins to active enzymes, a new amino-terminal isoleucine is generated and this residue forms an ion pair with the carboxyl group of the aspartic acid residue which is adjacent to the active serine (Sigler et al., 1968; Stroud et al., 1975). These reactions then lead to the charge relay system (Matthews et al., 1967; Sigler et al., 1968; Blow et al., 1969; Shotton and Watson, 1970) or a proton relay system (Kraut, 1977) which is characteristic of the pancreatic serine proteases. Factor XI also contains an aspartic acid six residues prior to the active site serine. In trypsin, this aspartic acid residue is located in the bottom of the binding pocket and forms an ion pair with a basic amino acid residue in the substrate (Mares-Guia and Shaw, 1965; Stroud et al., 1971; Ruhlmann et al., 1973; Blow et al., 1974; Sweet et al., 1974; Krieger et al., 1974). Accordingly, it seems probable that the aspartic acid occupying a similar position in factor XI_a gives this enzyme its specificity toward basic amino acids. This suggestion is consistent with the fact that factor XI_a cleaves a specific Arg-Ala and a specific Arg-Val bond in factor IX (Lindquist et al., 1978). It is also consistent with the esterase and amidase activity of factor XI_a for small substrates such as tosyl-L-arginine methyl ester, benzoyl-L-arginine ethyl ester, and *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginyl-*p*-nitroanilide.

Factor XI_a also has a marked increase in coagulant activity when compared with factor XI. An increase in coagulant activity during the activation of factor XI was only observed when the sample was preincubated with factor XI deficient plasma for 30 s prior to the addition of phospholipid and calcium ions. Under these conditions, the total factor XI activity expressed in the coagulant assay was much lower than that obtained after a 10-min preincubation. With the 10-min preincubation assay, however, no difference was noted in the coagulant activity of factor XI and factor XI_a.

The maximal clotting activity of factor XI_a still required the presence of kaolin in the factor XI deficient assay. Kaolin has no effect on the activation of factor IX by factor XI_a in an activation reaction containing purified components. Thus, more experiments are required to explain the effect of kaolin in the assay of factor XI employing deficient plasma.

The presence of two active sites per mole of factor XI_a represents, to our knowledge, the first case of a serine protease with two catalytic sites. The presence of two active sites in factor XI_a was clearly shown by inhibition studies employing increasing concentrations of antithrombin III (Figure 7). Similar results have also been obtained with bovine factor XI_a (K. Kurachi, unpublished results).

A dimeric structure for factor XI_a is analogous to that of seminal ribonuclease BS-1. This hydrolytic enzyme has a dimeric structure and the two subunits are linked by two disulfide bonds (D'Alessio et al., 1975).

The inhibition of human factor XI_a by antithrombin III confirms the earlier data of Damus et al. (1973) who employed partially purified preparations of human factor XI_a. In the present studies, it was also possible to demonstrate the formation of a complex between enzyme and inhibitor by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Furthermore, it was shown that the binding of the inhibitor to the enzyme occurred via the two light chains in factor XI_a which contain the catalytic site.

Human factor XI has an apparent molecular weight of 140 000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This value is probably high, however, since glycoproteins tend to bind less amounts of detergent resulting in high estimates of molecular weight (Segrest and Jackson, 1972). Accordingly, a molecular weight of 124 000 was employed for the composition data shown in Table I. This value was obtained previously for bovine factor XI by sedimentation equilibrium studies (Koide et al., 1977a). Thus, it is probable that the individual chains in human factor XI, like bovine factor XI, have a molecular weight of about 60 000. This value is consistent with the fact that the subunits of human and bovine factor XI comigrate on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (K. Kurachi, unpublished results) and the subunits of bovine factor XI have a molecular weight of 60 000 as determined by sedimentation equilibrium studies (Koide et al., 1977a). Thus, the molecular weights of the heavy and light chains of factor XI_a estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are also probably high and their correct values may be closer to 35 000 and 25 000, respectively. A summation of these two values would then give a molecular weight of about 60 000 for the subunits of factor XI_a. It is clear that some revisions for these molecular weight estimates will be necessary in the future when more accurate data become available.

In previous experiments, Wuepper (1972) and Bouma and Griffin (1976) have reported the cleavage of human factor XI into heavy and light chains during the activation. Also, Schiffman et al. (1975) and Griffin and Cochrane (1976) have found that the activation of factor XI by factor XII is stimulated by the presence of high molecular weight kininogen and kaolin. In these experiments, stoichiometric amounts of high molecular weight kininogen were required for optimal activation of factor XI (Griffin and Cochrane, 1976). Accordingly, it was suggested that high molecular weight kininogen and factor XII form a complex on the kaolin surface, and this leads to the activation of factor XI. Very high concentrations of factor XII relative to factor XI were employed in these experiments. Also, the factor XII was probably a single-chain preparation. In the present investigation, a homogeneous preparation of bovine factor XII_a was used as the enzyme. This enzyme is composed of a heavy and light chain held together by a disulfide bond(s) and readily activated factor XI at an enzyme-to-substrate ratio of 1 to 50. Under these conditions, the reaction occurred in the absence of high molecular weight kininogen and kaolin. Whether this reaction will also be stimulated by high molecular weight kininogen and kaolin remains to be tested.

Acknowledgments

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