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Mechanism of Activation of Bovine Factor XI by Factor XII and Factor XII_a†

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ABSTRACT: Factor XI (plasma thromboplastin antecedent) is one of several plasma proteins that participate in the contact phase of blood coagulation. Bovine factor XI (M_r 124 000) is a glycoprotein composed of two nearly identical polypeptide chains held together by a disulfide bond(s). The amino-terminal sequence of each of the two chains is Glu-Cys-Val-Thr-Thr-Leu-Phe-Gln-Asp-Ala-Cys-Phe-Lys-Gly-Gly-Asp-, a sequence which shows considerable homology with the amino-terminal sequence of bovine plasma prekallikrein. During the coagulation cascade, factor XI is converted by limited proteolysis to factor XI_a by either factor XII (Hageman factor) or factor XII_a. The activation of factor XI by factor XII requires the presence of sulfatide, dextran sulfate, or kaolin plus high molecular weight kininogen. In these reactions, factor XII, a zymogen, participates as an enzyme in the conversion of factor XI to factor XI_a. Accordingly, this reaction appears to be a very specific substrate-induced catalysis by a zymogen protein. Factor XII_a, a well-characterized serine protease, also activates factor XI in a reaction stimulated by sulfatide, dextran sulfate, or kaolin plus high molecular weight kininogen. During the activation of factor XI, each of the two chains of the zymogen is cleaved. This gives rise to factor XI_a composed of two heavy chains (M_r ~35 000 each) and two nearly identical light chains (M_r ~24 000 and ~26 000), and these four chains are held together by disulfide bonds. The heavy chains originate from the amino-terminal portion of the precursor molecule, while the light chains originate from the carboxyl-terminal portion of the precursor molecule. The amino-terminal sequence of each of the light chains is Ile-Val-Gly-Gly-Thr-Gln-Ala-Val-?-Gly-Glu-Trp-Pro-Tyr-Gln-Ile-. The light chains also contain an active-site serine residue. These data indicate that factor XI is converted to a serine protease by limited proteolysis, and this reaction, catalyzed by either factor XII or factor XII_a, readily occurs in the presence of sulfatide, dextran sulfate, or kaolin plus high molecular weight kininogen.

Factor XI (plasma thromboplastin antecedent),[1] factor XII (Hageman factor), prekallikrein, and HMW kininogen[2] participate in the early or contact phase of blood coagulation (Kaplan, 1978). When the coagulation process is initiated in the presence of a surface, a series of reactions are triggered, leading to an enzyme cascade and fibrin formation (Davie et al., 1979). The various clotting factors participating in this enzyme cascade are present in plasma in a precursor form, and most are converted to serine proteases during the coagulation process. This includes factor XI which has been extensively purified and characterized from human and bovine plasma (Koide et al., 1977a; Bouma & Griffin, 1977; Kurachi & Davie, 1977). Factor XI is composed of two similar or identical polypeptide chains, and these two chains are held together by a disulfide bond(s). The conversion of human factor XI to factor XI_a by factor XII_a has been studied in detail (Kurachi & Davie, 1977; Bouma & Griffin, 1977). This reaction involves the cleavage of an internal peptide bond in each of the two chains of the precursor molecule, giving rise to a serine protease called factor XI_a. Accordingly, human factor XI_a is composed of two identical heavy chains (M_r 35 000 each) and two identical light chains (M_r 25 000 each), and these four chains are held together by disulfide bonds. The activation of human factor XI, as well as the activation of factor XII and prekallikrein, is stimulated by the presence of kaolin and HMW kininogen (Griffin & Cochrane, 1976; Webster et al., 1976; Schiffman et al., 1977; Saito, 1977; Bouma & Griffin, 1977; Liu et al., 1977; Fujikawa et al., 1980).

In the present study, the mechanism of activation of bovine factor XI by factor XII as well as factor XII_a has been studied in detail. The data provide clear evidence that factor XII, a

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[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959). In the present studies, factor XII refers to the single-chain molecule (M_r 74 000) and factor XII_a refers to the two-chain molecule (M_r 74 000) held together by a disulfide bond(s).
[2] Abbreviation used: HMW kininogen, high molecular weight kininogen.

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single-chain zymogen, or factor XII_a, a two-chain molecule held together by a disulfide bond(s), catalyzes the activation of bovine factor XI by limited proteolysis. These reactions are stimulated by sulfatide, dextran sulfate, or kaolin plus HMW kininogen.

Experimental Section

Materials. Bovine factor XII and factor XII_a were purified to homogeneity as previously described (Fujikawa et al., 1977a,b). HMW kininogen was purified by a modification (Fujikawa et al., 1980) of the method of Komiya et al. (1974). Bovine antithrombin III was isolated as previously described (Kurachi et al., 1976). Rabbit antibody raised against bovine factor XII was prepared by the method of Fujikawa et al. (1977a) and pretreated with 1 mM diisopropyl fluorophosphate to inhibit any contaminating serine protease(s). Tosyl-L-arginine [^{3}H]methyl ester and sodium boro[^{3}H]hydride were purchased from Amersham/Searle, Arlington Heights, IL. Bovine brain sulfatide was kindly provided by Dr. S. Hakomori, Fred Hutchinson Cancer Research Center, Seattle, WA. 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. Heparin lithium salt (158 USP units/mg) was a product of Riker Laboratories, Inc., Northridge, CA. Polybrene (hexadimethrine bromide), benzamidine hydrochloride, and diisopropyl fluorophosphate were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Tritium-labeled diisopropyl fluorophosphate (0.25 mCi/0.051 mg in 0.25 mL), EN^{3}HANCE, and Aquasol were purchased from New England Nuclear, Boston, MA. Cyanogen bromide was obtained from Baker Chemical Co., Phillipsburg, NJ. Acrylamide, 2-mercaptoethanol, *N,N'*-methylenebis(acrylamide), and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Kodak Co., Rochester, NY. CM-Sephadex C-50, Sephadex G-150, Sephadex G-75, Sepharose 4B, and dextran sulfate (M_r ~500 000) were products of Pharmacia Fine Chemicals, Piscataway, NJ. Kaolin was obtained from Matheson Coleman and Bell, Norwood, OH, and sodium dodecyl sulfate was obtained from British Drug House, Poole, England. 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., 1977a). Heparin–agarose was prepared by the cyanogen bromide method of Fujikawa et al. (1973). Dextran sulfate was covalently linked to Sepharose 4B by the method of Pepper & Prowse (1977). Phosphorylase *b* and myosin were provided by Dr. E. Fischer in our department. The purified protease inhibitor from Russell's viper venom (RVV inhibitor II) was a gift from Dr. W. Kisiel in our department.

Bovine factor XI deficient plasma was provided by Dr. G. Kociba of The Ohio State University. Human Fitzgerald factor deficient plasma and human Fletcher factor deficient plasma were purchased from George King Biomedicals, Salem, NH. Human factor XII deficient plasma from a Hageman trait patient in the Seattle area was supplied by Dr. G. Schmer in our department. All other chemicals were commercial preparations of the highest quality available.

Methods. Protein concentrations were determined by absorption at 280 nm by employing an $E_{280}^{1\%}$ of 12.6 for bovine factor XI (Koide et al., 1977a); 12.6 was also employed for factor XI_a. A value of 14.2 was employed for factor XII and factor XII_a (Fujikawa et al., 1977a), 7.4 was employed for HMW kininogen (Komiya et al., 1974), 6.0 was employed for antithrombin III (Kurachi et al., 1976), and 5.5 was employed for RVV inhibitor II (Iwanaga et al., 1976). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as previously described (Kurachi & Davie, 1977) according to Weber & Osborn (1969) or Laemmli (1970). Samples were run at 7 mA/tube for 2.5 h by employing 6.0% gels in the Weber and Osborn system or 3 mA/tube for 5 h by employing 10% separation gels in the Laemmli system. When a 10% slab gel was employed in the Laemmli system, electrophoresis was performed at 4 mA for 15 h. Fluorography or scintillation autography of the slab gel was performed by the method of Bonner & Laskey (1974) or according to the manufacturer's instruction when EN^{3}HANCE was used.

Radiolabeling of the lysine residue(s) of factor XII and factor XII_a with tritium was carried out by a modification of the reductive alkylation procedure of Means & Feeney (1968) using [^{3}H]$NaBH_4$ and formaldehyde. By this method, the proteins were radiolabeled with a specific activity of 20 000–150 000 cpm/μg.

Automated Edman degradation was performed with a Beckman sequenator, Model 890C. The mode of operation of the instrument and the methods of sequence analysis were adaptations of the technique of Edman & Begg (1967) employing the dimethylbenzylamide system of Hermodson et al. (1972). Phenylthiohydantoin amino acids were identified and quantitated by high-pressure liquid chromatography (Bridgen et al., 1976; Ericsson et al., 1977). The sequence analysis of the light chain of factor XI_a was carried out on 1.6-mg samples of carboxymethylated protein, and the anaysis of the heavy chain was carried out on 1.9-mg samples of protein.

The activation of factor XI by factor XII or factor XII_a was carried out as follows. Twenty-five microliters of factor XI (0.43 mg/mL in 0.025 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl) was added to a negatively charged substance (kaolin, dextran sulfate, or sulfatide) in 0.018 mL of 0.10 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and incubated at 37 °C for 2 min. Factor XII_a (2.05 μL from a solution of 0.13 mg/mL in 0.025 M phosphate buffer, pH 6.0, and 0.15 M NaCl) or factor XII (2.05 μL from a solution of 0.13 mg/mL in 0.025 M phosphate buffer, pH 7.5, and 0.15 M NaCl) was then added to the reaction mixture. The final volume of the incubation mixture was 45 μL. Aliquots (5 μL) were removed at various times during the incubation and immediately mixed with 5 μL of antibody to factor XII to stop the reaction. The samples were then assayed for esterase activity by employing 12.5 mM tosyl-L-arginine [^{3}H]methyl ester as described below. In control experiments, it was shown that the antibody readily inactivated the coagulant activity and esterase activity of factor XII_a. Factor XI was also activated by the addition of 1.35 μL of [^{3}H]factor XII (0.2 mg/mL) or 15 μL of [^{3}H]factor XII_a (0.018 mg/mL) under the same conditions as those described above. The inhibition of bovine factor XI_a by antithrombin III was performed as previously described (Kurachi & Davie, 1977).

The large-scale preparation of diisopropylphosphoryl-factor XI_a was carried out as follows. Factor XI (13 mg) in 20 mL of 0.025 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl was added to 2 mL of factor XII_a (0.45 mg) in the same buffer, and the reaction mixture was incubated for 3 h. Aliquots were examined for esterase activity and also subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in order to follow the reaction. Fifty microliters of [^{3}H]diisopropyl fluorophosphate (0.013 mg containing 50 μCi) was then added to the reaction mixture, and the incubation was continued for 30 min at room temperature. The sample was then passed through a Sephadex G-50 column (2.5 × 30 cm) previously

equilibrated with 5% formic acid, and the radiolabeled sample was lyophilized.

The separation of the heavy chain and the light chains of bovine factor XI_a was carried out as follows. The radiolabeled diisopropylphosphoryl-factor XI_a (approximately 13 mg) was reduced and carboxymethylated in 2.5 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 to a Sephadex G-150 column (1.6 × 100 cm) previously equilibrated with 5% formic acid containing 3 M urea. The polypeptide chains were eluted with 5% formic acid containing 3 M urea. Aliquots (10 μL) from each fraction were added to 10 mL of Aquasol solution, and their radioactivity was measured in a Beckman liquid scintillation counter, LS-100C. The protein peaks containing the heavy and light chains were pooled separately and desalted by passing through a Sephadex G-50 column (1.6 × 20 cm) in 5% formic acid, followed by lyophilization.

The coagulant assay for factor XI was performed as previously described (Kurachi & Davie, 1977). The esterase assay for factor XI_a was carried out using tosyl-L-arginine [^{3}H]-methyl ester as the substrate according to the procedure of Roffman et al. (1970) as modified by Anderson et al. (1975). The reaction mixture (5 μL containing 1.1 μg or less of enzyme) was transferred into 5 μL of factor XII antibody solution to stop the activation reaction. Sixty microliters of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl was then added to the reaction mixture. Radiolabeled tosyl-L-arginine methyl ester (10 μL containing 50 nCi) was then added to each sample and mixed (final volume 80 μL), and 10 mL of Omnifluor scintillation liquid was added. Counting was started immediately in a Beckman Model LS-100C scintillation counter at room temperature, and the initial rate of hydrolysis of tosyl-L-arginine [^{3}H]methyl ester was recorded. Ester hydrolysis in the presence of buffer and factor XII antibody was measured as a blank. Factor XI_a had a specific activity of 17.0 μmol of tosyl-L-arginine methyl ester hydrolyzed per mg per min as assayed by this method.

Purification of Factor XI. Bovine factor XI was purified according to the procedure of Koide et al. (1977a) with the following modifications. (1) The 20–40% ammonium sulfate precipitate was dialyzed against water overnight, followed by dialysis against 0.02 M Tris-HCl buffer, pH 7.0, instead of 0.02 M Tris-HCl buffer, pH 7.2, containing 0.05 M NaCl. This procedure leads to the precipitation of considerable amounts of contaminating proteins and greatly improved the reproducibility of the first heparin–agarose column chromatography step. The heparin–agarose column was developed as previously described (Koide et al., 1977a). (2) The CM-Sephadex column was equilibrated with 0.02 M phosphate buffer, pH 5.8, containing 0.07 M NaCl, and the protein sample to be applied to this column was also dialyzed against the same buffer solution at pH 5.8. (3) After the protein sample was applied to the DEAE-Sephadex column, the column was washed with 0.05 M Tris-HCl buffer, pH 8.4, containing 0.12 M NaCl instead of 0.05 M Tris-HCl buffer, pH 8.4, containing 0.08 M NaCl. The gradient elution was formed with 200 mL of 0.05 M Tris-HCl buffer, pH 8.4, containing 0.12 M NaCl and 200 mL of 0.05 M Tris-HCl buffer, pH 8.4, containing 0.4 M NaCl. Factor XI thus purified was incubated with 1 mM diisopropyl fluorophosphate for 15 min at room temperature and passed through a Sephadex G-50 column (1.6 × 10 cm) equilibrated with the same buffer solution. The final preparation was homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with apparent molecular weights of 124 000 and 76 000 for the nonreduced and reduced samples, respectively. The specific activity of the final preparation of factor XI was 280 clotting units/mg. It contained no detectable esterase activity toward tosyl-L-arginine methyl ester.

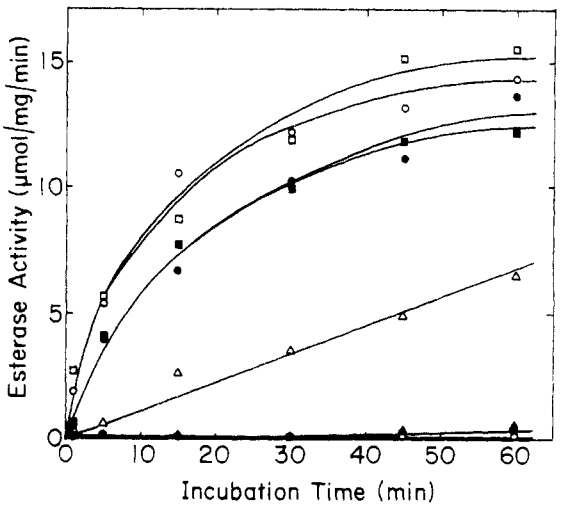

FIGURE 1: Time curve for the activation of factor XI by factor XII and factor XII_a in the presence of sulfatide and HMW kininogen. The reaction mixture contained 10.8 μg of factor XI, 0.27 μg of factor XII or factor XII_a, 5 μg of sulfatide, 0.19 μg of HMW kininogen, and 0.1 M Tris-HCl buffer, pH 7.5, containing 0.10 M NaCl in a final volume of 45 μL. The reaction was initiated by the addition of factor XII or factor XII_a and incubated at 37 °C. Aliquots (5 μL) were withdrawn at various times and immediately mixed with 5 μL of antibody to factor XII to stop the reaction. The samples were then quickly frozen at −25 °C until assayed for factor XI_a esterase activity. They were then thawed and added to tosyl-L-arginine [^{3}H]methyl ester as described under Methods. (□) Factor XI plus factor XII_a, sulfatide, and HMW kininogen; (○) factor XI plus factor XII, sulfatide, and HMW kininogen; (■) factor XI plus factor XII_a and sulfatide; (●) factor XI plus factor XII and sulfatide; (△) factor XI plus factor XII_a; (▲) factor XI plus factor XII; (○) factor XI plus sulfatide and HMW kininogen or factor XII plus sulfatide and HMW kininogen.

Results

Effect of Sulfatide or Dextran Sulfate on the Activation of Bovine Factor XI by Factor XII or Factor XII_a. It has been shown that kallikrein readily activates bovine factor XII in the presence of sulfatide or dextran sulfate (Fujikawa et al., 1980). Accordingly, it was of interest to examine the effects of these compounds on the activation of factor XI by factor XII or factor XII_a (Figure 1). Factor XII (the zymogen or single-chain molecule) readily activated factor XI in the presence of 0.11 mg/mL sulfatide (solid squares) and this rate was identical with that observed with the same concentration of factor XII_a (solid circles). The generation of factor XI_a was assayed by its esterase activity toward tosyl-L-arginine methyl ester. The addition of HMW kininogen (4.2 μg/mL) increased the initial rate of activation of factor XI about 50% with either factor XII (open circles) or factor XII_a (open squares). No lag phase was observed in the reactions with either factor XII or factor XII_a in the presence of sulfatide. In the absence of sulfatide, the activation of factor XI by factor XII was extremely slow (solid triangles), while the activation of factor XI by factor XII_a was substantial (open triangles). The last reaction went to completion in about 150 min. In these various experiments, an enzyme to substrate weight ratio of 1:40 was employed. These data suggest that factor XII in the presence of sulfatide is participating in the activation of factor XI as an enzyme and its specific activity is essentially identical with that of factor XII_a in this reaction.

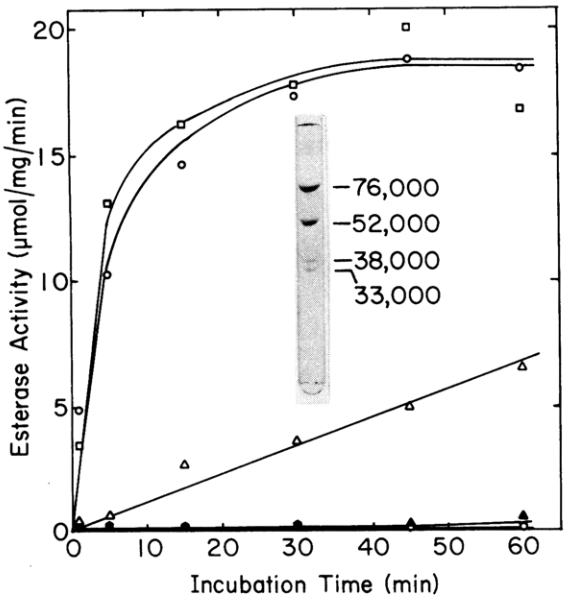

FIGURE 2: Time curve for the activation of factor XI by factor XII and factor XII_a in the presence of dextran sulfate. The reaction mixture contained 10.8 μg of factor XI, 0.27 μg of factor XII or factor XII_a, 5 μg of dextran sulfate, and 0.1 M Tris-HCl buffer, pH 7.5, containing 0.10 M NaCl in a final volume of 45 μL. The rest of the conditions are the same as those described in Figure 1. (□) Factor XI plus factor XII_a and dextran sulfate; (○) factor XI plus factor XII and dextran sulfate; (△) factor XI plus factor XII_a; (▲) factor XI plus factor XII; (○) factor XI plus dextran sulfate or factor XII plus dextran sulfate. The insert shows the sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of a reduced sample removed from the reaction mixture after 5 min of incubation. The anode was at the bottom of the gel.

Dextran sulfate (M_r 500 000) was also found to be active in the activation of factor XI by factor XII or factor XII_a (Figure 2). In these experiments, the rate of activation of factor XI by factor XII (open circles) was essentially identical with that of factor XII_a (open squares). No lag phase was observed in these reactions. The activation of factor XI by equal amounts of factor XII and factor XII_a gave the same results as with factor XII or factor XII_a alone. The dextran sulfate was employed at a concentration of 0.11 mg/mL in these experiments. HMW kininogen (4.2 μg/mL) did not influence the rate of activation of factor XI by factor XII or factor XII_a in the presence of dextran sulfate.

Factor XII which was adsorbed to and eluted from dextran sulfate–Sepharose did not activate factor XI. Furthermore, it had no detectable esterase activity toward tosyl-L-arginine methyl ester and was not inhibited by 0.005 M diisopropyl fluorophosphate or antithrombin III plus heparin. In these experiments, factor XII in 0.10 M Tris-HCl buffer, pH 7.5, containing 0.10 M NaCl was applied to a dextran sulfate–Sepharose column. The column was then washed extensively with the same buffer, and factor XII was eluted with 0.10 M Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl. These data indicate that factor XII required the presence of dextran sulfate during the activation of factor XI. Thus, the mere contact of factor XII to dextran sulfate was insufficient to convert it irreversibly to an active enzyme.

The activation of bovine factor XI by factor XII_a in the presence of dextran sulfate was then studied by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In these experiments, samples from a reaction identical with that shown in Figure 2 were removed at various times and examined in the presence and absence of 2-mercaptoethanol. In the absence of reducing agent, a single sharp band (apparent M_r 124 000) was observed for samples removed at 0, 30, and 50 min. This indicates that little, if any, change in the molecular weight of factor XI occurred during its conversion to factor XI_a. A difference in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of the protein was observed, however, after reduction of the samples. At zero time, a single sharp protein band (apparent M_r 76 000) was observed. This band is characteristic of reduced bovine factor XI (Koide et al., 1977a). During the first few minutes of the reaction, three new faster moving bands appeared with apparent molecular weights of 52 000, 38 000, and 33 000. The gel electrophoresis pattern for the 5-min sample is shown in the insert in Figure 2. After 30 min, the 76 000 molecular weight band disappeared completely and was converted into heavy and light chains. The appearance of the faster moving bands (one heavy chain and two light chains) occurred in parallel with the formation of factor XI_a esterase activity.

In similar experiments, the sodium dodecyl sulfate–polyacrylamide gel electrophoretic pattern was examined for the activation of factor XI by factor XII in the presence of dextran sulfate. The gel pattern for these experiments was identical with that observed for factor XII_a plus dextran sulfate. Furthermore, the same two light chains were observed for factor XI_a when it was generated in the presence of either factor XII or factor XII_a. These data indicate that bovine factor XI is converted to factor XI_a by the cleavage of each of the two chains of the precursor molecule by either factor XII or factor XII_a.

The activation of factor XI by factor XII is unusual in that bovine factor XII is a zymogen and has no detectable esterase activity toward tosyl-L-arginine methyl ester in the presence or absence of dextran sulfate, sulfatide, or kaolin, in contrast to factor XII_a. Furthermore, factor XII coagulant activity was not inhibited by 0.025 M diisopropyl fluorophosphate in the presence of dextran sulfate. Also, factor XII did not bind radiolabeled diisopropyl fluorophosphate in the presence of dextran sulfate. In additional experiments, it was also shown that the coagulant activity was not inhibited by antithrombin III plus heparin in the presence of dextran sulfate, sulfatide, or kaolin. Accordingly, it was important to establish whether or not factor XII was rapidly converted to factor XII_a by trace amounts of factor XI_a (Fujikawa et al., 1980) within the first few minutes of the activation reaction. In this situation, a lag phase might not be readily detected during the conversion of factor XI to factor XI_a. [^{3}H]Factor XII and [^{3}H]factor XII_a were examined by fluorography during the activation reaction in order to test for the possibility of an extremely rapid conversion of factor XII to factor XII_a early in the activation reaction. The [^{3}H]factor XII employed in these experiments had a specific activity in a clotting assay of 110 units/mg which is about 55% of that of the unlabeled molecule. It migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. The specific activity of the [^{3}H]factor XII_a was 190 units/mg which is about 60% of that of the unlabeled factor XII_a. [^{3}H]Factor XII_a migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol and appeared as a heavy and a light chain in the presence of 2-mercaptoethanol. The heavy chain (apparent M_r 52 000) contained nearly 90% of the radioactivity, while the light chain (apparent M_r 31 000) contained about 10% of the radioactivity.

A time course for the activation of factor XI by [^{3}H]factor XII or [^{3}H]factor XII_a in the presence of dextran sulfate is shown in Figure 3. The rate of activation of factor XI by

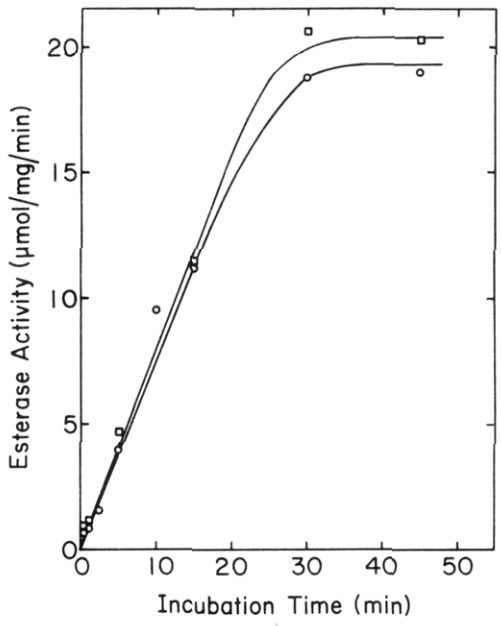

FIGURE 3: Time curve for the activation of factor XI by radiolabeled factor XII and factor XII_a in the presence of dextran sulfate. The experimental conditions were essentially the same as those described in Figure 2 except radiolabeled factor XII and factor XII_a were employed. (□) Factor XI plus factor XII_a and dextran sulfate; (○) factor XI plus factor XII and dextran sulfate.

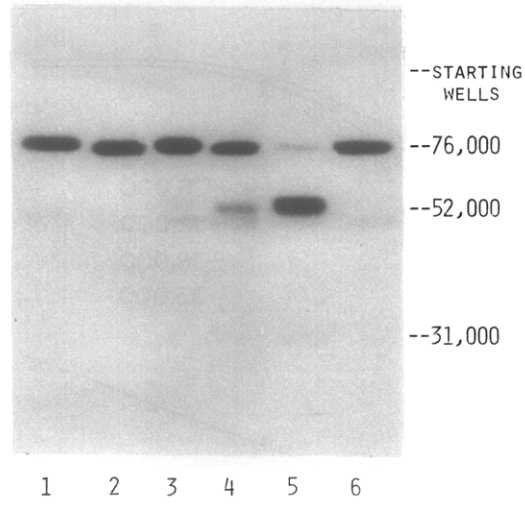

FIGURE 4: Fluorogram of the activation mixture of factor XI by radiolabeled factor XII. The experimental conditions were exactly the same as those described in Figure 3 except that the reaction mixture was increased twofold. At various time intervals, 20-μL aliquots were removed and mixed with 2 μL of 0.55 M diisopropyl fluorophosphate and frozen at −20 °C. Each aliquot contained ~14000 cpm of radioactivity. To each of the thawed samples were then added 10 μL of 10% sodium dodecyl sulfate and 5 μL of 2-mercaptoethanol, and the samples were immediately boiled for 1 min prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis employing the Laemmli system. The conditions for electrophoresis and fluorography are described under Methods. Lanes 1 and 6, radiolabeled factor XII; lane 2, 30-s sample; lane 3, 1-min sample; lane 4, 5-min sample; lane 5, 15-min sample.

[^{3}H]factor XII (open circles) or [^{3}H]factor XII_a (open squares) was nearly identical by employing an enzyme to substrate weight ratio of 1:40 in both reactions. As previously observed, no lag phase was detected with either [^{3}H]factor XII or [^{3}H]factor XII_a.

Aliquots were also removed during the activation of factor XI by [^{3}H]factor XII and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a slab gel in the presence of 2-mercaptoethanol. By this technique, it was possible to determine the relative amounts of factor XII and factor XII_a that were present in the early phase of the activation reaction since [^{3}H]factor XII (single chain) and factor XII_a (two chains) were easily identified by fluorography (Figure 4). Lanes 1 and 6 are control samples containing only reduced factor XII, while lanes 2–5 are samples removed during the activation reaction and reduced with 2-mercaptoethanol prior to electrophoresis. After 30 s or 1 min of incubation, more than 90% of the factor XII was present as a single-chain molecule (lanes 2 and 3, respectively). The percent of factor XII and factor XII_a was determined by scanning the X-ray film from the slab gel with a densitometer. After 5 min, 20% of the factor XI was converted to factor XI_a with either [^{3}H]factor XII or [^{3}H]factor XII_a, and no lag phase was observed (Figure 3). At this time, 65% of the factor XII was still present as a single-chain molecule (lane 4, Figure 4). These data provide good evidence for the proteolytic cleavage of factor XI by factor XII in the early phase of the activation reaction at a rate which is identical with that of factor XII_a. After 15 min of incubation, 10% of the [^{3}H]factor XII was present as a single-chain molecule, while 90% was present as the two-chain molecule (lane 5, Figure 4). Accordingly, the proteolysis of factor XI is catalyzed primarily by factor XII_a after the reaction is about 50% complete. At this time, the ratio of factor XI_a to factor XII is high (approximately 25:1 after 15 min), and this leads to the conversion of most of the factor XII to factor XII_a.

The activation of factor XI by [^{3}H]factor XII was then studied in the presence of RVV inhibitor II, a low molecular weight serine protease inhibitor from Russell's viper venom (Iwanaga et al., 1976). This inhibitor readily inactivated the esterase activity of factor XI_a but had no effect on the esterase activity of factor XII_a. A time course for the activation of factor XI by [^{3}H]factor XII in the presence of dextran sulfate and a 2.5 molar excess of inhibitor (relative to factor XI) is shown in Figure 5. The conversion of factor XI to factor XI_a was measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by densitometry of the stained gels (solid circles, Figure 5). The conversion of [^{3}H]factor XII to [^{3}H]factor XII_a (open circles, Figure 5) was measured by densitometry of a fluorogram similar to that shown in Figure 4. After 5 min of incubation, 28% of the factor XI was converted to factor XI_a while 94% of the factor XII was present as the single-chain molecule. After 15 min, 44% of the factor XI was converted to factor XI_a while 77% of the factor XII was present as the single-chain molecule. These experiments provide strong evidence for the proteolytic cleavage of factor XI by factor XII.

Effect of Kaolin and HMW Kininogen on the Activation of Bovine Factor XI by Factor XII or Factor XII_a. The effect of kaolin (0.3 mg/mL) and HMW kininogen (4.2 μg/mL) on the activation of bovine factor XI is shown in Figure 6. Factor XII readily activated factor XI in the presence of kaolin (solid circles). The rate of activation of factor XI by factor XII and kaolin was about 40% of that found with factor XII_a and kaolin (solid squares). The activation of factor XI by factor XII and kaolin was enhanced by the addition of HMW kininogen (open circles), and this rate was about 60% of that found with factor XII_a, kaolin, and HMW kininogen (open squares). In these experiments, no lag phase was observed in the activation of factor XI by factor XII or factor XII_a.

Factor XII does not activate factor XI in the absence of kaolin (solid triangles), in contrast to factor XII_a (open triangles). The optimal kaolin concentration in these experiments was found to be 0.2–0.45 mg/mL. Higher concentrations (0.8

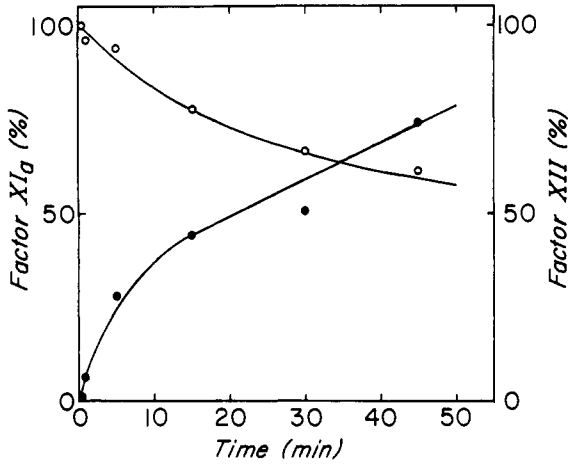

FIGURE 5: Time curve for the activation of factor XI by [^{3}H]factor XII in the presence of dextran sulfate and RVV inhibitor II. The reaction mixture contained 64.2 μg of factor XI, 1.6 μg of [^{3}H]factor XII, 15.4 μg of RVV inhibitor II, and 30 μg of dextran sulfate in 192 μL of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.10 M NaCl. The [^{3}H]factor XII was added to dextran sulfate and incubated for 2 min at 20 °C, and RVV inhibitor II was added. The reaction was initiated by the addition of factor XI and incubated at 37 °C. Aliquots (28 μL) were removed and the reaction was stopped by rapid freezing of the samples in a dry ice–ethanol bath. They were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of dithiothreitol employing 6% gels. The gels were then stained with 0.25% Coomassie brilliant blue R and destained in 7.5% acetic acid–5% methanol, and the protein bands were measured by densitometry to determine the concentration of factor XI$_a$. Aliquots (28 μL) were also subjected to slab gel sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of dithiothreitol employing 10% gels. After staining (Coomassie brilliant blue R) and destaining, the gel was prepared for fluorography with EN^{3}HANCE. After exposure to X-ray film for two days, the concentration of factor XII was determined by densitometry. (●) Factor XI$_a$; (○) factor XII.

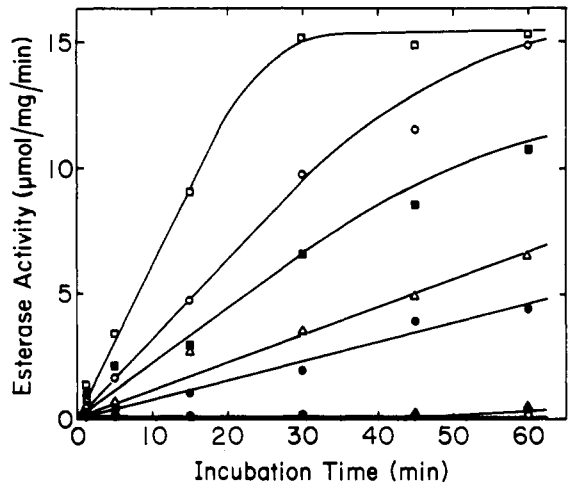

FIGURE 6: Time curve for the activation of factor XI by factor XII or factor XII$_a$ in the presence of kaolin plus HMW kininogen. The reaction mixture contained 10.8 μg of factor XI, 0.27 μg of factor XII or factor XII$_a$, 20 μg of kaolin, 0.19 μg of HMW kininogen, and 0.1 M Tris-HCl buffer, pH 7.5, containing 0.10 M NaCl in a final volume of 45 μL. The reaction was initiated by the addition of factor XII or factor XII$_a$ and incubated at 37 °C. The rest of the conditions are the same as those described in Figure 1. (□) Factor XI plus factor XII$_a$, kaolin, and HMW kininogen; (○) factor XI plus factor XII, kaolin, and HMW kininogen; (■) factor XI plus factor XII$_a$ and kaolin; (●) factor XI plus factor XII and kaolin; (△) factor XI plus factor XII$_a$; (▲) factor XI plus factor XII; (○) factor XI plus kaolin and HMW kininogen or factor XII plus kaolin and HMW kininogen.

mg/mL) or lower concentrations (0.05 mg/mL) decreased the activation rate about 50%. The optimal concentration of HMW kininogen was found to be 4–6.5 μg/mL. At higher concentrations (53 μg/mL) or lower concentrations (1.0 μg/mL), the enhancing effect of HMW kininogen was about 20% of that found with 4.2 μg/mL. The weight ratio of HMW kininogen to factor XII$_a$ under the optimal conditions ranged from 0.7 to 1.1. The weight ratio of HMW kininogen to factor XI under optimal conditions ranged from 0.018 to 0.027. These data suggest that HMW kininogen (M_r 76 000) is forming a 1:1 stoichiometric complex with factor XII$_a$ (M_r 74 000) during the activation of factor XI. This conclusion was also supported by experiments in which the concentration of factor XII$_a$ was reduced 10-fold. Under these conditions, the optimal concentration of HMW kininogen was also reduced approximately 10-fold. These experiments further support the conclusion that factor XII$_a$ and HMW kininogen may form a 1:1 molar complex during the activation reaction.

Inhibition of Bovine Factor XI$_a$ by Antithrombin III. Bovine factor XI$_a$ like human factor XI$_a$ (Damus et al., 1973; Kurachi & Davie, 1977) was rapidly inactivated by antithrombin III, and the rate of the inhibition was greatly accelerated by the presence of heparin. Complete inhibition of factor XI$_a$ esterase activity was achieved in the presence of heparin when the molar ratio of inhibitor to factor XI$_a$ was 2:1. In these studies, a molecular weight of 124 000 was employed for bovine factor XI$_a$ and a molecular weight of 56 000 was employed for antithrombin III. The formation of a stable complex between antithrombin III and factor XI$_a$ was demonstrated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and this complex was shown to involve both of the light chains of factor XI$_a$ which contain the active-site serine residue. These experiments indicate the presence of two active-site serine residues per mol of bovine factor XI$_a$, and each is inhibited by antithrombin III.

Isolation and Amino-Terminal Sequence of the Heavy and Light Chains of Bovine Factor XI$_a$. Bovine factor XI$_a$ is also readily inhibited by other serine protease inhibitors, such as diisopropyl fluorophosphate, and this inhibitor is bound to the active-site serine residue in the enzyme (Koide et al., 1977b). Accordingly, bovine factor XI$_a$ was labeled with radioactive diisopropyl fluorophosphate, and the diisopropylphosphoryl enzyme was reduced, S-carboxymethylated, and subjected to gel filtration on Sephadex G-150 to separate the heavy and light chains (Figure 7). Three protein peaks were obtained. The first peak contained a small amount of factor XI which was not converted to factor XI$_a$ during the activation reaction. The second peak contained the heavy chain (apparent M_r 52 000) of factor XI$_a$ and migrated as a major sharp band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (insert, left gel). The third peak migrated as a doublet (apparent M_r's 38 000 and 33 000) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (insert, right gel) and contained the light chains of factor XI$_a$. The light chains contained essentially all of the radioactivity (solid circles, Figure 7), indicating that they contain the active-site serine.

An amino-terminal sequence 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 glutamic acid and no other amino acids were detected. The amino acid sequence of the first 16 residues is shown in Figure 8 along with the amino-terminal sequence of human factor XI (Kurachi & Davie, 1977) and bovine plasma prekallikrein (Heimark & Davie, 1979). The yields of residues 1–16 were 0.5, 0.3, 0.8, 0.8, 0.8, 0.5, 0.3, 0.4, 0.2, 0.4, 0.2, 0.3, 0.2, 0.2, 0.2, and 0.1 equiv/35 000 g of protein, respectively. These data indicate that the two chains in bovine factor XI give rise to two identical heavy

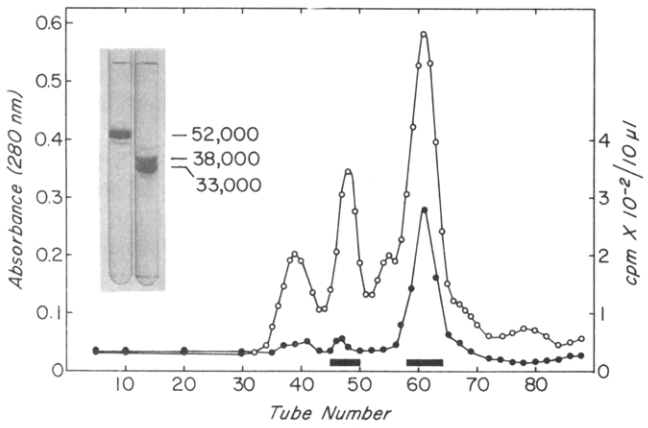

FIGURE 7: Gel filtration pattern of reduced and carboxymethylated diisopropylphosphoryl-factor XI_a. After the carboxymethylation reaction was completed, the reaction mixture (2.5 mL) was applied to a Sephadex G-150 column (1.6 × 100 cm) which was previously equilibrated with 5% formic acid containing 3 M urea. The column was eluted with the 5% formic acid containing 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 under Methods. (○) Absorbance; (●) radioactivity. Insert: the left gel shows a sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern for peak 2, and the gel on the right is for peak 3. Approximately 10 µg of protein was applied to each 6% gel. The anode was at the bottom of the gel.

chains in factor XI_a and each contains an amino-terminal glutamic acid residue. These experiments also indicate that the heavy chains of bovine factor XI_a originate from the amino-terminal region of the precursor protein since this sequence shows considerable homology (greater than 60%) with the amino-terminal region of human factor XI.

The amino-terminal residue of the mixture of two light chains was isoleucine, and no other amino acids were detected. The amino acid sequence of the first 16 residues of these chains was Ile-Val-Gly-Gly-Thr-Gln-Ala-Val-?-Gly-Glu-Trp-Pro-Tyr-Gln-Ile-. The yields for residues 1–16 were 0.6, 0.6, 0.7, 0.6, 0.4, 0.2, 0.2, 0.5, not determined, 0.4, 0.2, not quantitated, 0.3, 0.1, 0.2, and 0.1 equiv/25 000 g of protein, respectively. No residue was identified in position 9. This sequence shows considerable homology (greater than 75%) with the amino-terminal region of the light chains of human factor XI_a (Kurachi & Davie, 1977). Thus, it is clear that these chains originate from the carboxyl-terminal region of the two chains of the precursor molecule. Thus, bovine factor XI_a contains two identical heavy chains and two very similar light chains with the same amino-terminal sequence. Furthermore, the difference in apparent molecular weight for the two light chains is due to a difference in some portion of the polypeptide chain other than the amino-terminal region.

Discussion

The present data indicate that bovine factor XI (M_r 124 000) is composed of two nearly identical polypeptide chains (M_r ~60 000 each) held together by a disulfide bond(s) (Figure 9). The amino-terminal residue of the two polypeptide chains is glutamic acid. The amino-terminal sequence of bovine factor XI, as well as human factor XI, shows considerable homology with bovine prekallikrein (Figure 8), suggesting that these two plasma proteins have evolved from a common ancestor.

Bovine factor XI is readily converted to factor XI_a by factor XII or factor XII_a in the presence of sulfatide, dextran sulfate, or kaolin plus HMW kininogen. The activation of factor XI is due to the cleavage of an internal peptide bond in each of the two nearly identical chains (shown by the two arrows in Figure 9). This gives rise to a pair of heavy chains and a pair of light chains in factor XI_a. Thus, the mechanism of activation of bovine factor XI is essentially identical with that of human factor XI (Kurachi & Davie, 1977; Bouma & Griffin, 1977).

The heavy and the light chains of bovine factor XI_a have apparent molecular weights of 52 000 each and 38 000 or 33 000, respectively, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. These values are probably high, however, since glycoproteins tend to bind less amounts of detergents, resulting in high estimates of molecular weight (Segrest & Jackson, 1972). Accordingly, molecular weights of 35 000 and 25 000 were employed for the quantitation of the amino-terminal residues in the heavy and light chains of factor XI_a. A summation of these two values gives a molecular weight of about 60 000 for each of the subunits of factor XI, which is the same as the value determined by sedimentation equilibrium (Koide et al., 1977a). The two light chains show small differences in their size, and their correct values are probably about 24 000 and 26 000. Since the amino-terminal sequences of the two light chains are identical, it seems probable that their difference is due to the loss of a polypeptide fragment from the carboxyl-terminal region of one

FIGURE 8: Amino-terminal sequences of bovine factor XI, human factor XI, and bovine plasma prekallikrein. The sequence of bovine factor XI was determined from the heavy chain of factor XI_a. The sequences for human factor XI and bovine plasma prekallikrein were taken from Kurachi & Davie (1977) and Heimark & Davie (1979), respectively. Amino acid residues that are identical in all three proteins are shown in blocks. (*) Originally, residue 11 in human factor XI was reported as Gln (Kurachi & Davie, 1977), but this was in error (K. Kurachi, unpublished results).

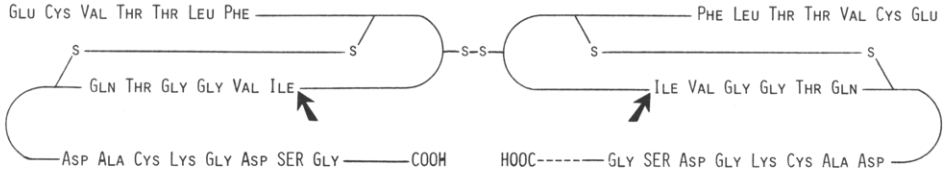

FIGURE 9: Partial structure of bovine factor XI. The two nearly identical chains are held together by a disulfide bond(s). The active-site serine is shown in capital letters. The two arrows indicate the site of cleavage in the two chains when the protein is converted to factor XI_a by factor XII or factor XII_a. The resulting four chains are held together by disulfide bonds. The number and exact location of these disulfide bonds are not known. The dotted line indicates a possible difference in the carboxyl-terminal region of one of the light chains of factor XI_a. The amino acid sequence surrounding the active-site serine was taken from Koide et al. (1977b).

of the light chains via minor proteolysis. Furthermore, it seems likely that this degradation is catalyzed by some contaminating plasma protease during the isolation of factor XI rather than by factor XII_a or factor XI_a during the activation reaction. This is suggested by the fact that the degradation reaction never goes to completion during an activation reaction when substantial levels of factor XII_a and factor XI_a are present.

The finding that the initial rate of activation of factor XI was catalyzed equally well by factor XII or factor XII_a in the presence of sulfatide or dextran sulfate was somewhat surprising. Bovine factor XII is a single-chain protein that lacks esterase activity and is not inhibited by diisopropyl fluorophosphate or antithrombin III plus heparin in the presence or absence of sulfatide or dextran sulfate. Accordingly, the activation of factor XI appears to be due to a very specific factor XI or substrate-induced catalysis by factor XII. Also, it seems likely that the catalytic unit that participates in this reaction is a complex between factor XII and sulfatide or dextran sulfate.

Saito (1977) has reported that no fragmentation of human factor XII occurred during the activation of factor XI in the presence of HMW kininogen and ellagic acid. Under the conditions of these experiments, apparently no cleavage of factor XII occurred during the reaction, even in the presence of the newly generated factor XI_a. Ratnoff & Saito (1979) have also reported amidase activity toward D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide for single-chain human factor XII previously exposed to ellagic acid–Sepharose. In the present study, no esterase activity was observed for bovine factor XII in the presence or absence of sulfatide, dextran sulfate, or kaolin plus HMW kininogen.

Substantial catalytic activity for zymogens or precursor proteins has been observed for several other plasma proteins. For instance, prothrombin complexed with staphylocoagulase has esterase activity toward tosylarginine methyl ester and the ability to clot fibrinogen (Hemker et al., 1975). Plasminogen complexed with streptokinase is capable of converting plasminogen to plasmin (Reddy & Markus, 1972). Also, this complex has an active-site serine which reacts rapidly with *p*-nitrophenyl *p*-guanidinobenzoate. Bovine factor VII, a single-chain zymogen (Radcliffe & Nemerson, 1975; Kisiel & Davie, 1975), has been shown to possess esterase activity (Zur & Nemerson, 1978) and was readily inhibited by diisopropyl fluorosphosphate (Radcliffe & Nemerson, 1975). The coagulant activity of this protein, however, is increased about 30-fold when it is converted to factor VII_a, a protease containing a heavy and a light chain held together by a disulfide bond(s) (Radcliffe & Nemerson, 1975, 1976; Kisiel et al., 1977). Accordingly, it appears likely that factor VII in the zymogen form has little or no activity toward specific protein substrates, such as factor X.

The activation of factor XI was also rapidly catalyzed by factor XII_a. The rate of activation of factor XI by factor XII_a was increased about twofold in the presence of kaolin and nearly sixfold in the presence of kaolin plus HMW kininogen. Under the optimal reaction conditions, the molar ratio between factor XII_a and HMW kininogen was about 1:1, suggesting that these two proteins may form an equimolar complex which is responsible for the activation of factor XI. A similar conclusion has been made by Griffin & Cochrane (1976), who employed human preparations in their studies. In the absence of kaolin, HMW kininogen had no effect on the activation of factor XI by factor XII_a or factor XII.

The activation of factor XI by factor XII in the presence of sulfatide is consistent with the fact that sulfatide readily initiates reactions leading to fibrin formation when incubated with normal platelet-poor citrated plasma supplemented with phospholipid and calcium (Fujikawa et al., 1980). Also, the clotting time for prekallikrein-deficient plasma was only a little longer than that for normal plasma in the presence of sulfatide. Therefore, sulfatide can lead to the initiation of blood clotting without an initial proteolytic activation of factor XII by kallikrein. This is consistent with the findings that factor XII or factor XII_a activates factor XI equally well, and thus the proteolytic activation of factor XII by kallikrein is mainly bypassed in the presence of sulfatide. The clotting time for HMW kininogen deficient plasma was substantially longer, however, than that for normal plasma in the presence of sulfatide (Fujikawa et al., 1980). This is consistent with the fact that the activation of factor XI by factor XII in the presence of sulfatide is stimulated by the addition of HMW kininogen (Figure 1).

The rate of activation of factor XI by factor XII in the presence of kaolin and HMW kininogen was about 50% of that found with factor XII_a (Figure 6). Accordingly, an initial conversion of factor XII to factor XII_a by proteolysis appears to be more important under these conditions. This is also indicated by the fact that the clotting time for normal plasma in the presence of kaolin is much faster than that of prekallikrein-deficient plasma. Under these conditions, the initiation of clotting appears to proceed primarily by the initial conversion of prekallikrein to kallikrein, followed by the conversion of factor XII to factor XII_a and factor XI to factor XI_a. Kaolin also influences clotting after the formation of factor XI_a, as shown by the fact that factor XI_a still requires kaolin to trigger coagulation and fibrin formation in normal or factor XI deficient plasma (Kurachi & Davie, 1977). This is probably due in part to the fact that the rate of activation of factor IX by factor XI_a is increased about 60% in the presence of kaolin (0.25 mg/mL) (K. Kurachi, unpublished results).

The present experiments have also shown a marked effect of dextran sulfate on the activation of factor XI by factor XII or factor XII_a. Dextran sulfate, however, has a heparin-like activity (Ricketts, 1952) which probably inhibits clotting via an effect on antithrombin III. This inhibitory effect of dextran sulfate was also demonstrated in a purified system employing antithrombin III and factor XI_a (K. Kurachi, unpublished results). Thus, the clotting cascade may be initiated in normal plasma by the addition of dextran sulfate but is quickly terminated by antithrombin III in the presence of this reagent.

Dextran sulfate, sulfatide, and kaolin also have some effect on the esterase activity of factor XI_a. For instance, dextran sulfate (0.2 mg/mL) enhanced the esterase activity of factor XI_a about 25%, while sulfatide (0.10 mg/mL) or kaolin (0.45 mg/mL) inhibited the esterase activity 25 and 15%, respectively (K. Kurachi, unpublished data). Since these effects are relatively minor, the primary effect of these three agents is on the conversion of factor XI to factor XI_a rather than on the esterase activity of the factor XI_a once it is formed.

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