Human Blood Coagulation Factor XI

PURIFICATION, PROPERTIES, AND MECHANISM OF ACTIVATION BY ACTIVATED FACTOR XII*

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Human blood coagulation Factor XI was purified in 20% yield from plasma by ion exchange chromatography. The specific clotting activity of purified Factor XI was 250 ± 20 units/mg implying that its concentration in normal citrated human plasma is 4 μg/ml. The purified Factor XI in its precursor form gave a single protein band on polyacrylamide gels in the presence of sodium dodecyl sulfate with an apparent molecular weight of 160,000 in the absence of the reduction of disulfide bonds, whereas in the presence of mercaptoethanol a single protein band at $M_r = 83,000$ was observed.

Proteolytic activation of $^{125I}$-Factor XI by a mixture of purified Factor XII, prekallikrein, high molecular weight kininogen and kaolin was demonstrated. The appearance of activated Factor XI activity was directly correlated with the extent of cleavage of $^{125I}$-Factor XI. The cleaved activated $^{125I}$-Factor XI retained its apparent molecular weight of 160,000 in the absence of reducing agents on sodium dodecyl sulfate polyacrylamide gels, whereas reduction of activated $^{125I}$-Factor XI yielded $M_r = 50,000$ and 33,000 fragments. When normal human plasma containing $^{125I}$-Factor XI was subjected to contact activation by kaolin, the $^{125I}$-Factor XI gave similar molecular weight profiles on sodium dodecyl sulfate gels in the absence and presence of reducing agents. These results show that the activation of human Factor XI both by purified activated Factor XII and by the contact activation system in plasma involves limited proteolytic cleavage of Factor XI yielding polypeptide chains of $M_r = 50,000$ and 33,000 which are held together by disulfide bonds.

Human blood coagulation Factor XI (plasma thromboplastin antecedent, PTA) was first identified in 1963 by Rosenthal et al. (1). This protein plays an important role in the intrinsic pathway of blood coagulation. The first recognized step in the coagulation of plasma after exposure to an activating surface such as kaolin or glass involves the activation of Factor XII (Hageman factor). Optimal activation of Factor XII is obtained in the presence of high molecular weight kininogen and prekallikrein (2). Activated Factor XII with high molecular weight kininogen as a cofactor can convert Factor XI to an active form (2, 3) which subsequently is capable of activating Factor IX (4, 5). Factor XI can also be converted into an active form by trypsin (6, 7) although this is not thought to be physiologically important.

Efforts to purify human and bovine Factor XI have been reported by several groups (5-14). In this paper, we describe a new purification method for Factor XI. In contrast to previous observations, precursor human Factor XI is obtained in a good yield and remains in precursor form upon storage. Evidence is presented here that the appearance of clot-promoting activity is associated with limited cleavage of Factor XI by surface-bound activated Factor XII both in a mixture of purified proteins and in human plasma.

MATERIALS AND METHODS

All chemicals obtained from commercial sources were the best grade available. Human Factor XII (80 clotting units/mg) was purified on DEAE-Sephadex A-50 and SP-Sephadex C-50 columns as described elsewhere (15, 16). Human high molecular weight kininogen (11 clotting units/mg) and prekallikrein (21 clotting units/mg) were purified by ion exchange chromatography. All purified proteins were judged to be >95% homogeneous on polyacrylamide gels.

Protein concentration was determined by the Lowry method (17) using bovine serum albumin (Sigma Chemicals) as a reference. pH measurements were performed at room temperature unless noted otherwise using a Radiometer pH meter, model 26 (Radiometer, Copenhagen). Conductivity was measured at room temperature with a Radiometer conductivity meter, type CDM 2d, using a 0.54-cm cell, type CDC 114.

Factor XI Clotting Assay

Factor XI clotting activity was measured by a kaolin-activated one-stage partial thromboplastin time method using human Factor XII-deficient plasma (George King Biomedical). The test sample was diluted 10-fold or more into Tri-buffered saline (0.15 M NaCl, 0.01 M Tris/C1, pH 7.4) containing 1 mg/ml of bovine serum albumin in order to overcome the inhibitors present in the buffers employed. Fifty microliters of the test sample, diluted appropriately, was incu-
Coagulation Factor XI: Isolation and Activation by Factor XII

Activated Factor XI (Factor XI), assays were performed by incubating the following for 1 min at 37°C in siliconized glass tubes (10 x 75 mm): 50 μl of test sample, 50 μl of cephalin solution, and 50 μl of Factor XI-deficient plasma (diluted 1:1 with Tris-buffered saline). The contents were then recalcified with 50 μl of 0.05 M CaCl₂, and the clotting time at 37°C was measured.

Prekallikrein clotting assays were performed by incubating the following for 30 s at 37°C in siliconized glass tubes (10 x 75 mm): 50 μl of the test sample, 50 μl of Fletcher-trait plasma (obtained through the help of Dr. C. Abildgaard, University of California, Davis), and 50 μl of a kaolin-cephalin suspension. The contents were then recalcified with 50 μl of 0.05 μM CaCl₂, and the clotting time at 37°C was measured.

Purification of Factor XI

Freshly prepared citrated human plasma was the starting material for isolation of Factor XI. Blood was collected from the antecubital vein of normal healthy volunteers into 50-ml plastic syringes and was rapidly mixed in centrifuge tubes with one-sixth volume acid/citrate/dextrose anticoagulant (1 liter contains 13.6 g of citric acid, 25 g of sodium citrate, 20 g of dextrose). The blood was centrifuged at 3,000 x g for 20 min at 20°C and the plasma obtained was then centrifuged again at 5,000 x g for 40 min at 20°C. Fresh frozen platelet-poor human plasma which has been stored at -70°C can also be used as starting material for isolation of Factor XI. Essentially identical results are obtained.

All purification steps were carried out in plasticware or siliconized glassware (Siliclad, Clay Adams) and at 4°C except for the first DEAE-Sephadex chromatography. Five grams of SP-Sephadex C-50 (Pharmacia) were allowed to swell at 56°C overnight in the starting buffer, 0.1 M NaCl, pH 8.1. The resin in a siliconized glass column (2.6 x 30 cm) gave a bed height of 19 cm. The resin was washed with 2 bed volumes of the starting buffer and the sample which had been dialyzed for 24 h against 4 liters of starting buffer, with two changes. The column containing the applied sample was then washed with 100 ml of starting buffer. Gradient elution was effected with 150 ml of starting buffer in the stirred proximal chamber and 250 ml of 0.02 M asparagine, 0.07 M NaCl, pH 9.0, in the distal chamber. Both buffers have the same conductivity of 8 mmho. The flow rate was 35 ml/h and 5-ml fractions were collected. The pH of the buffer and sample during dialysis and chromatography was maintained constant by placing a sodium hydroxide pellet trap in a two-hole rubber stopper at the air intake of a closed system. The pH of the effluent was measured immediately after emerging from the column. As seen in Fig. 1, Factor XI eluted between pH 9.7 and pH 9.0 before the bulk of the protein. The fractions containing Factor XI were pooled for the following step.

Step 3: SP-Sephadex Chromatography – An increasing salt gradient at constant pH was used to elute Factor XI from SP-Sephadex. Five grams of SP-Sephadex C-50 (Pharmacia) were swollen overnight at 56°C in the starting buffer, 0.02 M Tris, 0.01 M NaCl, pH 8.1. The resin in a siliconized glass column (2.6 x 30 cm) gave a bed height of 19 cm. The resin was washed with 2 bed volumes of the starting buffer and the sample which had been dialyzed for 18 h against 4 liters of starting buffer was applied. The column was then washed with 200 ml of starting buffer. Gradient elution was effected with 300 ml of starting buffer in the stirred proximal chamber and 300 ml of 0.02 M Tris, 0.15 M NaCl, pH 8.1, in the distal chamber. The flow rate was 25 ml/h and 5-ml fractions were collected. As seen in Fig. 2, the bulk of the protein did not bind to the column, whereas Factor XI was bound and eluted between 8- and 11-mmho conductivity.

Step 4: SP-Sephadex Chromatography – A second SP-Sephadex C-50 column was employed. Two grams of SP-Sephadex C-50 were allowed to swell overnight in the starting buffer, 0.1 M acetate, 0.15 M NaCl, pH 5.3. The resin was poured into a siliconized glass column (2.6 x 30 cm) to give a bed height of 7.5 cm. The resin was washed

| TABLE I: Purification of human Factor XI |
|---|---|---|---|---|
| Volume | Total protein (mg) | Total units of clotting activity | Specific clotting activity (units/mg protein) | Recovery (%) |
| Plasma | 800 | 85.8 | 960 | 0.0164 |
| DEAE-Sephadex | 420 | 4.4 | 672 | 0.15 | 70 |
| SA-Sephadex, pH 8.0 | 20 | 1.08 | 510 | 0.47 | 53 |
| SP-Sephadex, pH 5.3 | 30 | 0.0099 | 182 | 209 | 19 |

The specific clotting activity of purified Factor XI was determined to be 250 ± 20 clotting units/mg of protein when protein was quantitated directly by amino acid analysis (21-23) or 200 ± 20 clotting units/mg of protein using the Lowry method (17) with a bovine serum albumin standard.

ml of starting buffer before application of the pool of Factor XI from the previous column which had been dialyzed for 24 h against 4 liters of starting buffer, with two changes. The column containing the applied sample was then washed with 100 ml of starting buffer. Gradient elution was effected with 150 ml of starting buffer in the stirred proximal chamber and 250 ml of 0.02 M asparagine, 0.07 M NaCl, pH 9.0, in the distal chamber. Both buffers have the same conductivity of 8 mmho. The flow rate was 35 ml/h and 5-ml fractions were collected. The pH of the buffer and sample during dialysis and chromatography was maintained constant by placing a sodium hydroxide pellet trap in a two-hole rubber stopper at the air intake of a closed system. The pH of the effluent was measured immediately after emerging from the column. As seen in Fig. 1, Factor XI eluted between pH 9.7 and pH 9.0 before the bulk of the protein. The fractions containing Factor XI were pooled for the following step.

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\(^{2}\) The abbreviations used are: Factor XI, the active procoagulant form of Factor XII; SDS, sodium dodecyl sulfate; Factor XII, the active procoagulant form of Factor XII (Hageman factor).
Coagulation Factor XI: Isolation and Activation by Factor XII

Polyacrylamide Gel Electrophoresis

The procedure was carried out on 7.5% gels (6 mm NaCl, pH 5.3, essentially 0.1 M Tris) to give a bed height of 2.5 cm. The column was washed with 10 bed volumes and subsequently incubated with 5 ml diisopropyl phosphorofluoridate (Sigma Chemicals) for 5 min. Then either 10 ml of kaolin (10 mg/ml) in Tris-buffered saline or 10 ml of buffer alone were added and the reaction mixture was incubated for 5 min at 37°C. At this point, a 5-ml aliquot was withdrawn and added to 50 ml of a 3% SDS solution in the presence or absence of 3% mercaptoethanol. The sample was placed in a boiling water bath for 7 min and then analyzed on SDS-polyacrylamide gels.

Preparation of Human Factor XI—The purification steps and the yield for a typical preparation of human Factor XI are shown in Table I. The first DEAE-Sephadex column is used to separate Factor XI in the γ fraction from the bulk of plasma proteins. At pH 8.3 at 2-mM conductivity, Factor XI does not adhere to this resin. In the following two steps, the QAE-Sephadex column and the SP-Sephadex column at pH 5.3 partially separate Factor XI from γ-globulin, prekallikrein, plasminogen proactivator, and β-glycoprotein I. With the SP-Sephadex column at pH 5.3, complete separation from these proteins was obtained. In some preparations, traces of γ-globulin were still present after this column and these could be removed using concanavalin A-Sepharose affinity chromatography. This technique is also very suitable for concentration of the Factor XI preparations. An overall purification of 12,000-fold was observed. The final solution of Factor XI contained no measurable (less than 0.01 unit/ml) activated Factor XI or prekallikrein as measured in Factor XI or Fletcher factor activities. An overall purification of 12,000-fold was observed. The final solution of Factor XI contained no measurable (less than 0.01 unit/ml) activated Factor XI or prekallikrein as measured in Factor XI or Fletcher factor clotting essays. Based on the observed specific activity of 550 clotting units/mg, the concentration of Factor XI in plasma is 4 μg/ml. Polyacrylamide Gel Electrophoresis of Factor XI—A single protein band (Fig. 4) was observed in SDS-polyacrylamide gels in the absence of reducing agents, corresponding to an apparent molecular weight of 160,000. After reduction of Factor XI, a single protein band with an apparent molecular weight of 83,000 was observed (Fig. 4). One variable aspect of SDS-gel

Amino Acid Analysis

Fifteen-microgram samples in duplicate from two different preparations were hydrolyzed in vacuo with 6 N HCl at 105°C for 24, 48, and 72 h, and the hydrolysates were analyzed on a Beckman 121M amino acid analyzer (21-23).

Rate of Cleavage of 125I-Factor XI

The following mixture was incubated for varying times at 37°C: 10 μl of Tris buffer, 5 μl (0.8 μg) of Factor XII, 5 μl (1.0 μg) of high molecular weight kininogen, 5 μl (0.05 μg) of human albumin, 10 μl (0.034 clotting unit, −0.17 μg) of Factor XI, 5 μl (0.005 clotting units, −0.025 μg, 0.5 μCl) of 125I-Factor XI, and 10 μl (20 μg) of kaolin. The Tris buffer was 0.1 M Tris, 0.05 M NaCl, pH 7.4. All protein reagents were in 0.5 mM acetate, 0.5 mM EDTA, 0.15 M NaCl, pH 5.0. The kaolin was in Tris-buffered saline. At the various incubation times, a 25-μl aliquot of the reaction mixture was removed using concanavalin A-Sepharose affinity chromatography and eluted with affinity chromatography. This technique is also very suitable for concentration of Factor XI, activity. The extent of cleavage of the 125I-Factor XI was determined by counting the 125I. Factor XI in 1.2-mm gel slices obtained from the SDS-polyacrylamide gels and by integrating the radioactivity in the Mr = 50,000 and 92,000 peaks and expressing this as the percentage of the radioactivity of the Mr = 83,000 peak present at zero minute.

Cleavage of 125I-Factor in Plasma during Contact Activation

Five microliters of 125I-Factor XI (0.5 μCl, 0.005 clotting unit, −0.025 μg of protein) plus 5 μl of Tris-buffered saline were added to 10 μl of normal human plasma or Factor XII-deficient plasma in plastic tubes. Then either 10 μl of kaolin (10 mg/ml) in Tris-buffered saline or 10 μl of buffer alone were added and the reaction mixture was incubated for 8 min at 37°C. At this point, a 5-μl aliquot was withdrawn and added to 50 μl of a 3% SDS solution in the presence or absence of 3% mercaptoethanol. The sample was placed in a boiling water bath for 7 min and then analyzed on SDS-polyacrylamide gels. Results

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Radiolabeling of Factor XI

Factor XI was labeled with 125I using the chloramine-T method (19). Radioactivity was measured with a Packard 320 γ-spectrometer. The 125I-Factor XI containing from 4 to 16 μCi/μg retained its procoagulant activity after it had been subjected to the radiolabeling procedure.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out on 7.5% gels (6 mm × 8 cm) essentially according to the general method of Weber and Osborn (20). The gels were stained for protein with Coomassie blue.
analysis of Factor XI was that, in some experiments, some $M_r = 83,000$ material appeared on gels in the absence of added 2-mercaptoethanol. Further studies showed that, in the absence of reducing agent, a 100°, 5-min treatment of the SDS-Factor XI mixture would promote this breakdown while a 37°, 1-h treatment of a similar sample yielded only $M_r = 160,000$ material. The presence of 8 M urea in the SDS-Factor XI sample incubated at 37° for 1 h did not produce any $M_r = 83,000$ material in the absence of mercaptoethanol. Thus, human Factor XI appears to contain two similar, if not identical, $M_r = 83,000$ polypeptide chains covalently linked by disulfide bonds.

**Amino Acid Composition of Factor XI**—The amino acid composition of Factor XI is shown in Table II. The relative high content of basic amino acids may account for the reported isoelectric point near 9.1 (8).

**Mechanism of Activation of Factor XI by Activated Factor XII**—The rate of activation and proteolytic cleavage of Factor XI by purified activated Factor XII was studied. In the presence of kaolin, Factor XI was incubated with a mixture of purified human Factor XII, high molecular weight kininogen, and prekallikrein for varying times at 37° with each protein at its approximate plasma concentration. This combination of purified reagents at these concentrations has been shown to reconstitute very effectively the contact phase activator of Factor XI (2). At varying incubation times, aliquots of the mixture were analyzed for the extent of cleavage of $^{125}$I-Factor XI and for Factor XI clotting activity. During the incubation, $^{125}$I-Factor XI was cleaved as shown on SDS-polyacrylamide gels in the presence of reducing agent. No cleavage of Factor XI was observed if either Factor XII or high molecular weight kininogen was omitted. The $M_r = 83,000$ polypeptide chain disappears with concordant appearance of $M_r = 50,000$ and 33,000 polypeptides (Fig. 5A). As seen in Fig. 5B, the extent of cleavage of $^{125}$I-Factor XI was directly correlated with the

**TABLE II**

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<th>Amino acid composition of human Factor XI</th>
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<td>Residues per 100 residues</td>
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<td>Lysine</td>
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<td>Alanine</td>
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**Fig. 4.** Human coagulation Factor XI on 7.5% SDS-polyacrylamide gels. The gel run in the absence of reducing agent contains 7 μg of protein; the gel run in the presence of reducing agent contains 4 μg of protein.

**Fig. 5.** Correlation of the kinetics of activation of human Factor XI with its cleavage by activated Factor XII. A, the extent of cleavage of $^{125}$I-Factor XI is seen for various indicated incubation times in these radioactivity profiles of 7.5% SDS-polyacrylamide gels. B, the appearance of Factor XIa activity is correlated with the cleavage of Factor XI. For details, see "Materials and Methods."
appearance of procoagulant Factor XI activity. In the absence of reducing agent, the cleaved $^{125}$I-Factor XI retained its apparent $M_r = 160,000$ on SDS-polyacrylamide gels.

In other experiments, activation of Factor XI by trypsin in solution resulted in similar cleavage products as produced by surface-bound activated Factor XII in the presence of high molecular weight kininogen and kallikrein. Similar observations of trypptic cleavage were reported by Wuepper (7).

Cleavage of $^{125}$I-Factor XI in Plasma—The molecular weight pattern of $^{125}$I-Factor XI in plasma subjected to contact activation was studied in order to see whether contact activation which produces Factor XI activity involved limited proteolytic cleavage of Factor XI as seen above using purified proteins. Normal human plasma containing $^{125}$I-Factor XI was incubated in the presence or absence of kaolin for 8 min at 37°C, since activated partial thromboplastin time clotting assays typically employ such an incubation. The data seen in Fig. 6A show that in the absence of kaolin or contact activation, the $^{125}$I-Factor XI in normal plasma exhibits its native molecular weight of 83,000. Contact activation of plasma due to the presence of kaolin results in the cleavage of $^{125}$I-Factor XI to yield $M_r = 50,000$ and 33,000 chains held together by disulfide bonds (Fig. 6A). The radioactivity profiles seen in Fig. 6, A and B, showing the cleavage of $^{125}$I-Factor XI during contact activation in plasma are essentially identical to the 0- and the 20-min profiles seen in Fig. 5A for the activation of Factor XI by purified reagents. In order to test whether the cleavage of $^{125}$I-Factor XI in plasma during contact activation was dependent on Factor XII, identical experiments were performed using Factor XII-deficient plasma. The results seen in Fig. 6, C and D, show that no cleavage of $^{125}$I-Factor XI occurred in the absence or presence of kaolin. Thus, just as the appearance of Factor XI activity during contact activation of plasma is dependent on Factor XII, so also is the limited specific proteolytic cleavage of Factor XI.

**DISCUSSION**

Human coagulation Factor XI plays an important role in the intrinsic pathway of the blood coagulation sequence (26-28) as a potent activator of Factor IX (4, 5). In the initiation of the intrinsic pathway, after exposure of plasma to an activating surface (i.e. glass, kaolin), Factor XII is activated in the presence of prekallikrein and high molecular weight kininogen. Recently, a proposed molecular mechanism responsible for contact activation was described (2). The current hypothesis is that a surface-bound complex between Factor XII and high molecular weight kininogen is formed which places Factor XII into a conformation which is highly susceptible to proteolytic cleavage and that this proteolytic cleavage is responsible for activation of Factor XII. This surface-bound complex between active Factor XII and high molecular weight kininogen is a potent activator of Factor XI, prekallikrein, and plasminogen proactivator. The newly formed kallikrein can reciprocally activate more Factor XII in other bound Factor XII-high molecular weight kininogen complexes, thereby augmenting the amount of active Factor XII and consequently the activation of Factor XI, prekallikrein, and plasminogen proactivator. A deficiency of prekallikrein, known as Fletcher trait (7, 29, 30), or of high molecular weight kininogen known variously as Flaujeac (31), Fitgerald (32, 33), or Williams trait (34), results in severely defective contact activation reactions.

The existence of alternative mechanisms for the activation of Factor XI have been postulated because of the absence of a bleeding tendency in Factor XII-deficient patients (35). Evidence for the existence of such a mechanism has been shown by Walsh (36). For example, collagen can induce a coagulant activity in platelets, which initiates intrinsic coagulation (36). This activity is independent of Factor XII but is dependent on Factor XI.

Attempts to purify human and bovine Factor XI have been reported by several groups (5-14). The new purification method described here differs significantly from these reports. Highly purified Factor XI is isolated in its precursor form in a relatively high yield and is stable upon storage at 4°C. The isolated Factor XI has an apparent molecular weight of 160,000 as determined by SDS-polyacrylamide gel electrophoresis, and it is composed of two very similar or identical polypeptide chains with a molecular weight of 83,000 which are held together by disulfide bonds. Similar molecular weight values have been previously reported for bovine (5, 12) and human (0-11, 14) Factor XI.

The observed specific clotting activity of purified Factor XI of 250 clotting units/mg of protein suggests that the concentration of Factor XI in normal human plasma is approximately 4 µg/ml. The specific clotting activity of purified human Factor XII is 80 clotting units/mg of protein (15) and the concentration of Factor XII in normal human plasma as measured by immunologic techniques is 24 µg/ml (15, 37). Thus, in plasma the concentration of the substrate, Factor XI, is one-third to one-sixth that of its activator, Factor XII. For this reason, studies of the activation of Factor XI both in a purified system and in plasma were performed under conditions in which Factor XII was in excess over Factor XI. The studies reported here show that activation of Factor XI by a mixture of Factor XII, high molecular weight kininogen and prekallikrein at their respective plasma concentrations in the presence of kaolin is associated with limited proteolytic cleavage of Factor XI. In the presence of reducing agents, two fragments of Factor XI are observed on SDS-polyacrylamide gels with apparent molecular weights of 50,000 and 33,000. Factor XI in the absence of reducing agent retains a molecular weight of 160,000, indicating that the fragments are held together by disulfide bonds. Cleavage of human Factor XI by human Factor XII or fragments or by trypsin yielded similar polypeptide fragments (7, 38). The kinetic studies correlating the rates of cleavage and activation of Factor XI indicate that surface-bound active Factor XII converts Factor XI from a precursor to a clot-promoting agent through a very limited
proteolytic modification. The studies of the cleavage of $^{125}$I-Factor XI in normal plasma during contact activation suggest that the same limited proteolytic cleavage of Factor XI occurs in plasma under conditions which generate Factor XI$\alpha$. As expected, this limited cleavage of Factor XI in plasma is dependent on the presence of Factor XII, since addition of kaolin to Factor XII-deficient plasma, which does not generate Factor XI$\alpha$, does not result in any cleavage of $^{125}$I-Factor XI. Previously it was shown that the rate of activation of Factor XI during contact activation in plasma requires limited proteolytic cleavage.

A schematic drawing of human Factor XI is seen in Fig. 7. The two-chain form of Factor XI is converted to a four-chain Factor XI$\alpha$ by trypsin or by Factor XII$\alpha$. For simplicity, intermediate three-chain forms of the molecule which must exist are not depicted. Preliminary experiments using $^{35}$Sdiisopropyl phosphorofluoridate ($^{35}$SDFP) indicated the uptake of the $^{35}$Sdiisopropyl phosphoryl group by the $M_r = 33,000$ fragment, suggesting that this fragment contains the active site. If the two subunits of Factor XI are identical, Factor XI$\alpha$ at $M_r = 160,000$ may contain two active sites. Further studies on this point are in progress.

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