Activation of Bovine Factor IX (Christmas Factor) by Factor XI\textsubscript{a} (Activated Plasma Thromboplastin Antecedent) and a Protease from Russell’s Viper Venom*

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Bovine factor IX (Christmas factor) is a coagulation protein present in plasma in a precursor or inactive form. It is a glycoprotein ($M_\text{r} = 55,000$) composed of a single polypeptide chain. Factor IX in the presence of calcium ions is converted to factor IX\textsubscript{a} by factor XI\textsubscript{a} (activated plasma thromboplastin antecedent) or a protease present in Russell’s viper venom. The activation of factor IX by the protease from Russell’s viper venom is due to the cleavage of a single internal Arg-Val peptide bond in factor IX. This leads to the formation of factor IX\textsubscript{a}, a protein with the same molecular weight as the precursor. Factor IX\textsubscript{a} is composed of two polypeptide chains held together by a disulfide bond(s), and these two chains have an NH$_2$-terminal sequence of Tyr-Asn-Ser-Gly- and Val-Val-Gly-Gly-. The mechanism of activation of factor IX catalyzed by the protease from Russell’s viper venom differs from that catalyzed by factor XI\textsubscript{a}. The latter enzyme activates factor IX in a two-step reaction. In the first step, an internal Arg-Ala bond is cleaved, and in the second step, an Arg-Val bond is cleaved. The second cleavage leads to the liberation of an activation peptide from the NH$_2$-terminal portion of the heavy chain to produce factor IX\textsubscript{a}. The critical step in the activation of factor IX by both pathways is the cleavage of the same Arg-Val peptide bond and the formation of a new NH$_2$-terminal sequence of Val-Val-Gly-Gly- in the heavy chain of the enzyme which contains the active site serine residue. Factor IX\textsubscript{a} has approximately 50% of the specific activity of factor IX\textsubscript{a} in a coagulant or esterase assay, and both enzymes are inhibited by antithrombin III. A number of other purified plasma proteases were also examined for their ability to activate factor IX. Factor X\textsubscript{n} (activated Stuart factor) catalyzed a slow but definite activation of factor IX, while the activation of factor IX by bovine kallikrein, thrombin, or factor XII, (activated Hageman factor) was negligible.

Factor IX (Christmas factor)$^*$ is a vitamin K-dependent

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EXPERIMENTAL PROCEDURES

Materials – Bovine factor IX and factor X\textsubscript{n} were purified to a homogeneous state as previously described (13, 14). Bovine thrombin and bovine plasma kallikrein were kindly provided by Drs. W. Kisiel and R. Heimark in our laboratory. Bovine factor XII was purified by the method of Fujikawa et al. (15), and bovine factor XI\textsubscript{a} was kindly provided by Dr. K. Kurachi in our laboratory. Bovine thrombin, kallikrein, factor XII\textsubscript{a}, and factor XI\textsubscript{a} were homogenous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Russell’s viper venom was obtained from the Miami Serpentarium, Miami, Florida, and was purified by the procedure of Schiffman et al. (16) or by the method of Kisiel et al. (17). The specific activity was about 5000 units/mg of protein (17) and both preparations gave identical results. Bovine antithrombin III was isolated by the method of Thaler and Schmer (18) and was kindly provided by Dr. K. Kurachi. Carboxypeptidase A and B were purchased from Worth-
gington and pretreated with 1 × 10⁻³ M diisopropyl phosphorofluoridate (DFP) before use.

Sephadex G-100, G-50, SP-Sephadex C-50, and Sepharose 4B were products of Pharmacia. N,N'-methylenebisacrylamide, 2-mercaptoethanol, and N,N,N′,N″-tetramethylethylenediamine were purchased from Eastman. Acrylamide was obtained from Bio-Rad Laboratories. Baker Chemical Co. supplied 4-vinylpyridine which was distilled before use. Benzoyl-L-arginine ethyl ester labeled with [³H]Hethanol was synthesized in our department by Eric Fodor, and tosyl-L-arginine [¹⁴C]methyl ester was a gift from Dr. J. Felsenfeld. The toluenol scintillant was prepared by dissolving 15 g of Omnifluor (New England Nuclear) in 3.8 liters of toluene. Cephalin (rabbit brain extract) was obtained from Sigma. The content of one vial was suspended in 100 ml of 0.15 M NaCl and frozen in aliquots. To make a concentrated solution, the lyophilized material was suspended in 10 ml of saline. Sodium heparin (20,000 units/ml) was purchased from Invenex, San Francisco, Calif.

Methods - Protein concentrations were determined by absorption at 280 nm employing an ε₉ₐ₀ of 14.9 for factor IX and 14.3 for factor X. Factor X was measured by its coagulant activity as determined by amino acid analysis after hydrolysis of the sample in 6 N HCl. The sequence analysis on Peak I (Fig. 3) was carried out on 6 mg of protein. These analyses were each carried out in duplicate.

The assay of factor IX, coagulant activity was performed according to the method of Fujikawa et al. (7). After activation of factor IX by thrombin or factor XI, the reaction mixture was diluted 10-fold with 0.1 M phosphate buffer (pH 6.0) containing 0.05 M NaCl. Each diluted sample was then applied to a small column (0.3 × 2 cm) of DEAE-Sephadex A-50 which had been previously equilibrated with 0.1 M phosphate buffer (pH 6.0) containing 0.05 M NaCl. The column was washed with 8 ml of the same buffer to elute the thrombin activity. Factor IX was then eluted with 5 ml of 0.1 M phosphate buffer (pH 6.0) containing 0.3 M NaCl and assayed for coagulant activity. Factor X activity remained bound to the column under these conditions. For the activation of factor IX by factor XI or kallikrein or factor XII, the reaction mixtures were diluted and assayed directly.

The esterase activity of factor IX was determined using benzoyl-L-arginine [³H]Hethyl ester or tosyl-L-arginine [¹⁴C]methyl ester according to the method of Roffman et al. (28) as described by Kurachi et al. (29). Ten microliters of sample were transferred into a counting vial and 20 µl of 0.67 mM benzoyl-L-arginine ethyl ester or 1 mM tosyl-L-arginine methyl ester solution in dry dimethylformamide was then added and mixed. Ten milliliters of Omnifluor scintillation fluid were added and counting was started immediately in a Beckman LS-100C scintillation counter at room temperature.

The protease from Russell's viper venom was coupled to Sepharose 4B by the method of Cuatrecasas (31). Activated Sepharose 4B was prepared by reacting 10 g of cyanogen bromide with 50 ml of washed, packed Sepharose at 25° for 15 min. Thirty micromoles of the partially purified protease from Russell's viper venom in 50 ml of 0.1 M NaHCO₃ (adjusted to pH 9.0) were added to the activated Sepharose 4B and the mixture was stirred slowly for 20 h at 4°. The RVV-X-Sepharose was then washed with 0.05 M Tris/HCl buffer (pH 8.0) containing 1 M NaCl, followed by 0.05 M Tris/HCl buffer (pH 4.0) containing 1 M NaCl. This washing cycle was repeated three times.

Factor X was prepared by activating bovine factor X with RVV-X-Sepharose. In these experiments, factor X (2 mg) was incubated with 0.5 ml of RVV-X-Sepharose in 0.05 M Tris/HCl buffer (pH 8.0) for 30 min. The reaction was stopped by the addition of 1 ml of 0.1 M EDTA and the reaction mixture was lyophilized. The lyophilized sample was then reduced and pyridyldethylylated by the method of Friedman et al. (24), and salt and excess reagent were removed by gel filtration on a Sephadex G-50 column (1.8 × 50 cm) previously equilibrated with 0.1 M phosphate buffer (pH 6.0) containing 0.3 M NaCl in the presence of 0.05 M CaCl₂.

The separation of the two chains of factor IX was carried out in the following manner. A reaction mixture containing 12 mg of factor IX, 0.5 mg of Russell's viper venom protease, 5 mM CaCl₂, and 50 mM Tris/HCl (pH 8.0) in a total volume of 10 ml was incubated at 37° for 30 min. The reaction was stopped by the addition of 1 ml of 0.1 M EDTA and the reaction mixture was lyophilized. The lyophilized sample was then reduced and pyridyldethylylated by the method of Friedman et al. (24), and salt and excess reagent were removed by gel filtration on a Sephadex G-50 column (1.8 × 50 cm) previously equilibrated with 0.1 M phosphate buffer (pH 6.0) for 30 min. The protein was eluted by a gradient formed from 150 ml of 7 M urea containing 9% formic acid and 0.2 M NaOH and applied to an SP-Sephadex column (1.5 × 25 cm) previously equilibrated with the same buffer. The protein was eluted by a gradient formed from 150 ml of 7 M urea containing 9% formic acid and 0.2 M NaOH and 150 ml of 7 M urea containing 9% formic acid and 0.8 M NaOH. The two protein components were pooled separately and desalted by passing each sample through a Sephadex G-50 column (1.9 × 50 cm) in 9% formic acid followed by lyophilization. The activation peptide released during the activation of factor IX by factor XI was isolated as previously described (7).

The inhibition of factor IX by bovine antithrombin III was carried out by mixing a plasm in a concentration of 3.9 × 10⁻⁸ M with antithrombin III in 0.025 M Tris/HCl buffer (pH 8.0) containing 10 mM NaCl and 100 units of heparin as previously described (30). The mixture was incubated at 37° for 90 min and assayed for activity by the esterase assay.

RESULTS

Activation of Bovine Factor IX by a Protease from Russell's Viper Venom - A time curve for the activation of factor IX by the Sepharose-bound protease of Russell's viper venom is shown in Fig. 1. In these experiments, factor IX was activated in the presence of 0.01 M CaCl₂ and 0.05 M Tris/HCl buffer (pH 8.0). Factor IX was measured by its coagulant activity (open circles) and its esterase activity toward benzoyl-L-arginine ethyl ester (closed circles). These two activities increased concurrently and reached a maximum after 10 min incubation.

Similar experiments employing the soluble protease from Russell's viper venom showed that the same rate of reaction, as measured by esterase activity, could be obtained when the weight ratio of enzyme to factor IX was 1 to 20. In the absence of CaCl₂ or when Sepharose 4B was added alone, no increase in either coagulant or esterase activity was observed.

¹The abbreviation used is: DFP, diisopropyl phosphorofluoridate.
A possible cleavage of a peptide bond in factor IX after activation by the protease from Russell's viper venom was tested by carrying out sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments on aliquots of the reaction mixture. Fig. 2 shows the gel pattern for factor IX before and after activation. Factor IX migrates as a single polypeptide chain in the absence or presence of 2-mercaptoethanol (Gels 1 and 2, respectively). After activation, a protein band is observed with the same migration as factor IX (Gel 3). These experiments indicate that the activated factor IX (referred to as factor \( I_{Xa} \)) has essentially the same molecular weight as its precursor. In the presence of 2-mercaptoethanol, factor \( I_{Xa} \) appears as two bands of smaller molecular weight (Gel 4). These experiments suggest that the activation of factor IX by the protease from Russell's viper venom results from the hydrolysis of a single internal peptide bond. Factor \( I_{Xa} \) migrates at a rate identical with factor IX (\( M_r \) = 55,400), but contains two chains of similar size held together by a disulfide bond(s). The migration of the faster moving band (referred to as the valine chain) is the same as the heavy chain of factor \( I_{Xa} \) formed in the presence of factor XI (Gel 6). The molecular weight of this polypeptide chain was found to be 27,300 by sedimentation equilibrium (7).

In addition to the major protein band seen in the gel of factor \( I_{Xa} \), a very minor band is evident with an apparent lower molecular weight (Gel 3, Fig. 2). This minor band migrates in the same position as factor \( I_{Xa} \) formed in the presence of factor XI (Gel 5) and has been called factor \( I_{Xa, b} \). Factor \( I_{Xa} \) and factor \( I_{Xa, b} \) can be separated by gel filtration activity. Factor \( I_{Xa} \) was observed in all preparations of factor \( I_{Xa} \), but accounted for less than 5% of the total factor IX observed on gels after activation with the protease from Russell's viper venom.

Characterization of Two Chains of Factor \( I_{Xa} \) — The above experiments indicate that the protease from Russell's viper venom activates factor IX by a mechanism different from that catalyzed by factor XI. Thus, it was important to characterize the two chains of factor \( I_{Xa} \) in order to identify the specific peptide bond which was split during the activation reaction. Accordingly, factor \( I_{Xa} \) was reduced, pyridylethylated, and chromatographed on SP-Sephadex (Fig. 3). Two major peaks were observed. The protein fractions in each peak region were pooled separately, desalted on a Sephadex G-50 column, and lyophilized. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of the two peaks are shown in Fig. 4. Gel 1 is Peak II from the SP-Sephadex column and corresponds to the valine chain or faster moving component of reduced factor \( I_{Xa} \). Gel 2 is a mixture of Peak I and Peak II and corresponds to the doublet seen for reduced factor \( I_{Xa} \) (Gel 4, Fig. 2). Gel 3 is Peak I and corresponds to the tyrosine chain or slower moving component of reduced factor \( I_{Xa} \).

Each chain from factor \( I_{Xa} \) was then subjected to automated Edman degradation in order to determine its NH₂-terminal sequence. Peak I contained an NH₂-terminal tyrosine residue, and no other amino acids were detected. The NH₂-terminal sequence and equivalents per 25,500 g of glycoprotein were as follows: tyrosine (0.3), asparagine (0.1), serine (not quantitated), and glycine (0.4). This sequence is identical with that of factor IX (7) and indicates that this polypeptide chain is derived from the NH₂-terminal end of the precursor molecule. Peak II contained an NH₂-terminal valine residue, and no other amino acids were detected. The NH₂-terminal sequence and equivalents per 27,000 g of glycoprotein were as follows: valine (0.6), valine (0.8), glycine (0.7), and glycine (0.3). This sequence is identical with that of the heavy chain of factor \( I_{Xa} \) formed by factor XI (7) and indicates that this polypeptide chain is derived from the COOH-terminal end of the precursor molecule.

The method for the separation of the two chains of factor \( I_{Xa} \) on SP-Sephadex was initially developed by Dr. Kotiti Titani in our department.
Factor IX Activation

FIG. 3. Elution profile for the S-pyridylethyl chains of factor IX from an SP-Sepahex column. Factor IX (10 mg) was activated with the protease from Russell's viper venom and the S-pyridylethyl derivatives were prepared as described under "Methods." The S-pyridylethyl peptides were then separated by chromatography on an SP-Sepahex C50 column (1.5 × 30 cm) equilibrated in 9% formic acid, 0.2 M NaOH, and 7 M urea. Elution was carried out by a gradient formed from 150 ml of equilibration buffer and 150 ml of 9% formic acid, 0.8 M NaOH, and 7 M urea (pH 3.8) at a flow rate of 12 ml/h. Peaks I and II were pooled as shown by the bars.

The amino acid compositions of the tyrosine and valine chains of factor IX were then determined (Table I). The amino acid composition of the tyrosine chain is shown in Column 1 and is essentially identical with the summation of the tyrosine chain of factor IX and the activation peptide (Column 2). The amino acid composition of the valine chain is shown in Column 3 and is essentially identical with that of the valine chain of factor IX (Column 4) (7). Furthermore, a summation of the amino acids in the tyrosine chain and the valine chain of factor IX (Column 5) agrees well with the amino acid composition of the precursor factor IX molecule (Column 6). These data also support the conclusion that no activation peptide is split from factor IX when it is converted to factor IX by the protease from Russell's viper venom. These data also support the conclusion that the activation of factor IX results from the cleavage of a single internal peptide bond in the precursor protein.

The COOH-terminal residue of the tyrosine chain of factor IX was then examined after incubating this polypeptide with carboxypeptidase A or B. The amino acids released by these two enzymes were then analyzed by the amino acid analyzer. Arginine (1.05 mol/mol of tyrosine chain) and small amounts of serine and lysine were found after incubation with carboxypeptidase B for 24 h at 37°C. Only traces of amino acids were released by carboxypeptidase A treatment under essentially identical conditions. These data indicate that the tyrosine chain of factor IX has an arginine as its COOH-terminal residue. From these data and the NH-terminal analysis of the valine chain of factor IX, it is evident that the protease from Russell's viper venom hydrolyzes a single, specific arginyl-valine peptide bond during the conversion of factor IX to factor IX.

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the S-pyridylethyl fragments of factor IX isolated by chromatography on SP-Sepahex. The two S-pyridylethyl chains were isolated as shown in Fig. 3 and incubated at 37°C for 4 h with 2% sodium dodecyl sulfate containing 5% 2-mercaptoethanol and 3 M urea and then applied to 8.5% polyacrylamide gels. Gels 1 and 3 contain 15 μg of Peaks II and I, respectively. Gel 2 contains 15 μg of both Peaks I and II. The anode was at the bottom of the gels.

Comparison of Activation of Factor IX by the Protease from Russell's Viper Venom and Factor XI -- A time curve for the activation of factor IX by factor XI employing an enzyme:substrate ratio of 1:50 is shown in Fig. 5 (open circles). In this experiment, the activation of factor IX leveled off in approximately 25 min. Factor IX was the only product formed in this reaction as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Gel 5, Fig. 2). The specific activity of factor IX in the clotting assay, however, was approximately twice that obtained after activation of factor IX by the protease from Russell's viper venom (open triangles). As previously noted, factor IX was the predominant product (>95% by sodium dodecyl sulfate-polyacrylamide gels) formed in the activation of factor IX by the protease from Russell's viper venom. The addition of factor XI to an aliquot of the latter reaction mixture (shown by the arrow) resulted in an immediate increase in clotting activity (solid triangles), and this clotting activity was now essentially identical with that obtained by the activation of factor IX by factor XI. The conversion of factor IX to factor IX was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Factor IX formed by the protease from Russell's viper venom was also separated from traces of factor IX by gel filtration on a Sephadex G-100 column (1.6 × 80 cm). This preparation of factor IX had a specific activity in the clotting...
Factor IX Activation

Amino acid compositions of tyrosine and valine chains of bovine factor IXα

Expressed in residues per mol of glycoprotein.

<table>
<thead>
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<th>Components</th>
<th>Tyrosine chain of factor IXα</th>
<th>Tyrosine chain of factor IXα plus activation peptide</th>
<th>Valine chain of factor IXα</th>
<th>Valine chain of factor IXα</th>
<th>Tyrosine chain plus Valine chain of factor IXα</th>
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* From Fujikawa et al. (7)

† Determined as S-pyridylethylcysteine by the method of Friedman et al. (24).

‡ The 4.0 residues of leucine which were originally reported to be present in the activation peptide (7) was in error; the correct value was 4.4.

Table I

Identification of COOH-terminal Amino Acid in Tyrosine Chain of Factor IXα and Activation Peptide—In our previous studies on the activation of factor IX with factor Xa, two cleavage sites in the precursor molecule were reported (7). In this reaction, factor XIa hydrolyzed an internal peptide bond yielding an inactive factor IX intermediate (factor IXα) containing two chains held together by a disulfide bond(s). The heavy chain contained an NH2-terminal alanine residue. In the second step, an activation peptide was split from the NH2-terminal end of this chain giving rise to factor IXα. In order to further identify the specific bond split in the first reaction, the tyrosine chain of factor IXα was prepared as previously described (7) and incubated with carboxypeptidase B for 24 h at 37°C. Only traces of amino acids were released by carboxypeptidase B. This indicates that the tyrosine chain of factor IXα has an arginine as the COOH-terminal residue. Thus, the conversion of factor IXα to factor IXa or factor IXα to factor IXα is due to the cleavage of a specific arginyl-alanine peptide bond.

Tyrosine chain plus Valine chain of factor IXα

In order to further identify the specific bond split in the second reaction, the activation peptide was incubated with carboxypeptidase A or B and the amino acids released were again analyzed by the amino acid analyzer. Arginine (0.31 mol/mol of activation peptide) was the only amino acid found after incubation with carboxypeptidase B for 24 h at 37°C. Only traces of amino acids were released by carboxypeptidase A.

Effect of Other Serine Proteases on Factor IX—The activation of bovine factor IX by homogeneous preparations of thrombin, factor Xa, plasma kallikrein, and factor XII was also examined to compare these enzymes with factor XIa and the protease from Russell’s viper venom. In these experiments, a high enzyme:substrate ratio (1:20) was employed. In order to assay for factor IXa in the presence of thrombin or factor Xa, aliquots of the reaction mixtures were applied to small DEAE-Sephadex columns to separate factor IXa from thrombin or factor Xa prior to assay (see “Methods”). As shown in Fig. 6.

This conclusion has been confirmed by the amino acid sequence data of Drs. K. Katayama and K. Titani who have shown the COOH-terminal sequence of the tyrosine chain of factor IXα to be Lys-Lys-Leu-Thr-Arg (personal communication).
The data presented in this manuscript indicate that bovine factor IX is converted to an enzyme by the cleavage of a specific internal Arg-Val peptide bond. This bond is readily cleaved by the protease from Russell's viper venom, leading to factor IX₇₉ (lower pathway, Fig. 6). This reaction is followed by a very slow conversion of factor IX₇₉ to factor IX₈₇. Whether the latter reaction is catalyzed by the protease from Russell's viper venom or by factor IX₈₇ is not known. It does occur readily, however, in the presence of factor XI₈ and calcium ions.

The activation of factor IX by factor XI₈ and calcium ions involves a two-step mechanism (upper pathway, Fig. 6). In the first step, an Arg-Ala bond is cleaved giving rise to factor IX₈₇. This protein has no enzymatic activity. In a second slower step, factor IX₈₇ is converted to factor IX₈₇ in the presence of factor XI₈ and calcium ions. In this reaction, an Arg-Val bond is cleaved giving rise to factor IX₉₇ and an activation peptide. This reduces the molecular weight of the precursor from 55,000 to approximately 45,000. The clotting and esterase activities of factor IX₇₉ and factor IX₈₇ differ in that factor IX₇₉ has about 2-fold the specific activity of factor IX₈₇. Thus, the presence of the activation peptide on the COOH-terminal end of the tyrosine chain of factor IX₇₉ decreases the biological activity of this enzyme by approximately 50%. Both factor IX₇₉ and factor IX₈₇, however, are readily inhibited by antithrombin III forming a 1:1 molar complex of enzyme and inhibitor.

The sequence of the active site of factor IX is homologous with many other serine proteases, including pancreatic trypsin (32). Thus, it seems probable that the basic mechanism for the proteolytic activation of bovine factor IX involves an ion pair formation between the newly formed NH₂-terminal valine residue and the carboxyl group of the aspartic acid residue adjacent to the active site serine. This activation mechanism would be analogous to that of the pancreatic enzymes (33, 34) which involve a charge relay network (33, 35-37) or a proton relay system (38) in their active sites.

The activation of factor IX by the protease from Russell's viper venom represents the third plasma protein activated by RVV-X. The other two plasma proteins known to be activated by this protease are human and bovine factor X (7, 13, 39-42) and bovine protein C (17). With factor X, a specific Arg-Ile bond is cleaved, and with protein C, a specific Arg-Val bond is cleaved during the activation reaction. RVV-X is different from a second protease in Russell's viper venom which activates factor V (16).
In the present experiments, no activation of bovine factor IX was observed by thrombin. These results are in contrast to those published recently by Irwin and Mammen (9). The reasons for these differences are not known. Also, essentially no activation of bovine factor IX was observed by factor X, or kallikrein. Factor IX was activated, however, by factor X, in the presence of calcium ions and phospholipid confirming the earlier data of Kalousek et al. (8). In this reaction, factor X, was separated from factor X, before assaying for factor IX, coagulant activity. Thus, it appears very unlikely that the activation of factor IX by factor X, is of any physiological importance since prothrombin is activated at a far faster rate by factor X, than is factor IX.

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Activation of bovine factor IX (Christmas factor) by factor XIa (activated plasma thromboplastin antecedent) and a protease from Russell's viper venom.

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