Purification of Factor VII from Bovine Plasma

REACTION WITH TISSUE FACTOR AND ACTIVATION OF FACTOR X*

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SUMMARY

Factor VII has been purified up to 330,000-fold from bovine plasma by adsorption on, and elution from, barium sulfate, anion exchange chromatography, gel filtration, and affinity chromatography on benzamidine coupled to Sepharose. The purified material is inhibited by diisopropylphosphorofluoridate both in its native state and after the formation of a complex with tissue factor in the presence of calcium ions. The Factor VII-tissue factor complex, unlike native Factor VII, has coagulant activity; this activity results in a complex proteolytic activation of Factor X to Xa, as shown by gel electrophoresis in the presence of sodium dodecyl sulfate, and by assay of the Factor Xa produced.

It has been concluded that Factor VII, unlike Factor X and prothrombin, circulates in the plasma as an active enzyme which has an absolute requirement for tissue factor in order to develop proteolytic activity towards Factor X.

In a previous study (1) it was shown that Factor VII is the only plasma component of the bovine tissue-factor pathway that is inhibited by diisopropylphosphorofluoridate (DFP). We now report the purification of the DFP-sensitive protein from bovine plasma. This protein corrects the defect in human plasma congenitally deficient in Factor VII.

The fact that bovine Factor VII is inhibited by DFP before the formation of a complex with tissue factor is in marked contrast with the inability of two other members of the "prothrombin group," Factor X and prothrombin, to be inactivated by DFP. Furthermore, the present studies show no detectable change in the molecular weight of Factor VII on its formation of a complex with tissue factor. This is again different from the activation of Factor X and prothrombin, both of which release large peptides (2-6). Thus, although Factor VII has certain biological and physical similarities to Factor X and prothrombin, it is strikingly different in that it apparently exists in plasma not as a zymogen, but in a partially active form.

The tissue factor-Factor VII complex is shown to activate Factor X directly, and furthermore, it appears that the activation of Factor X by the complex proceeds through at least two steps before the formation of the final product.

EXPERIMENTAL PROCEDURE

Materials

Sodium dodecyl sulfate, cadaverine (1,5-diaminopentane), diisopropylphosphorofluoridate, Tris ("Trizma base"), and p-aminoazobenzamidine-HCl were obtained from Sigma, St. Louis, Mo. N-(1-Naphthyl)-ethylenediamine-HCl and phenylmethylene sulfonyl fluoride (PMSF) were from Schwarz-Mann, Orangeburg, N. Y. The latter was used as a 0.1 M solution in dry isopropyl alcohol. [14C]DFP was a product of Amersham-Searle, Arlington Heights, Ill., and used as a solution of 0.8 mCi per ml (approximately 10 mCi) in isopropyl alcohol. Cyanogen bromide was obtained from Eastman, Rochester, N. Y. 1-Cyclohexyl-3-(2-morpholinioethyl)-carbodiimide methyl-p-toluene sulfonate was a product of Aldrich, Milwaukee, Wis. Guanidine hydrochloride (ultrapure) was obtained from Heico, Delaware Water Gap, Pa. Acrylamide and N,N'-methylene-bisacrylamide were products of Eastman, and recrystallized from chloroform and ethanol, respectively. Urea (Fisher Scientific Co.) was deionized before use. Sepharose 4B, DEAE-Sephadex A50, and Sephadex G-100 were products of Pharmacia, Piscataway, N. J.

Barium sulfate eluate from bovine plasma was prepared by the method of Nemerson and Esnouf (1) by the New England Enzyme Center, Boston, Mass. Purified Factor X was generously provided by Drs. Paul Bajaj and K. G. Mann and stored in 50% glycerol at -20 °C. Factor X-deficient plasma was obtained from Diagnostic Reagents Ltd., Thame, Oxon, England. Factor VII-defcient plasma was prepared from citrated bovine plasma (Pentex, Kankakee, III.) by the method of Nemerson and Clyne.1 1 g of Celite was suspended in 100 ml of plasma, and 0.1 ml of DFP slowly added with stirring. The suspension was stirred for 1 hour at 22 °C and the Celite removed by centrifugation. The plasma was stored at -20 °C before use. On the addition of bovine brain thromboplastin (7) and Ca++, the clotting time is more than 2 min (cf. 13 s for normal bovine plasma). Tissue factor apoprotein was purified from bovine brain by the method of Nemerson and Pitlick (8) and stored in 50% glycerol at -20 °C. Before use it was dialyzed against 0.375 M NaCl-50

Factor VII was assayed by a one-stage clotting time at 37°C. Samples, 50 ml (diluted in 0.1 M NaCl-50 mM Tris-Cl, pH 7.5), were mixed with 50 ml of Factor VII-deficient plasma and 50 ml bovine brain thromboplastin. CaCl2, 25 mM (50 ml), was then added and the clotting time, t, recorded. The assay was standardized with serial dilutions of normal bovine plasma, giving a linear plot of log t against log (concentration Factor VII). Normal citrated bovine plasma was defined as containing 100 units of Factor VII per ml. Factor X was assayed by the method of Denson (9). Activated Factor X was assayed by the method of Jobin and Ensonf (10) except that Factor X-deficient plasma was used as a substrate.

Factor X-converting Activity—Factor VII and tissue factor incubated in the presence of 5 mM Ca2+ develop coagulant activity. This reaction was stopped as required by the addition of 1/50 volume of 0.12 M sodium citrate, and the sample diluted in 0.1 M NaCl-50 mM Tris-Cl, pH 7.5, for assay: 0.1 ml diluted sample was added to 0.1 ml Factor VII-deficient plasma enriched with mixed brain phospholipid (11). CaCl2, 25 mM (0.1 ml), was then added and the clotting time, t, recorded. A plot of log t against log (concentration tissue factor-Factor VII complex) was linear. The activity units determined are arbitrary and are expressed as a percentage of maximum.

Sodium Dodecyl Sulfate Gel Electrophoresis—Electrophoresis was performed in 1.5-mm vertical slab gels which held up to 12 samples (12), according to the method of Fairbanks et al. (13), except that the gel contained 0.1% sodium dodecyl sulfate and 5 M (deionized) urea, and the samples 2% sodium dodecyl sulfate and 5 M urea. Electrophoresis was carried out at 30 mA per gel (approximately 12 ma per cm2 cross-sectional area). The gels were stained for 12 hours with 0.1% Coomassie blue in 25% isopropanol-10% acetic acid, and destained in 14% methanol-7% acetic acid at 35°C. The gels were dried to a thin film before autoradiography on Kodak RP/R54 x-ray film.

 Determination of Protein—Protein was estimated by measurement of the extinction at 280 nm, it being assumed that A280nm = 10.

Synthesis of Affinity Matrix—p-Aminobenzamidine was coupled to activated Sepharose 4B via a nine-carbon chain by the method of Cuatrecasas (14): 200 g of packed Sepharose 4B were activated with 40 g of CNBr dissolved in 40 ml of cold dimethylformamide.

1,5-Diaminopentane, 35 ml, was added to the washed activated Sepharose (pH maintained at 9.5), and the mixture left for 16 hours at 4°C. This derivative was then succinylated with 80 g of succinic anhydride (pH maintained at 6.0) for 16 hours at 4°C. Carboximidide, 18 g, was added to the washed succinylated derivative (suspended in H2O) at pH 4.75. p-Aminobenzamidine, 3.6 g, was then added and the reaction allowed to proceed for 5 hours at 22°C (pH maintained at 4.75), then for 16 hours at 4°C (15). The resulting material was washed extensively with 2 M NaCl, then with 4 M guanidine HCl. Even after prolonged washing, material absorbing at 280 nm was leached from the affinity matrix.

The extent of coupling of the p-aminobenzamidine to the substituted agarose was determined by hydrolyzing washed 1-ml aliquots of the matrix in 6 N HCl for 22 hours at 110°C in vacuo. The hydrolysate was dried and taken up in 3.0 ml of 0.1 N NaOH; particulate oxidation products of the agarose were removed by filtration. The free p-aminobenzamidine was diazotized and coupled to N-(1-naphthyl)-ethylenediamine according to the method of Goldbarg and Rutenberg (16). The reaction product gave an absorption maximum at 555 nm (ε = 45,000) and was quantified at that wavelength. The matrix used in these experiments contained 5 to 6 moles of p-aminobenzamidine per ml of settled Sepharose.

Purification of Factor VII—Factor VII was partially purified by DEAE-Sephadex chromatography of a barium sulfate eluate as described by Nemerson and Ensonf (1). Subsequent steps were carried out at 4°C. The DEAE-eluate was concentrated by ultrafiltration (Amicon; PM-10 membrane) to 100 ml and applied at 80 ml per hour to a column (2.5 x 36 cm) of Sephrose-benzamidine equilibrated in 0.2 M guanidine HCl-0.2 M NaCl-50 mM Tris-Cl, pH 7.5. The protein was eluted at 80 ml per hour with a linear 3-liter gradient, from 0.2 to 0.85 M guanidine HCl, in 0.2 M NaCl-50 mM Tris-Cl, pH 7.5. The fractions containing Factor VII were pooled, concentrated by ultrafiltration to 8 ml, and applied at 15 ml per hour to a column (2.5 x 90 cm) of Sephadex G-100 (medium) previously equilibrated in 0.2 M guanidine HCl in 0.2 M NaCl-50 mM Tris-Cl, pH 7.5. The column, fitted with upward-flow adaptors, was developed at the same flow rate with the same solvent. The fractions containing Factor VII were pooled, concentrated to 50 ml by ultrafiltration, and applied to a column (1.3 x 19 cm) of Sepharose-benzamidine, previously equilibrated in the same solvent, at 18 ml per hour. The protein was eluted at the same flow rate with a linear 600-ml gradient, from 0.3 to 0.9 M guanidine HCl, in 0.2 M NaCl-50 mM Tris-Cl, pH 7.5. The fractions containing Factor VII were pooled, titrated to pH 5.8 with 2 M acetic acid, and dialyzed against 0.1 M NaCl-10 mM acetic acid, 100 mM sodium citrate, and stored at -20°C.

RESULTS

Purification of Factor VII—Factor VII was partially purified by adsorption to, and elution from, barium sulfate, followed by DEAE-Sephadex chromatography (Fig. 1). The chromatography completely resolved Factor VII from Factor X, but effected only a partial separation from prothrombin. Preparative gel electrophoresis of the Factor VII-containing fractions (1) has yielded a preparation in which Factor VII is approximately 2000-fold purified. Analysis of this material by sodium dodecyl sulfate gel electrophoresis showed about 90% of the protein in one band (apparent molecular weight of 65,000) and a minor component with an apparent molecular weight of 60,000. Upon reaction with tissue factor, the major band was cleaved, yielding a product with a lower apparent molecular weight (Fig. 2). To identify the DFP-sensitive component previously described (1), the tissue factor-Factor VII incubation mixture was treated at 0, 15, and 30 min with [3H]DFP, subjected to electrophoresis in sodium dodecyl sulfate gels, and radioautographed. It was found that the radioactivity did not correspond to a stained band, and it therefore appeared that the DFP-sensitive protein was a trace constituent of this preparation (Fig. 2). The apparent molecular weight (nonsolvent) of the radioactive band is approximately 55,000. In the presence of 1% 2-mercaptoethanol this falls to approximately 37,000 (Fig. 8).

The apparent proteolysis of the major component is blocked by pretreatment of the preparation with DFP (1). Thus, the proteolysis of the major protein was used to evaluate potential
Fraction number

FIQ. 1 (left). Barium sulfate eluate, 2 liters, was chromatographed on DEAE-Sephadex A50 by batch and column procedures according to the method of Nemerson and Esnouf (1). The protein was eluted with a linear 4-liter gradient, from 0.14 M to 0.45 M NaCl, in 0.1 M Tris-Cl, pH 7.5. Fractions, 25-ml, were collected and assayed for Factors VII and X. The fractions indicated by arrows were pooled (DEAE-eluate).

FIG. 2 (center). Partially purified Factor VII (0.5 mg per ml) was incubated with tissue factor (0.2 mg per ml) and 5 mM Ca²⁺ in 50 mM Tris-Cl, pH 7.5, at 37°C. At the times shown 0.15 ml was removed into 10 μl of 0.18 M sodium citrate to stop the reaction. [²⁵P]DFP, 2 μl (0.8 mCi per ml), was added to each aliquot and the mixtures allowed to stand for 1 hour at 22°C. The reaction was stopped by the addition of an equal volume of 4% sodium dodecyl sulfate-10 M urea to each sample, followed by heating at 100°C for 4 min. Each sample, 70 μl (nonreduced), was applied to the gel.

FIG. 3 (right). Partially purified Factor VII (0.5 mg per ml) and tissue factor (0.15 mg per ml) were incubated with 5 mM Ca²⁺ in 50 mM Tris-Cl, pH 7.5, for 30 min at 37°C in the presence of various compounds present at the concentrations shown. The control mixture was sampled at 0 and 30 min, and the remaining mixtures at 30 min, into an equal volume of 4% sodium dodecyl sulfate-10 M urea, and heated at 100°C for 4 min. Each sample, 60 μl (nonreduced), was applied to the gel. Electrophoresis was from right to left.

FIG. 4. (left). The DEAE-Sephadex eluate (Fig. 1) was concentrated to 100 ml by ultrafiltration and chromatographed on benzamidine-Sepharose as described in the text. Fractions, 20-ml, were collected, the extinctions measured at 280 nm (○), and assayed for Factor VII activity (□). The fractions containing Factor VII were pooled as indicated by arrows (benzamidine Pool 1).

FIG. 5 (center). The benzamidine Pool 1 (Fig. 4) was concentrated by ultrafiltration to about 8 ml and chromatographed on a column of Sephadex G-100 as described in the text. Fractions, 5-ml, were collected, the extinctions measured at 280 nm (○), and assayed for Factor VII (□). The fractions indicated by arrows were pooled (G-100 pool).

FIG. 6 (right). The G-100 pool (Fig. 5) was applied directly to a column of benzamidine-Sepharose and chromatographed as described in the text. Fractions, 8-ml, were collected, their extinctions measured at 280 nm (○), and assayed for Factor VII (□). The fractions indicated by arrows were pooled and concentrated to 25 ml by ultrafiltration.

Fig. 1 (left). Barium sulfate eluate, 2 liters, was chromatographed on DEAE-Sephadex A50 by batch and column procedures according to the method of Nemerson and Esnouf (1). The protein was eluted with a linear 4-liter gradient, from 0.14 M to 0.45 M NaCl, in 0.1 M Tris-Cl, pH 7.5. Fractions, 25-ml, were collected and assayed for Factors VII and X. The fractions indicated by arrows were pooled (DEAE-eluate).

Fig. 2 (center). Partially purified Factor VII (0.5 mg per ml) was incubated with tissue factor (0.2 mg per ml) and 5 mM Ca²⁺ in 50 mM Tris-Cl, pH 7.5, at 37°C. At the times shown 0.15 ml was removed into 10 μl of 0.18 M sodium citrate to stop the reaction. [²⁵P]DFP, 2 μl (0.8 mCi per ml), was added to each aliquot and the mixtures allowed to stand for 1 hour at 22°C. The reaction was stopped by the addition of an equal volume of 4% sodium dodecyl sulfate-10 M urea to each sample, followed by heating at 100°C for 4 min. Each sample, 70 μl (nonreduced), was applied to the gel.

Fig. 3 (right). Partially purified Factor VII (0.5 mg per ml) and tissue factor (0.15 mg per ml) were incubated with 5 mM Ca²⁺ in 50 mM Tris-Cl, pH 7.5, for 30 min at 37°C in the presence of various compounds present at the concentrations shown. The control mixture was sampled at 0 and 30 min, and the remaining mixtures at 30 min, into an equal volume of 4% sodium dodecyl sulfate-10 M urea, and heated at 100°C for 4 min. Each sample, 60 μl (nonreduced), was applied to the gel. Electrophoresis was from right to left.
DFP was then added both to the complex and to the pure Factor VII in the presence of calcium ions and incubated for 1 hour at 37°C. It was of interest to know whether purified Factor VII and tissue factor-Factor VII complex has proteolytic activity. That the tissue factor-Factor VII complex has proteolytic activity is confirmed by disc gel electrophoresis (Fig. 7). This protein contained less than 50 units of Factor VII per mg, and was not significantly labeled by [32P]-labelling time on the addition of bovine brain thromboplastin and Ca2+. We consider that this is probably because of the solution of agarose polymer containing substituted benzamidine. If this were the case, the concentration of eluted protein solutions by ultrafiltration would result in the concentration of the soluble agarose, and hence inaccuracies in protein determination.

Second, in a previous preparation, material judged to be 70% pure by sodium dodecyl sulfate gel electrophoresis had a specific activity of 460,000 units per mg. This, however, lost activity on storage, showing that the pure active material is labile. It is therefore thought that the final purification achieved (Table I) represents a minimum figure, owing to overestimation of the protein concentration, and to decay of Factor VII activity. From the activity of partially purified preparations it is thought that fully active pure Factor VII would have a specific activity of about 660,000 units per mg, corresponding to a concentration of eluted protein solutions by ultrafiltration would result in the concentration of the soluble agarose, and hence inaccuracies in protein determination.

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On the addition of purified Factor VII to human Factor VII-deficient plasma (kindly provided by R. Goldstein, New York Medical College) to a concentration of 100 units per ml, the clotting time on the addition of bovine brain thromboplastin and Ca2+ was shortened from more than 2 min to 24 s. This correction is incomplete, presumably owing to the species specificity of the clotting factors involved, notably bovine Factor VII and human Factor X.

The major component of the Factor VII preparation obtained by disc gel electrophoresis (Fig. 2) has been completely separated from Factor VII by chromatography on benzamidine-Sepharose, and shows a single band on sodium dodecyl sulfate gel electrophoresis (Fig. 7). This protein contained less than 50 units of Factor VII per mg, and was not significantly labeled by [32P]-DFP, nor did it show Factor X, Factor IX, or prothrombin activity.

Reaction of Tissue Factor and Factor VII—It is clear from Fig. 2 that the tissue factor-Factor VII complex has proteolytic activity, and it was of interest to know whether purified Factor VII undergoes proteolysis on the formation of the complex. Saturating amounts of tissue factor were added to Factor VII in the presence of calcium ions and incubated for 1 hour at 37°C. [32P]DFP was then added to the complex and to the pure Factor VII, and allowed to react for 30 min at 22°C. The samples were added to sodium dodecyl sulfate-urea, subjected to electrophoresis on a sodium dodecyl sulfate gel, and autoradiographed (Fig. 8). The protein stain is not shown, as a tissue factor protein band obscured the Factor VII protein band on the gel. It is clear that on the formation of the active complex there is no change in the mobility of the [32P]-DFP-Factor VII band in either the reduced or the nonreduced samples. It may be noted that the active complex apparently contains more [32P] than the native Factor VII at the same concentration, which suggests that the active complex is more sensitive than native Factor VII to DFP.

Although pure Factor VII and Factor VII in bovine plasma are inhibited by DFP, no coagulant activity is detectable until the formation of a complex with tissue factor in the presence of Ca2+. We therefore determined whether the development of coagulant activity is accompanied by a corresponding change in the reactivity of Factor VII toward DFP and PMSF, the latter also being an active site-directed inhibitor of serine proteases (17). The rates of inactivation of purified Factor VII and the Factor VII-tissue factor complex by these inhibitors were determined. As shown in Fig. 9, on the formation of the active complex the half-time of inactivation by 2 mM DFP (at pH 7.5, 22°C) falls from 2.5 to 2.5 min, and that of inactivation by 2 mM PMSF from 24 to 12 min. This change in the rates of inactivation by active site-directed irreversible inhibitors, although confirming that there is probably a corresponding change in the reactivity or accessibility of the active site, is extremely small compared to the change in the coagulant activity of Factor VII which results from its reaction with tissue factor.

Action of Tissue Factor-Factor VII Complex on Factor X—Earlier observations led to the proposal that the tissue-factor pathway of blood coagulation involves the proteolytic activation of Factor X to Xa by an enzymatically active complex of tissue factor plus Factor VII plus Ca2+ (18). Therefore, the activation of Factor X by this complex, formed from purified tissue factor and Factor VII, was followed by sodium dodecyl sulfate gel electrophoresis and by assay of the Xa produced. Purified bovine Factor X was treated with 5 mM DFP to inactivate any traces of Factor VII in the preparation and dialyzed to remove the residual DFP. This material, at a concentration of 0.4 mg per ml, was incubated (37°C, pH 7.5) with the tissue factor-Factor VII complex in the presence of 5 mM Ca2+, and the reaction followed by the removal of aliquots for the assay of Xa (Fig. 10) and for sodium dodecyl sulfate gel electrophoresis (Fig. 11). A second incubation, with Factor X at a concentration of 0.8 mg per ml, was carried out for the study of reduced samples by sodium dodecyl sulfate gel electrophoresis (gel not shown).

It is clear that as proteolysis proceeds Xa activity develops.
chain. The right-hand channel in Fig. 11 shows the activation of Factor X by Russell's viper venom in the presence of Ca²⁺; the major product is evidently of very similar apparent molecular weight to one of the intermediates which appear on activation by the tissue factor-Factor VII complex.

**DISCUSSION**

The tissue factor pathway of coagulation has been thought to be initiated by the formation of an enzymatically active complex of Factor VII and tissue factor (18). In a previous study, it was postulated that tissue factor complexed with a DFP-sensitive protein forming a proteolytic intermediate (1). Further, it was shown that on prolonged incubation a protein which comprised the bulk of the preparation was cleaved to a species of lower molecular weight. We have now separated the major protein from the DFP-sensitive protein (Fig. 7), and while we have confirmed the proteolysis of the major protein, it is now clear that it does not possess Factor VII activity. The formation of the proteolytic complex, however, has been shown by these observations and, significantly, by the proteolytic activation of Factor X (Fig. 11).

Factor VII has been purified to essential homogeneity (Fig. 7). The maximum purification achieved, 330,000-fold, indicates that Factor VII is a trace protein in plasma. This purification was attained in an earlier preparation without a gel filtration step and the material was 70% pure, as estimated following sodium dodecyl sulfate gel electrophoresis. Thus, the concentration of Factor VII in bovine plasma is of the order of 150 μg per liter. There are two similar proteins in plasma which are required for the tissue factor pathway, Factor X and prothrombin, but both are present in much higher concentration, approximately 9 and 120 mg per liter, respectively. Owing to its low concentration, Factor VII could not be purified using conventional techniques. Further, as previously shown and noted above, Factor VII co-purifies with another protein through several steps including DEAE-Sephadex chromatography and preparative gel electrophoresis.

The use of benzamidine coupled to agarose as a chromatographic medium enabled us to resolve these proteins (Fig. 4). The selection of benzamidine as the ligand for affinity chromatography was based on the ability of p-aminobenzamidine to inhibit the proteolytic activity of the tissue factor-Factor VII complex. Surprisingly, the benzamidine-agarose also bound the zymogens prothrombin and Factor X. The mechanism of this binding is not clear, but the benzamidine-agarose does not appear to function as a simple anion exchange medium for several reasons. First, the order of elution from these columns is different from that observed when DEAE-Sephadex, also an anion exchanger, is used. For example, Factor X elutes before prothrombin from the former, and much later from the latter. Secondly, the capacity of the benzamidine-agarose (substituted at 5 to 6 μmoles per ml) is considerably greater than that of DEAE-Sephadex (substituted at approximately 150 μmoles per ml). Finally, prothrombin was not eluted from the benzamidine-agarose by 0.5 M NaCl, but did elute sharply with guanidine hydrochloride of equivalent ionic strength. It may be, therefore, that the guanidino group, which closely resembles the amidino group, specifically displaced the bound proteins. This would imply that the column was functioning as a true affinity material.

Factor VII exists in plasma in a DFP-sensitive form. This sensitivity toward DFP is enhanced by the formation of a complex with tissue factor, the complex being inactivated by DFP.
FIG. 8 (upper). Sodium dodecyl sulfate gel electrophoresis and autoradiography of Factor VII and the tissue factor-Factor VII complex labeled with \[^{32}P\]DFP. Tissue factor, 1.2 mg, was centrifuged (130,000 X g, 1 hour) and the pellet suspended in 0.5 ml of Factor VII (0.3 mg per ml) in 5 mM CaCl\(_2\)-50 mM Tris-Cl, pH 7.5 at 37\(^\circ\) for 60 min. The reaction was stopped by the addition of 5/\(_4\) volume of 0.12 M sodium citrate, and 5/\(_4\) volume of \[^{32}P\]DFP (0.8 mCi per ml in isopropyl alcohol) added. After 30 min at 22\(^\circ\) the reaction was stopped by the addition of an equal volume of 4\(^\circ\)/\(_{10}\) sodium dodecyl sulfate-10 M urea. An aliquot of this mixture was reduced with 1\(^{1/2}\)% 2-mercaptoethanol (12 hours at 37\(^\circ\)). A control sample of Factor VII at the same concentration was labeled with \[^{32}P\]DFP in the same way. Each sample, 50 \(\mu\)l, was applied to the gel, which was dried and autoradiographed. (1) Protein stain of 8 \(\mu\)g (nonreduced) Factor VII; (2) autoradiogram of nonreduced Factor VII; (3) autoradiogram of nonreduced tissue factor-Factor VII complex; (4) autoradiogram of reduced Factor VII (1\(^{1/2}\)% 2-mercaptoethanol); (5) autoradiogram of reduced tissue factor-Factor VII complex (1\(^{1/2}\)% 2-mercaptoethanol).

FIG. 9 (lower). The inactivation of Factor VII and of the tissue factor-Factor VII complex by 2 mM DFP and PMSF. (a) Samples of purified Factor VII (300 units per ml in 0.1 M NaCl-50 mM Tris-Cl, pH 7.5, containing ovalbumin at 1.1 mg per ml) were incubated at 22\(^\circ\) in the presence of 5/\(_4\) volume of isopropyl alcohol, 0.1 mM DFP, or 0.1 mM PMSF in isopropyl alcohol. At the times shown aliquots were removed and diluted 5/\(_4\) before assay for Factor VII activity (● and ○). The assay was calibrated with serial dilutions of the starting material. (b) Purified Factor VII (800 units per ml) and tissue factor (approximately 100 \(\mu\)g per ml) were incubated in the presence of 5 mM Ca\(^{2+}\) (0.1 M NaCl-50 mM Tris-Cl, pH 7.5) for 60 min at 37\(^\circ\), and the reaction stopped by the addition of 5/\(_4\) volume of 0.12 M sodium citrate. Samples of this mixture were incubated at 22\(^\circ\) in the presence of 5/\(_4\) volume of isopropyl alcohol, 0.1 mM DFP, or 0.1 mM PMSF in isopropyl alcohol. At the times shown aliquots were removed and diluted 5/\(_4\) before assay of the tissue factor-Factor VII complex (▲ and △). The assay was calibrated with serial dilutions of the starting material.

FIG. 10 (upper). The activation of Factor X by the tissue factor-Factor VII complex. Purified Factor VII (0.24 \(\mu\)g per ml) and tissue factor (0.15 mg per ml) were incubated at 37\(^\circ\) for 30 min in 5 mM CaCl\(_2\)-50 mM Tris-Cl, pH 7.5. This mixture, 0.13 ml, was added to 0.5 ml of Factor X (0.4 mg per ml in 5 mM CaCl\(_2\)-50 mM Tris-Cl, pH 7.5) at 0\(^\circ\) and the zero time sample removed. The reaction mixture was then incubated at 37\(^\circ\), and 10 \(\mu\)l aliquots removed at the times shown and added to 1 ml of 12 mM sodium citrate at 0\(^\circ\). These samples were further diluted 1/\(_4\) into 0.1 M NaCl-50 mM Tris-Cl pH 7.5 before assay for Factor X.

FIG. 11 (lower). Sodium dodecyl sulfate-gel electrophoresis of the activation of Factor X. Aliquots of the Factor X-activation mixture described in Fig. 10 were removed at the times shown into an equal volume of 4\(^\circ\)/\(_{10}\) sodium dodecyl sulfate-10 M urea and boiled for 4 min. A sample of Factor X was also activated by crude Russell's viper venom: 0.6 \(\mu\)l of Factor X (0.4 mg per ml) in 5 mM CaCl\(_2\)-50 mM Tris-Cl, pH 7.5 were added to 0.2 \(\mu\)l of crude venom (10 \(\mu\)g per ml) and incubated at 37\(^\circ\) for 25 min. The reaction was stopped by the addition of an equal volume of 4\(^\circ\)/\(_{10}\) sodium dodecyl sulfate-10 M urea, and boiled for 4 min. Each sample, 50 \(\mu\)l (nonreduced), was applied to the gel.
about four times as fast as native Factor VII. This change is very small compared to the change in coagulant activity, which is not measurable before the formation of the complex. PMSF, another active site-directed irreversible inhibitor, inactivates the complex only twice as fast as it inactivates Factor VII alone.

The change in sensitivity to DFP and PMSF on formation of the Factor VII-tissue factor complex also cannot be compared with the change that occurs on the activation of the pancreatic zymogens chymotrypsinogen and trypsinogen, the respective enzymes being inactivated by DFP 4 to 6 orders of magnitude more rapidly than the zymogens (19). Factor X and prothrombin, too, are insensitive to DFP, whereas the respective enzymes are inactivated by 5 mM DFP (20, 21).

The active site of the Factor VII-tissue factor complex which catalyzes the activation of Factor X is in Factor VII: (a) treatment of Factor VII with DFP inhibits its potential proteolytic activity, whereas treatment of tissue factor does not (1); and (b) both Factor VII and the tissue factor-Factor VII complex, when treated with [32P]DFP, show the 32P to be incorporated into Factor VII, as judged by sodium dodecyl sulfate gel electrophoresis (Fig. 8). However, the formation of the complex does not apparently involve any change in the molecular weight of Factor VII, whereas the activations of both Factor X and prothrombin, members with Factor VII, of the "prothrombin group," involve large changes in molecular weight (Fig. 11)(2, 3, 5). It is possible that there is a small change in molecular weight, comparable to that on the activation of the pancreatic zymogen, but this would be detectable only by structural studies. To this end we are now attempting to recover Factor VII from the tissue factor-Factor VII complex.

From the foregoing discussion it is clear the "activation" of Factor VII, on its formation of a complex with tissue factor, is not immediately comparable either with the activation of Factor X and prothrombin, or with that of the pancreatic zymogens. It is, however, similar to the activation of Factor Xa by the complex of Factor V plus Ca++ plus phospholipid. This has been shown to increase the coagulant activity of Factor Xa at least 1000-fold (22), and, although there are no data on the rate of inactivation by DFP of the prothrombin-converting complex, it is known that Factor Xa alone is inactivated by DFP. Therefore, we conclude that Factor VII circulates as an active enzyme that has an absolute requirement for a specific lipoprotein, tissue factor, to develop proteolytic activity toward Factor X. Furthermore, from the data available, we consider that the formation of the tissue factor-Factor VII complex probably does not involve a large change in the conformation or accessibility of the active site of Factor VII, but rather that tissue factor orients Factors VII and X to facilitate the cleavage of Factor X by Factor VII.

In accord with its remarkably low concentration in plasma, Factor VII (as the tissue factor complex) is extremely active in catalyzing the activation of Factor X: for example, 25 μg of Factor VII activated 200 μg of Factor X to 50% of maximum activity in about 4 min at 37°. It is notable that, whereas the activation of Factor X catalyzed by the coagulant protein of Russell's viper venom appears to involve proteolysis of a single bond (4), the activation catalyzed by Factor VII is more complex. Three major products are seen to be derived from Factor X (Fig. 11). As noted earlier, at least two of these must possess coagulant activity. From these data, we cannot deduce the sequence of the proteolytic cleavages observed; nor can we say whether all the cleavages are due to the action of the tissue factor-Factor VII complex or whether some are the result of the action of a form of Factor Xa. It is of interest, however, that the multiple cleavages are limited to the heavy chain of Factor X, which is the chain cleaved by the venom protein (4). The multiple forms of Factor Xa are being studied collaboratively with Drs. P. Bajaj and K. G. Mann of the Mayo Clinic and will be reported later.

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Jolyon Jesty and Yale Nemerson


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