The Dual Role of Factor VII in Blood Coagulation

INITIATION AND INHIBITION OF A PROTEOLYTIC SYSTEM BY A ZYMOGEN

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Blood coagulation as currently conceived proceeds via a stepwise sequence of proteolytic activations. In each step, an inert zymogen is converted to an active protease by limited proteolysis (1). It is not yet clear how the system is triggered, i.e., under what conditions the first cleavage occurs, and what catalyzes it. Our laboratory has previously adduced evidence, albeit circumstantial, indicating that the single-chain Factor VII is a likely candidate to initiate coagulation without prior proteolysis. It is well established that Factor VII, an enzyme proteolytically derived from Factor VII (2), can start this process by attacking Factor X (3, 4) and Factor IX (5, 6). These reactions require the presence of Ca" and tissue factor. The latter, a catalytically inert lipoprotein derived from certain cell membranes, is an obligatory cofactor for the coagulant activity of Factor VII (3, 4) and has recently been purified to homogeneity (7). It is, however, uncertain whether Factor VII acquires proteolytic capability only when it is itself cleaved, or whether it possesses inherent coagulant activity in the zymogen state. We have previously demonstrated the esterase activity of the purified zymogen (8) and have shown that Factor VII and Factor VII, react stoichiometrically with DFP (5), which is incorporated into the same peptide in each species (9); in bovine plasma, Factor VII is the only coagulation factor inactivated by DFP (10). In a direct clotting assay, the zymogen exhibits approximately 1-2% of the coagulant activity of Factor VII (2). However, as the zymogen preparations probably contain trace amounts of enzyme (the two differ only in one peptide bond, and are difficult to separate), we could not unambiguously attribute this activity to the zymogen.

In this paper we report experiments designed to circumvent the ambiguity and to examine the coagulant activity of Factor VII zymogen. If the proteolytically active species is a complex of tissue factor and enzyme, then an active zymogen must also bind the cofactor. When tissue factor is limiting, i.e., in lower concentration than the total of Factor VII and Factor VII, the low activity zymogen would act as an inhibitor by competing with high activity enzyme for tissue factor sites. We report experiments testing this hypothesis and, indeed, the concept of a proteolytically active complex. We further evaluate quantitatively the avidity of inert (derivatized) Factor VII for tissue factor sites. We show the following: 1) Factor VII inhibits the activation of "H-labeled Factor IX by Factor VII, when tissue factor is limiting, indicating that enzyme and zymogen compete for the cofactor. In contrast, when tissue factor is in excess, the activities are additive. 2) Disopropylphosphoryl derivatives of Factor VII and Factor VII, both inhibit the radioassay for Factor VII, tissue factor, the K (inhibition being 2.8 ± 0.4 and 2.6 ± 0.2 nM, respectively. 3) The rate of incorporation of ["H]DFP by these proteins is insensitive to trace contamination; pseudo-first order rate constants were calculated for the incorporation of 2 mM DFP into Factor VII and Factor VII, These were 0.032 ± 0.001 min-' and 0.130 ± 0.003 min', respectively. Inhibition rates of the coagulant activity of the two proteins were also determined in 2 mM DFP. The inhibition kinetics and the rate constants of incorporation were used to calculate the intrinsic coagulant activity of the zymogen. It was nearly 0.8% that of the enzyme. 4) Factor VII was rendered virtually free of Factor VII, by incubation with 2 mM DFP for over 6 half-lives of Factor VII. At this point, Factor VII had approximately 0.8% of the activity of the enzyme. The coagulant activity decayed with the rate constant of the zymogen, 0.033 ± 0.003 min'-. 1-2% of the activity of its two-chain derivative

and previously undescribed mechanism of regulating a proteolytic system.

† The abbreviations used are: DFP, disopropylfluorophosphate; DIP, diisopropylphosphoryl; IIGA-CH 3 Cl, 2-Glu-Ch-Ar-cholesterol methyl ketone; NaCl/tris·Alb buffer, 100 mM NaCl in 50 mM Tris·HCL, pH 7.5, containing 0.1% bovine serum albumin; SDS, sodium dodecyl sulfate; TF, tissue factor.

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VII and Factor VII, for tissue factor, using a kinetic approach. From another set of experiments designed to assess the reactivity of the zymogen, using DFP-inhibition kinetics coupled with a direct clotting assay for Factor VII, we conclude that the zymogen is active. As our conclusions depend on the results of all the catalytically active species in the tissue factor-dependent reactions, we have also examined the recent proposal that Factor Xa, a known proteolytic activator of both bovine (2) and human (11, 12) Factor VII, functions as a nonproteolytic modifier, forming an active complex with Factor VII and tissue factor (13). The evidence presented here does not corroborate this model, and indicates that Factor Xa functions as a proteolytic activator, not as a modifier.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ile-Glu-Gly-Arg(CH$_2$Cl)$_2$-2HCl was the generous gift of Dr. Elliott Shaw of the Brookhaven National Laboratory, Upton, NY. Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) was purchased from Ortho Diagnostics, Raritan, NJ. DFF (2-amino-2-hydroxymethyl-1,3-propanediol) base, bovine serum albumin, and sodium heparin were products of Sigma. Bovine lecithin (phosphatidylcholine) and synthetic phosphatidylserine were products of Supelco. Sodium [H]$^1$ borohydride, 5-10 Ci/mmol, was from New England Nuclear, as were the toluene-based liquid scintillation mixtures, Formula-963 and Aquasol. Reagents for column chromatography were obtained from Pharmacia, and for gel electrophoresis from Bio-Rad. All other reagents were of analytical grade, and obtained from standard sources.

**Proteins**—Bovine coagulation factors were purified from barium citrate eluate of plasma. Factor VII was prepared and activated essentially as described by Radcliffe and Nemerson (14), and its concentration was determined from the absorbance at 280 nm, $A_{570}^{	ext{max}} = 129.9$, taking $M_r = 53,000$ (9). Factor IX was initially purified by the method of Fujikawa et al. (15) and was further freed from small amounts of prothrombin and Factor VII by preparative acrylamide disc-gel electrophoresis (15); its concentration was determined from $A_{280}^{	ext{max}} = 14.9$ (17), $M_r = 55,400$ (15). Factor X was purified and activated following Jesty and Esnouf (18) and the concentration of Factor X, was calculated from $A_{280}^{	ext{max}} = 9.4$ (18) and $M_r = 42,600$ (18). Crude thromboplastin was purified from acetone-dried bovine brain powder by extraction with buffered saline according to Quick et al. (19). Tissue factor, purified as described by Bach et al. (7), was kindly provided by Dr. Ronald Bach of the Mount Sinai School of Medicine. Mixed brain phospholipids were prepared by extraction with chloroform/methanol by the method of Bell and Alton (20), or by extraction with heptane/butanol (21).

**Titration of Proteins**—Factor IX was labeled by reductive tritiation of oxidized sialic acid residues, employing the method of Van Lenten and Ashwell (22), as previously described (6), and Factor VII was labeled in the same way.

**Preparation of DIP-Factor VII**—150 pl of Factor VII containing 300 pg of protein was freshly desalted, to free it from the benzamidine (10 mm) in which it is routinely stored, over a Sephadex G-25 column (6 ml) equilibrated with Tris-buffered saline (50 mm Tris-Cl, pH 7.5, 100 mm NaCl). 0.5 ml of Factor VII was then made 5 mM in DFP by adding 5 pl of a 0.5 M solution of DFP in dry isopropl alcohol. The mixture was allowed to stand at room temperature, and the decrease in Factor VII activity was followed by clotting assay. Over 50% of the activity was lost in 15 min. After 3 h, when no coagulant activity was detectable, the protein was separated from reaction products on a Sephadex G-25 column (15 ml) equilibrated with Tris-buffered saline. The eluted fractions were located by the absorbance at 280 nm and stored at $-18^\circ C$ in small aliquots. [H]$^1$DFP incorporated per mol of Factor VII was calculated as previously (9).

**RESULTS**

**The Effects of Tissue Factor Concentration on the Activity of Factor VII, in the Presence of Excess Factor VII Zymogen**—Fig. 1 (top) shows that Factor VII (single-chain zymogen) inhibits the activity of Factor VII, assayed on [H]$^1$-labeled Factor IX, in the presence of a limiting concentration of tissue factor. In contrast, when tissue factor is in 5-fold excess over

![Graph](http://www.jbc.org/)

**Fig. 1.** The effect of tissue factor concentration on the activity of Factor VII, in the presence of excess Factor VII zymogen. Factor VII was initially incubated with tissue factor and 5 mM CaCl$_2$ in NaCl/Tris-Alb buffer, pH 7.5, at 37 °C for 60 s. Then, Factor VII, and [H]$^1$-labeled Factor IX was added simultaneously at zero time, and eight 25-pl subsamples were withdrawn at 60-sec intervals (A), or at 30-sec intervals (I), for the counting of acid-soluble tritium. The final concentrations of the reactants, in an assay volume of 250 pl, were [H]$^1$-labeled Factor IX, 200 nm, Factor VII, 0.2 nm, Factor VII, from 0.2 to 6.0 nm, CaCl$_2$, 5 mm, and tissue factor, 0.1 nm (A), or 1.0 nm (I).
Factor VIIa, the activity initially increases when Factor VII is added (Fig. 1, bottom). At high concentrations of the added zymogen, when the tissue factor again becomes limiting and falls below one-half with respect to the total Factor VII, the activity decreases with additional Factor VII. The concentration of Factor VII in these experiments is well below the estimated physiological level, 10–20 nM (2, 26). In other experiments (data not shown) where the tissue factor concentration was further reduced to one-tenth that of Factor VII, over 50% inhibition was seen when the Factor VII/Factor VII ratio was as low as 1, or smaller. This inhibition of Factor VII by Factor VII, and its reversal at high tissue factor concentrations, can be explained in terms of competition between a highly active species, Factor VII, and a minimally active (or inert) Factor VII, each of which binds the cofactor (TF). This situation may be formally expressed as follows:

\[ \text{TF} + \text{VII} \rightleftharpoons \text{TF-VII} \]

As shown above, when the tissue factor concentration is increased above saturation levels the addition of Factor VII enhances the activity. It is not clear if this increase is an expression of the intrinsic activity of Factor VII itself, or of a small quantity of Factor VII, contaminating the preparation. Alternatively, the increased activity may originate in Factor VII activated in the course of the assay, although we have sought to minimize this risk by using the 3H-labeled Factor IX assay rather than the more efficient activation of 3H-labeled Factor X. The newly formed Factor IX, being 2.8 ± 0.4 and 2.0 ± 0.2 nM for DIP-Factor VIIa or DIP-Factor VII, respectively. The concentration of DIP-Factor VII or DIP-Factor VIIa employed in this titration ranged from 0.2 to 20 nM, where Factor VIIa was held at 1 nM, 5-fold over tissue factor. This experiment might have been complicated by significant cleavage of DIP-Factor VII to DIP-Factor VIIa, had it occurred during the period (6 min) of the rate determinations. Extensive conversion seemed unlikely because the progress plots were fully linear, not curving as would be expected if a modifier were formed in the course of the assay. The possibility was examined directly, however, by incubating [3H]DIP-Factor VII, at two concentrations, in the same assay mixtures used for extracting the K_m data, but employing unlabeled Factor IX. Fig. 3 shows the radioactivity profiles of samples from these mixtures which were analyzed at several intervals by SDS-polyacrylamide gel electrophoresis, as described under “Experimental Procedures.” It is seen clearly that under the conditions used to study the inhibition no significant cleavage of the tritiated DIP-Factor VII is detected, thus confirming that DIP-Factor VII is nearly as potent an inhibitor of Factor VIIa as DIP-Factor VII. This observation leads us to infer that Factor VII zymogen and its

**Figure 2. The inhibition of Factor VII by DIP-Factor VII and DIP-Factor VIIa.** The inhibition of 3H-labeled Factor IX (200 nM) was followed in the presence of Factor VII (1 nM), tissue factor (0.2 nM), and CaCl_2 (5 mM) in NaCl-Tris-Alb buffer, pH 7.5, at 37 °C. DIP-Factor VII (A) or DIP-Factor VIIa (C) were added at the indicated concentrations, and the reagents were equilibrated for 1 min before the addition of CaCl_2 to initiate the reaction.

**Table 1**

The effect of DIP-Factor VII and DIP-Factor VIIa on the steady state kinetic parameters of the activation of 3H-labeled Factor IX by Factor VII.

<table>
<thead>
<tr>
<th>Addition</th>
<th>K_m (nM)</th>
<th>V_max (nM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>49.20 ± 7.96 (16)</td>
<td>4.12 ± 0.14</td>
</tr>
<tr>
<td>DIP-Factor VII</td>
<td>42.32 ± 15.88 (13)</td>
<td>0.86 ± 0.06</td>
</tr>
<tr>
<td>DIP-Factor VIIa</td>
<td>34.53 ± 5.66 (12)</td>
<td>0.92 ± 0.03</td>
</tr>
</tbody>
</table>
activated form have very similar binding affinities for tissue factor.

The Rates of Incorporation of Labeled DFP into the Two Molecular Forms of Factor VII—The intrinsic catalytic activity of Factor VII was evaluated by comparing the rates of incorporation of tritiated DFP into the zymogen and enzyme, and from the kinetics of the inhibition by DFP of the coagulant activity of Factor VII. Fig. 4 describes the progress of the incorporation of [3H]DFP (2 mM) into single- and two-chain Factor VII at 25 °C. The pseudo-first order rate constants for the incorporation of the inhibitor by Factor VII and Factor VII?, are 0.032 ± 0.001 min⁻¹ and 0.130 ± 0.003 min⁻¹, respectively, corresponding to half-times of incorporation of 21.6 and 5.3 min, respectively. These rates of incorporation are insensitive to cross-contamination of each species with traces of the other, and the actual degree of contamination of the single-chain Factor VII preparation with two-chain Factor VII, was negligible, as judged from SDS-polyacrylamide gel electrophoresis of reduced samples (not shown). This fact suggests that the rates of incorporation reflect the true rates of the reaction of DFP with the two species.

Determination of the Rate Constants of the Inactivation of Factor VII Coagulant Activity by DFP—The inhibition of the coagulant activity of Factor VII and Factor VII?, by 2 mM DFP was determined at 25 °C. For the assay, all the timed subsamples were diluted to the same extent, and the gradual lengthening of the clotting time was measured. Since tissue factor is thus kept constant and in excess, the loss of procoagulant activity directly reflects the loss of active sites, and the competitive effects of the DIP-Factor VII formed can be neglected. In a number of control experiments, it was found that: (a) DFP does not affect the assays at concentrations up to 200 μM, and samples were diluted so that the level was far below this; (b) the half-life of DFP in the buffer used for the assays was measured by pH-stat to be 9 h at 25 °C; accordingly, during an 80-min inhibition study the level of DFP would change by less than 10%; (c) the half-life of Factor VII, was found to be independent of the protein concentration over the range from 0.02 to 2 mg/ml. When the concentration of DFP was varied over a range from 0.5 to 5 mM, the half-life of Factor VII, varied linearly. This indicates that pseudo-first order reaction conditions obtain. The curve-fitting routines were performed using Marquardt’s algorithm for nonlinear least squares (Tektronix part 4050A10) on a Tektronix 4051 computer. Data are presented ±1 S.E.

The inhibition of the coagulant activity of Factor VII, by 2 mM DFP at 25 °C (Fig. 5) was found to proceed with a pseudo-first order rate constant of 0.127 ± 0.004 min⁻¹ (t₁/₂ = 5.5 min), in agreement with the incorporation kinetics. In contrast, the time course of the inhibition of the activity of the single-chain Factor VII preparation in the presence of 2 mM DFP (Fig. 5) is not described by a single exponential, thus indicating the presence of at least two species. As the two known species are Factor VII and Factor VII?, it seemed reasonable to describe the inhibition plot using the double exponential equation

\[ y = C_1 e^{-k_1 t} + C_2 e^{-k_2 t} \]

where \( y \) is the measurable activity at time \( t \), \( C_1 \) and \( C_2 \) are the coefficients describing the fractional contributions of Factor VII and Factor VII?, respectively, and \( k_1 \) (0.032) and \( k_2 \) (0.130) are the values of the rate constants for the incorporation of [3H]DFP into Factor VII and Factor VII?, respectively, as described in the previous section. Table II presents the contribution of each species calculated for five sets of experimental data which were fit to the double exponential equation. These data show that approximately one-third of the measured activity is attributable to the zymogen per se. A good fit was reproducibly obtained.

The same data can be used to calculate the theoretical maximum activatability of Factor VII, using the following...
The computed fractional contributions to coagulant activity by Factor VII and Factor VIIₐ in a Factor VII preparation inhibited by DFP

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Factor VII</th>
<th>Factor VIIₐ</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% of total</td>
<td>% of total</td>
</tr>
<tr>
<td>1</td>
<td>37.6 ± 2.0</td>
<td>62.4 ± 3.1</td>
</tr>
<tr>
<td>2</td>
<td>39.8 ± 2.1</td>
<td>59.9 ± 3.9</td>
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<tr>
<td>3</td>
<td>32.4 ± 1.4</td>
<td>67.5 ± 3.3</td>
</tr>
<tr>
<td>4</td>
<td>34.5 ± 2.1</td>
<td>65.5 ± 3.2</td>
</tr>
<tr>
<td>5</td>
<td>36.9 ± 2.3</td>
<td>63.1 ± 3.5</td>
</tr>
<tr>
<td>Mean</td>
<td>36.2 ± 1.28 S.E.</td>
<td>63.7 ± 1.31 S.E.</td>
</tr>
</tbody>
</table>

where \( y = (b - C_b)/C_1 \)

The Extent of Activatability of Factor VII During the Interaction with DFP: A Measure of Factor VII Intrinsic Activity—The biphasic inhibition profile described above indicates that single-chain Factor VII has coagulant activity, and that it is contaminated with minor amounts of Factor VIIₐ. Therefore, we attempted to determine the actual level of coagulant activity of the single-chain form by removing the contaminating traces of Factor VII, with DFP. In the experiments summarized in Table III, a Factor VII preparation was reacted with 2 mM DFP (at 25 °C), and aliquots were withdrawn at 10-min intervals, then diluted 100-fold to essentially stop the reaction with DFP. A subsample was immediately mixed with Factor Xₐ, phospholipids, and CaCl₂, and the degree of activation of each sample was determined. Since Factor VIIₐ is inactivated by DFP under these conditions, with a half-life of 5.5 min (Fig. 5), after 33 min (corresponding to 6 half-lives) most of it is eliminated (1.6% of initial level remaining). Therefore, the degree of activation of the later timed samples (Table III) should reflect the true degree of activatability of single-chain Factor VII, i.e., can serve to calculate the inherent Factor VII activity. The data show that after 40 min the activatability stays constant at approximately 110- to 130-fold the base-line. These results lead to the inference that Factor VII does possess coagulant activity, which is approximately 0.8% of the activity (units/ml) of Factor VIIₐ. Moreover, inspection of Table III reveals that the activity present after 40 min of reaction with 2 mM DFP decays with a rate constant virtually identical to the one determined for the incorporation of [³H]DFP into single-chain Factor VII (Fig. 4), thus suggesting that it is the true parameter of the inactivation reaction. The rate constant for the decay of Factor VII activity in the subsamples taken at 40, 50, 60, 70, and 80 min (Table III, Column 2) is 0.034 ± 0.003 min⁻¹ (Table III, Column 3). The identity of these numbers further indicates that all the activity after 40 min derives from the zymogen, thus validating the approach taken in this experiment.

The Activation of Factor VII by Catalytic Amounts of Factor Xₐ.—In the preceding sections we described a number of properties that distinguish the activity of the single-chain zymogen from that of its two-chain derivative, Factor VIIₐ. We have been engaged for several years in defining in detail the kinetic characteristics of Factor VIIₐ, which evidently is, in conjunction with tissue factor, the major active species in initiating coagulation. We have further directed our efforts at a better characterization of the properties of the zymogen Factor VII, regarding it as essential for the understanding of...
the initiation mechanism. We therefore felt it necessary to probe a recent report which hypothesized a major role in the initiation of the extrinsic pathway for a putative ternary complex between Factor X, Factor VII, and tissue factor (13). Accordingly, we have reexamined the conditions of the activation of Factor VII by Factor X, because it seemed to us that the experimental data offered in support of the ternary complex were actually indicative of incomplete activations and inappropriately chosen assay conditions. Moreover, although the *sine qua non* of the demonstration of a non-proteolytic activation is the recovery of non-proteolyzed zymogen, such proof was not provided (13). We evaluated the effect of Factor X, in an assay of Factor VII on *H*-labeled Factor IX by varying the concentration of Factor X, from 0 to 300 nM. Fig. 6 illustrates the results of this experiment, both in terms of the reaction rates and in terms of the lag period that elapses before these rates are attained. Three panels give the progress plots directly (Fig. 6, A to C). It is clear that the lag period shortens dramatically when picomolar quantities of Factor X, are added, whereas the maximum rates of substrate hydrolysis are constant (Fig. 6D). In fact, further experiments showed that these maximal final rates of Factor IX hydrolysis were the same as the initial rates seen when a fully activated preparation of an identical concentration (0.12 nM) was used instead of Factor VII. This Factor VII, had been activated by cleavage, in the presence of 1:1500 parts Factor X,; thus, the residual Factor X, in the assay is below 0.15 pm.

The fate of *H*-labeled Factor VII at low concentrations in the presence of Factor X, was also directly examined by SDS-polyacrylamide gel electrophoresis (Fig. 7). In order to ensure that the Factor VII was quantitatively incorporated into tissue factor complexes, excess tissue factor was employed, 5 nM, to 1 nM *H*-labeled Factor VII, and 1 nM (Fig. 7B) or 0.3 nM (Fig. 7C) Factor X, The radioactivity profiles clearly show that proteolytic cleavage of *H*-labeled Factor VII proceeds rapidly even at these low concentrations. Nearly 50% of the *H*-labeled Factor VII is cleaved in 5 min (Fig. 7B), well within the time frame of the assays described in Fig. 6. At a Factor X, concentration of only 0.3 nM (one-third that of the Factor VII employed), approximately 50% cleavage was also seen within 10 min (Fig. 7C).

The Effect of Factor VII on the Peptidase Activity of Factor X,—In a further attempt to verify the claims in favor of the putative ternary complex (13), we have assayed the activity of Factor X, on the peptide anilide, Benzoyl-Ile-Glu-Gly-Arg-pNA (S-2222), in the presence of Factor VII, phospholipids, and Ca*++* (Table IV). We have used approximately the same conditions under which competitive inhibition of the Factor X, peptidase by a partially pure preparation of Factor VII, at plasma concentration, was described (13). The results summarized in Table IV indicate that no such inhibition is apparent in the presence of a highly purified Factor VII, up to a concentration of 5- or 10-fold the plasma level. At 120 nM Factor VII, a drop of 10% in Factor X, peptidase activity is displayed measurable activity.

![Fig. 6. The effect of Factor X, on the assay of the activation of *H*-labeled Factor IX by Factor VII. The assay was performed in the presence of *H*-labeled Factor IX, 0.46 μM, Factor VII, 0.19 nM, tissue factor, 7.2 nM, and CaCl2, 5 mM, in NaCl/Tris-Alb buffer, pH 7.5, at 37 °C, in the absence (A) or presence of Factor X, 93 pm (B), or 295 pm (C). D gives the secondary plots of the velocities and lag times as functions of the Factor X, concentration in a series of similar experiments covering the range between B and C.](http://www.jbc.org/)

![Fig. 7. Gel electrophoresis profiles of *H*-labeled Factor VII incubated in the presence of Factor X, tissue factor, and CaCl2. *H*-labeled Factor VII, 1.2 nM, was incubated in the presence of tissue factor, 5 nM, Factor X, 1.2 nM (A, B), or 0.3 nM (C), and CaCl2, 5 mM, in 50 mM Tris-Cl, pH 7.5, containing 100 mM NaCl and 0.01% bovine serum albumin, in a total volume of 18 ml. 4-ml samples were removed at zero time (A), before adding the CaCl2, and after 5 min (B) or 10 min (C), reduced and prepared for electrophoresis as described under "Experimental Procedures."](http://www.jbc.org/)
The inhibition of Factor VII by IGGA-CH_{2}Cl. The reaction was performed in the presence of 4 μM IGGA-CH_{2}Cl, 10 μM Factor VII, 10 μM tissue factor, and 5 mM CaCl_{2} in NaCl/Tris-Alb buffer pH 7.5, at 37 °C (10). At the indicated intervals, 25-μl aliquots were withdrawn and diluted 10-fold into prewarmed assay mixtures containing 200 nM T_{-}H-labeled Factor IX in the same buffer. The activation reactions were initiated by adding fresh CaCl_{2} (5 mM) and were followed as described under "Experimental Procedures." Controls contained no IGGA-CH_{2}Cl (C), or 4 μM IGGA-CH_{2}Cl and mixed brain lipids, 0.2 mg/ml, substituting for tissue factor (A).

seen, which might be due to the competition for Factor X, between the two substrates, the anilide and Factor VII.

The Inactivation of Factor VII, by IGGA-CH_{2}Cl—Further support for the postulated ternary model was inferred from the observation that high concentrations of IGGA-CH_{2}Cl inhibit the hydrolysis of Factor IX by Factor VII, tissue factor is the presence of Factor X, (13). IGGA-CH_{2}Cl mimics the NH_{2}-terminal sequence adjacent to the bond cleaved in prothrombin by Factor X, and the inhibitor is highly specific for Factor X, which is inactivated with a half-life of 14.4 min by 25 nM IGGA-CH_{2}Cl at pH 7.0, 25 °C (28). It has been shown, however, that the specificity of this type of compound for homologous enzymes is a matter of degree (29). Therefore, we tested directly the sensitivity of Factor VII, to IGGA-CH_{2}Cl. Fig. 8 shows that in the presence of 4 μM IGGA-CH_{2}Cl, the half-life of Factor VII, is 44 min at 37 °C, pH 7.6, when saturated with tissue factor. It is noteworthy that tissue factor is required for the inhibition. Thus, when only a mixture of lipids is present in the incubation mixture, the effect of the inhibitor is negligible.

DISCUSSION

The data presented in this paper confirm our previous speculations that Factor VII can initiate coagulation without undergoing proteolysis. We believe that the arguments we present provide cogent, if not incontrovertible, support for this assertion. This laboratory has long been engaged in developing a detailed model of the tissue factor pathway of coagulation, and our working hypothesis has been that the catalytic species in this pathway is a complex of tissue factor with Factor VII or Factor VII, while it is clear that Factor VII, an enzyme derived by limited proteolysis from Factor VII, is catalytically active (2, 4), the role of Factor VII in initiating coagulation has been ambiguous. All preparations of Factor VII, both bovine (2, 26) and human (11, 12), examined by us and others have displayed activity in coagulation assays, but this activity is very low, approximately 1-2% that of the fully activated two-chain Factor VII. The sensitivity of the various assays to trace contamination by a more active enzyme makes it impossible to distinguish between a zymogen contaminated with 1% of an enzyme and a zymogen possessing 1% of the activity of the enzyme. In consequence, although after cleavage the activity of Factor VII, increases 50- to 80-fold in coagulation assays, the assignment of the base-line 1-2% activity to the zymogen could not be rigorously made. To clarify this issue we now add several lines of evidence to previous studies which have indicated the unusually reactive nature of Factor VII. Previously, we have shown that: (a) Factor VII readily incorporates DFP into the same peptide as does Factor VII, with the same stoichiometry, 0.9 mol/mol of protein (9); (b) Factor VII has significant esterase activity, expressed in a $k_{\text{cat}}$ for an arginine ester that is 50% that of the enzyme, although the $k_{\text{cat}}$ for the zymogen is 17-fold higher (8). We now present additional evidence for the activity of the zymogen: (c) there is only a 4-fold difference in the rates of DFP incorporation between Factor VII, zymogen and enzyme (Fig. 4), contrasted with known differences of several orders of magnitude for homologous serine zymogen/enzyme pairs, e.g. trypsinogen/trypsin (30); (d) analysis of the DFP-inhibition kinetics allowed us to calculate the fractional activity of the zymogen (Table III); (e) the residual Factor VII in a Factor VII, free preparation is consistently activatable to a level predicted by the analysis in (d) (Table III); (f) Factor VII inhibits the radioassay for Factor VII, in a manner consistent with displacement of the more active Factor VII, from tissue factor sites (Fig. 1). In other words, Factor VII zymogen binds the cofactor previously shown to be obligatory for the coagulant activity of Factor VII, (3, 4), thus meeting a precondition for proteolytic activity to proceed. Furthermore, we show that (g) the avidity of derivatized (inactivated) zymogen and enzyme for the cofactor is very similar (Fig. 2). Taken together, these experiments lead us to conclude that Factor VII does possess intrinsic activity prior to proteolytic activation.

A major experimental and conceptual problem has been the design of experiments which would be unaffected by trace contamination of the zymogen with enzyme. The measurement of the rate of incorporation of tritiated DFP meets this requirement. It was thus possible to generate valid rate constants for the interaction of the single-chain zymogen as well as of the two-chain Factor VII, with DFP (Fig. 4). Employing these rate constants when monitoring the loss of coagulant activity induced by DFP, we could estimate the relative activities of the zymogen and the enzyme and, consequently, the contamination with enzyme of the particular Factor VII preparation studied. The biphasic decay of the activity of the zymogen (Fig. 5) is consistent with a model in which the zymogen is contaminated with approximately 1.5% enzyme. Although curve-fitting procedures obviously do not rule out other contributions, the assumption of a model in which only Factor VII and Factor VII, are biologically active seems justified in view of the purity of the preparations employed in this study, and of the absence of any demonstrable tissue factor-dependent additional enzymes that can activate Factor IX. The ternary model recently postulated to consist of Factor X, Factor VII, and tissue factor is not tenable according to our data, as discussed below. From the rate constants of DFP incorporation by the two species we could predict that when Factor VII is incubated with DFP for a period equivalent to over 6 half-lives of Factor VII, the residual, virtually pure Factor VII should be approximately 120 to 130-fold activatable. This prediction was clearly borne out as shown in Table

![Figure 8](http://www.jbc.org/)
III. These two experiments, namely, the determination of the rate of inactivation of the zymogen and the measurement of the activatability of the residual Factor VII, although based on somewhat different assumptions, are internally consistent and show the zymogen to have significant proteolytic activity, approximately 0.8% that of the enzyme.

When a low activity zymogen and a high activity enzyme are together in the presence of a limiting amount of an obligatory cofactor—tissue factor—it follows that the zymogen would act as an inhibitor. This prediction was borne out as depicted in Fig. 1, in which increasing concentrations of the zymogen are shown to decrease the velocity of the reaction. In contrast, when the activator concentration is high with respect to enzyme and zymogen, both species would be saturated and their activities would be additive, as is also illustrated in Fig. 1. Indeed, the effects are additive until the zymogen exceeds the tissue factor concentration, at which point it again becomes inhibitory. This experiment could not be rigorously quantified, because minor contamination of the zymogen with enzyme cannot be excluded. However, the displacement of active Factor VII from tissue factor sites is clearly suggested by experiments utilizing derivatized, catalytically inert Factor VII, and Factor VII. The DIP-derivatives of these species act as potent inhibitors of Factor VII. From the data presented in Fig. 2 we were able to calculate a $K_{i/2}$ of inhibition, and both species showed virtually identical characteristics. These findings suggest that Factor VII and Factor VII, have essentially equal affinities for tissue factor. Further, this experiment supports our previous speculations about the mechanism of action of tissue factor: the true catalytic species in this reaction is a complex of tissue factor and enzyme. Thus, if the derivatized species were to displace active enzyme from tissue factor, the system would behave as if there were less catalyst present; it would exhibit a decrease in $V_{max}$. This prediction too is borne out in Table I, in which it is shown that DIP-Factor VII and DIP-Factor VII, decrease the $V_{max}$ but do not affect the $K_m$ of the reaction. These experiments provide a compelling argument both for the mode of action of tissue factor and for the inherent activity of the zymogen. Detailed kinetic and equilibrium studies for deriving the binding constants for the various forms of Factor VII to tissue factor are currently in progress.

We do, however, have one reservation concerning the measurement of Factor VII coagulant activity. As the zymogen is converted to its enzymatic derivative by at least three enzymes generated in the process of coagulation, i.e. Factor X, (2, 11, 12), Factor XI1, (31, 32), and thrombin (2), it is possible that this conversion occurs during the course of the assay. However, the excellent fit of the data to the double exponential equation (Fig. 5) implies that such a conversion would have occurred independently of the concentration of either Factor VII or DIP-Factor VII present. Since the coagulation assays were performed with highly dilute solutions of Factor VII, 0.5–2 nM, a very low $K_m$ for the activation of Factor VII would be indicated. Further, the DIP-Factor VII present would have to be kinetically silent and not act as an alternative substrate competing with Factor VII for the activating enzymes. As neither of these considerations appears likely, we do not believe that the coagulation assay has generated misleading results.

While we believe that the tissue factor pathway consists of one certain catalytic species, the Factor VIIa-tissue factor complex, and of one likely species, the Factor VII-tissue factor complex, data were recently published in support of a third species, a ternary complex of tissue factor, Factor VII and Factor X, (13). As our work requires a complete catalogue of all the catalytic species in this pathway, we explored the possibility that this putative ternary complex is active in coagulation. We wish to point out that our experiments have been performed employing highly purified tissue factor and Factor VII, whereas for the experiments in support of the ternary complex less pure reagents were used. The evidence adduced in favor of the ternary complex may be summarized as follows: (i) When Factor X, was added to a reaction consisting of Factor VII, tissue factor and ‘H-labeled Factor IX, the velocity of the formation of activated Factor IX was proportional to the amount of Factor X, added, and then reached saturation. Our data do not agree. Fig. 6 shows that in the absence of added Factor X, a lag period of approximately 12 min is observed before any discernible hydrolysis of Factor IX occurs. As Factor X, is added, this lag period shortens and is inversely proportional to the Factor X, concentration. We interpret this as previously (2): Factor X, cleaves Factor VII to Factor VIIa, thus indirectly accelerating the reaction. In fact, Fig. 7B shows that even at very low concentrations of Factor VII (1.2 nM), tissue factor (5 nM), and Factor X, (1.2 nM) are incubated, significant cleavage of Factor VII occurs within 5 min. (ii) The complex between Factor VII and Factor X, is tight, and therefore Factor VII is a potent inhibitor of the peptidase activity of Factor X, but when we repeated the experiment as reported, using the indicated concentrations of Factor X, and lipids and even increasing the Factor VII concentrations, we were unable to observe significant inhibition of the peptidase activity of Factor X, by Factor VII (Table IV). We note that even at a Factor VII concentration greater than 7-fold that present in plasma, only 10% inhibition is observed. This inhibition can be explained by the presence of competing substrates for Factor X, Factor VII, and Benzoyl-IGGA-pNA (S-2222). It is of interest that Neshim et al. (33) recently showed that the very complex which accelerates prothrombin activation, namely, Factor V, in the presence of phospholipids and Ca++, represses the activity of Factor X, on the anilide. (iii) A known irreversible inhibitor of Factor X, IIGG-CH2Cl (28), appeared to inhibit the hydrolysis of ‘H-labeled Factor IX in the presence of Factor VII and Factor X, and this inhibition was reversed by additional Factor X,. This curious phenomenon was observed only at uncharacteristically high concentrations of the inhibitor; in principle, it conflicts with the previous argument. We have shown above, however, that at these high concentrations Factor VII, is inhibited (Fig. 8). This preparation is the fully cleaved, two-chain Factor VIIa, which had been converted by 1/1500 parts of Factor X, in the presence of phospholipids and Ca++. In the incubation mixture, the concentration of Factor VII, is 1 nM, and of tissue factor, 10 nM; residual Factor X, is approximately 0.7 nM, and cannot be considered to have any role in the assay, where the reactants were further diluted 10-fold. The slight reversibility reported in the presence of fresh Factor X, (13) could have originated in new rapidly formed Factor VIIa. (iv) Factor VII appeared to inhibit its own activation, and the inhibition was reversed by dilution into fresh assay mixtures. As we have shown above (Fig. 1), the inhibition of the activity of Factor VII, by Factor VII is an intrinsic property of the tissue factor-Factor VII/VII system. In fact, the “dilution” of a sample of the assay mixture into a fresh reaction mixture containing additional tissue factor simply allowed more of the Factor VII, present to form a catalytically active complex with tissue factor, thus mimicking a “reversal by dilution” effect.

In conclusion, we show that the observations leading to the ternary model hypothesis are not corroborated by studies of highly purified and characterized Factor VII, and that the reasoning in constructing the model does not take into account the critical interaction between Factor VII and tissue factor.
It might be argued further that if the ternary complex is the critical operational mode in vivo, evolution might have dispensed with the proteolytic activation of Factor VII. But this clearly is not the case. Previous reports from this laboratory (2) have shown the rapid proteolytic conversion of Factor VII to Factor VII, by Factor Xa in ratios as low as 1/1000-1/2000; further, the activity of Factor VII, increases in parallel with this conversion, and the degree of activity is proportional to the quantity of Factor VII (two-chain) produced (2). Similar observations were reported for human Factor VII (11, 12). It is of note that in the paper proposing the ternary model, uncleaved Factor VII was not shown to be recovered from its "complex" with Factor Xa. (13). Significantly, Factor Xa is not the only proteolytic activator of Factor VII. Factor VII, is also formed by Factor XII, (31, 32), Factor IXa, (27), and thrombin (2), suggesting that its production serves a physiological requirement.

The system consisting of tissue factor-Factor VII/VIIa evidently functions as an efficient self-regulatory mechanism in the initiation of coagulation; each component of the system is integral to its normal operation. The ability of the zymogen to bind tissue factor appears unique to Factor VII. No record exists, as far as we are aware, to suggest that any other coagulation zymogens interact with the cofactors of their derivative enzymes. Moreover, activated Factor V, identified as the platelet binding site for Factor Xa, is distinctly specific for the enzyme and will not bind Factor Xa (34).

The question of the intrinsic activity of the zymogen Factor VII notwithstanding, the clear demonstration that Factor VII competes with Factor VII, for tissue factor introduces a subtle and previously undescribed form of control of coagulation, with interesting implications for hemostasis. This regulatory mode stems from a situation in which a high activity species exists, as far as we are aware, to suggest that any other coagulation zymogen is involved in a regulatory mechanism. This system allows the system to be primed by a weak stimulus, but to respond with maximal efficiency only when the stimulus grows sufficiently strong. Since, during the initial period Factor VII would be in very large excess over Factor VII, this mechanism effectively ensures against a "wild" response to an accidental or insignificant signal. On the other hand, once sufficient amounts of Factor VII are formed, coagulation can proceed very rapidly. Given the characteristic amplification pattern of the coagulation system (35, 36), even a small amount of Factor VIIa is adequate to clot plasma very quickly because of the efficiency of the subsequent conversions of Factor X, prothrombin, and fibrinogen.

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