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Kinetics of Activation of Human Prothrombin. Use of a Fluorescein-Labeled Derivative To Obtain Kinetic Constants as a Function of Factor V Concentration and Activation State†

Sidonie A. Morrison

ABSTRACT: Human prothrombin labeled with fluorescein to a level of 4 mol of dye/mol of protein was confirmed to be a normal substrate for prothrombinase by several criteria and used as a fluorogenic substrate for studies of the kinetics of prothrombin activation in a purified model system. In investigations of the response to modulation of prothrombinase activity the concentrations of the enzymatic component, activated factor X, and the cofactor, factor V, were varied in turn and the corresponding rates of fluorescence increase determined. When factor Xa was the variable, a hyperbolic relation of activation rate to [factor Xa] was seen at a limiting level of factor V(a), which with appropriate curve fitting gave an apparent K_d of 0.059 ± 0.0001 nM. As the factor V concentration was raised, the apparent saturation became progressively less, and at 10 nM factor V the relationship to [factor Xa] was linear. Results of a complementary experiment with factor V as the variable also showed saturation. When the level of prothrombin was varied at constant [factor Xa] and different, fixed levels of factor V, the relation of rate to prothrombin concentration was hyperbolic in all cases. At a high, saturating concentration of factor V—about one-tenth the plasma level—the apparent K_m was 0.28 ± 0.02 μM prothrombin, and the apparent V_{max} was 19.9 ± 0.8 mol s^{-1} (mol of factor Xa)$^{-1}$. Of the parameters V_{max} was the more sensitive to [factor V], dropping 7-fold with a 125-fold decrease, whereas the K_m varied in random fashion by only 60%. This variation of V_{max} reflects the amount of factor Xa bound to factor V when the latter is limiting in concentration. Even at very low concentrations of factor V it made no difference to the rates of activation that could eventually be reached whether the factor V was activated with thrombin beforehand or left to activate in situ. However, reactions in which factor V was strongly rate limiting while the concentration of prothrombin was high were characterized by lags without proteolysis of several minutes before activation began, and that maximum rate was achieved. In contrast, lags were essentially absent at low prothrombin levels. Experiments to compare the progress curves obtained when factor V had been preactivated with thrombin under various conditions showed that the lags resulted from a protective effect of prothrombin on the activation of factor V, most pronounced when the factor V level was low. One consequence of this "substrate inhibition" is that the system is damped until a threshold level of activation has been reached and/or a minimum availability of prothrombinase has been exceeded.

Thrombin is a crucial enzyme in hemostasis, with half a dozen roles to play. There is, therefore, a strong impetus to understand the kinetics and control of its formation as precisely as possible, and studies with this as one of the goals have been conducted by several investigators (Nesheim et al., 1979a; Kosow & Orthner, 1979; Rosing et al., 1980; Dahlback & Stenflo, 1980). In all cases, the strategy has been to use probes for the appearance of product (i.e., thrombin) active sites such as a chromogenic peptide substrate or a fluorogenic, reversible, active site inhibitor (Nesheim et al., 1979b). The rates measured in such experiments as a function of prothrombin concentration, and the kinetic constants derived, therefore

† From the Division of Hematology, Department of Medicine, State University of New York at Stony Brook, Long Island, New York 11794. *Received February 18, 1983.* This work was supported in part by National Institutes of Health Grants HL 22955 and AM 19185 and by an American Heart Association Grant-in-Aid with funds supplied in part by the Suffolk County, NY, affiliate. S.A.M. is the recipient of Research Career Development Award K04-00478 from the National Heart, Lung and Blood Institute.

0006-2960/83/0422-4053$01.50/0

apply to the overall two-step activation.

The very multiplicity of its actions makes the formation of thrombin from its precursor, prothrombin, difficult to measure rigorously in a complex system such as plasma. This is because any substrate or other probe added to measure the generation of product active sites must compete with all the other substrates and binding sites for thrombin that are present and may interfere with the actions of thrombin. One way around this difficulty would be to measure release of the other, amino-terminal product of activation, fragment 1.2 (Suttie & Jackson, 1977). Assays of this type have proved useful in kinetic investigations of the activations of factor IX and factor X in plasma (Jesty & Silverberg, 1979) and to measure the cleavage of fragment 1 from prothrombin by thrombin (Silverberg, 1979). All these methods have the drawback that they are discontinuous, i.e., they require time-consuming subsampling. They also rely on being able to extract the activation fragment preferentially from reaction mixtures, a condition unlikely to be met by fragment 1.2 release because of its large size.

It is clear, then, that a considerable advantage could be gained by monitoring prothrombin consumption rather than the appearance of thrombin per se. As a step toward this goal I have prepared a derivative of human prothrombin that is covalently labeled with fluorescein, through a coupling group that permits a greater extent of labeling than can be achieved with fluorescein isothiocyanate (Fass & Mann, 1973). The cleavage of the substrate by human factor Xa in the presence of factor V, phospholipids, and Ca^{2+} is accompanied by an increase in the fluorescence intensity at the emission peak of fluorescein. This can be followed while activation is in progress in a purified system or in plasma and used to obtain kinetic information. The attachment of the probe has no apparent effects on the kinetic behavior or activation pathway of the protein even though the product of activation has selectively lost its activity toward fibrinogen. The use of the fluorogenic substrate has permitted the determination of kinetic constants for the activation of human prothrombin in a purified system under initial rate conditions, information not hitherto available for the human protein. In addition, an apparent binding constant was derived with factor V present at a limiting level that is assumed to define the interaction of this constituent of prothrombinase with factor Xa. The constants obtained, which pertain to a mixed human and bovine system, are in the event rather similar to those measured—using very different techniques—by Lindhout et al. (1982), Rosing et al. (1980), and Nesheim et al. (1979b) in studies of the bovine proteins.

Experimental Procedures

Materials. Trizma base, Coomassie Brilliant Blue R, serum albumin (bovine, type 6, fatty acid free) fibrinogen (bovine type 1), ethylenediaminetetraacetic acid (disodium salt) (EDTA),[1] Russell's viper venom, bovine factor deficient plasmas, and soybean trypsin inhibitor (Kunitz, type 1) were from Sigma. Citrated fibrinogen-deficient human plasma (lot no. GK101-N12) with a prothrombin time of >10 min and factor IX deficient plasma were purchased from George King. Factor V deficient plasma for clotting assays was made by dialyzing oxalated bovine plasma (Pel-Freeze) vs. 100 volumes of NaCl/Tris/1 mM EDTA overnight. DCTAF [5-[(3,5-dichlorotriazinyl)amino]fluorescein] came from Molecular Probes Inc., Junction City, OR. DTNB was from Pierce. The prothrombin complex concentrate that was the source of factor X(a) was a gift from Dr. Charles Heldebrant, Alpha Therapeutics. Quinine sulfate dihydrate and cyanogen bromide were from Aldrich. S-2238 and S-2222, chromogenic substrates for thrombin and factor Xa, are the products of Hellena Laboratories. Poly(ethylene glycol) 6000 came from J.T. Baker. Standard 1 M calcium chloride solution and sodium dodecyl sulfate were from Gallard Schlesinger. Sephadex, Sephacryl, and Sepharose products are from Pharmacia. Phosphatidylcholine and phosphatidylserine were purchased from Supelco. Dextran sulfate agarose came from Bethesda Research Laboratories. All other chemicals were reagent grade.

Methods. Human prothrombin was isolated from fresh-frozen blood bank plasma by chromatography on DEAE-Sephadex (Morrison & Esnouf, 1973) and freed of traces of factor IX and factor X activities by chromatography on dextran sulfate–agarose (Pepper & Prowse, 1977) and treatment with a rabbit antiserum to factor X covalently bound to agarose beads.[2] The prothrombin was >95% homogeneous by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and contained no factor X, factor IX, or factor VII activity by clotting assays. The protein at 2 mg/mL contained no detectable protein C after activating with the Russell's viper venom coagulant protein and testing for inhibition in a factor Xa assay (Comp & Esmon, 1979). *Human factor X* was prepared by a method to be described elsewhere.[2] It was activated with Russell's viper venom coagulant protein coupled to Sepharose beads (Jesty et al., 1974) by incubating 0.36 mg of factor X/mL with the beads (15% v/v) for 20 min at 37 °C in NaCl/Tris/5 mM $CaCl_2$. The progress of activation was followed by rate assays of factor Xa by using 0.5 mM S-2222 as the substrate and monitoring *p*-nitroaniline formation at 405 nm. Activation was terminated by adding 10 mM EDTA and then centrifuging out the venom protease. The factor Xa was diluted to 40 nM with NaCl/Tris/1% poly(ethylene glycol), filtered through a 0.2-μm filter (Millipore), and stored in 0.2-mL portions at −70 °C. The coagulant activity of the preparation remained stable for at least 6 months. *Bovine factor V* was prepared by the method of Nesheim et al. (1981b) and stored at −20 °C in 50% glycerol. The specific activity of the preparation (~10 units/mg) could be enhanced ~20-fold (after dilution to 1 unit/mL) by incubating for 10 min with 1 unit of bovine thrombin/mL, but the factor V was used without prior activation in the present experiments except where noted.

Factor V activities were measured in a one-stage coagulation assay and are based upon an unactivated bovine plasma standard of 1 unit/mL and a plasma concentration of 0.1 μM (Nesheim et al., 1981b). Factor Xa concentrations are also based on coagulant activity, standardized with concentrations of human factor X of between 0.1 and 2 nM activated with Russell's viper venom coagulant protein in situ in a two-stage assay. Prothrombin coagulation assays were done as before (Silverberg, 1979). Prothrombin concentrations were determined for the normal protein by absorbance at 280 nm ($A^{1\%,1cm}$ = 13.8; Kisiel & Hanahan, 1973) and those for fluorescein-labeled prothrombin, after reduction and denaturation, by a colorimetric reaction with DTNB with normal prothrombin

[1] Abbreviations: NaCl/Tris, Tris-buffered saline, 0.1 M NaCl/0.05 M Tris, pH 7.5; Tris, tris(hydroxymethyl)aminomethane; DCTAF, 5-[(3,5-dichlorotriazinyl)amino]fluorescein; DTNB, Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid); PS:PC, equimolar mixture of dioleoylphosphatidylserine and phosphatidylcholine prepared by sonication; EDTA, ethylenediaminetetraacetic acid; STI, soybean trypsin inhibitor (Kunitz, type 1); HFL-II, human prothrombin labeled (under limit conditions) with 4 mol of aminofluorescein/mol of protein.

[2] S. A. Morrison and J. Jesty, unpublished results.

as the standard (Habeeb, 1972).

To label prothrombin with fluorescein, a 50 mM solution of DCTAF in 50% ethanol/water was added to prothrombin at 2.5–5 mg/mL of 0.2 M borate buffer, pH 9.0, to a final concentration of 1 mM. After 30-min incubation on ice with stirring, exhaustive dialysis vs. NaCl/Tris/1 mM EDTA, at least five changes of 500 volumes each, was carried out to ensure complete removal of unbound dye. When the labeling reaction was not quenched in any way, the derivative, "HFL-II", contained 4.0 mol of fluorescein/mol according to its absorbance at 496 nm, by using $\epsilon = 6.34 \times 10^4$ for fluorescein. Since this extinction coefficient may decrease somewhat when fluorescein is covalently bound (Nairn, 1976), the incorporation figure may be slightly underestimated. Use of the nomograph of Wells et al. (1966), suitably modified for prothrombin, also gave an incorporation of 4–5 mol of fluorescein/mol of prothrombin. No fluorescein could be separated from dialyzed HFL-II by chromatography on Sephacryl S-300 in either 1% sodium dodecyl sulfate or 0.1% Brij 35: by this criterion the protein is considered free of noncovalently bound dye. It was stored in NaCl/Tris/1 mM EDTA at −70 °C. All manipulations of the protein were carried out in darkness or dim light.

Activations of HFL-II were done at 37 °C in NaCl/Tris/5 mM $CaCl_2$/1% poly(ethylene glycol) or in mixtures containing fibrinogen-deficient citrated human plasma (80% v/v) recalcified with 15 mM calcium chloride. A phospholipid dispersion of equimolar phosphatidylserine and phosphatidylcholine was made by sonication as previously described (Silverberg, 1980). In rate determinations factor Xa was added last to start the reaction, after a 10-min preincubation of the other reactants in the dark at 37 °C. In plasma experiments, calcium was added 15 s before factor Xa. Fluorometry was done in a Travenol Laboratories (Aminco) SPF 500 ratio instrument with a 250-W xenon lamp. In all experiments a 4800 Å × 100 Å half-bandwidth narrow pass filter was present in the excitation beam. In rate determinations the emitted light was passed through a similar 5200-Å narrow band filter (Corion). Fluorescence measurements were normalized to a quinine sulfate standard (λ_{ex} 350 nm, λ_{em} 450 nm), 0.1 μg/mL of 0.1 N sulfuric acid, except where indicated otherwise.

For the derivation of initial rates, the conditions could in many cases be manipulated so that the fluorescence response of HFL-II to activation gave progress curves with linear portions lasting at least 2 min and representing less than 10% consumption of the substrate. In some faster reactions, however, substrate depletion had become significant in that time, with resulting downward curvature of the progress curves. These curves consequently had to be fitted to obtain an initial slope. The method chosen was to use a polynomial, cubic function as the model and take the x coefficient as the initial rate. This technique, while a simple one (Atkins & Nimmo, 1973), has statistical drawbacks (Fernley, 1974). In fact, the correlation coefficient for each fit was usually at least 0.99, indicating that this was an adequate model of the progress of all but the most rapid reactions. However, no mechanistic implication is intended in its use (Colquhoun, 1971). Fitting also permitted calculation of fluorescence at the start of the reaction, F_0, not always directly measurable in fast reactions because of the difficulty of mixing enzyme into the cuvette and beginning measurements in less than 20 s. For experiments in which [HFL-II] was constant, results are expressed as the fractional increase in fluorescence, $\Delta F/F_0$, or as the rate of that increase, $\Delta F/(F_0 t)$. By measurement of the final "yield" accompanying activation judged complete by another criterion (e.g., S-2238 assay), the fractional change corresponding to complete activation of the substrate, $\Delta F_f/F_0$, could be determined. For a given batch of HFL-II, $\Delta F_f/F_0$ was independent of [HFL-II] over the range 2–150 μg/mL, although it varied between 0.30 and 0.40 among different batches. Therefore, any fluorescence change could be expressed as a fraction of the total increment possible and, hence, as a fraction of the known initial concentration of substrate.

Weighted linear regressions were done with a Digital Minc microcomputer. For extraction of K_M values, the rate data were fitted to rectangular hyperbolas by using a weighted nonlinear regression program and a Hewlett-Packard 9820A desk top calculator. All rates are the means of at least three (usually four) determinations.

The derivation of K_d and V_{max} for the apparent binding of factor Xa (the variable component) to factor V present at a fixed limiting concentration $[C]_0$ was as follows. If we assume the rate of prothrombin activation, v, to be dependent only upon the concentration of a Xa·V (E·C) complex, i.e., neither free C nor free X has activity, then

$$v = V_{max}[E{\cdot}C]/[C]_0 \quad (1)$$

The dissociation constant, K_d, for binding of E to C is defined as

$$K_d = ([E]_0 - [E{\cdot}C])([C]_0 - [E{\cdot}C])/[E{\cdot}C]$$

which has the form of a quadratic equation in [E·C] (Rodbard & Feldman, 1975), i.e.

$$[E{\cdot}C]^2 - ([E]_0 + [C]_0 + K_d)[E{\cdot}C] + [E]_0[C]_0 = 0$$

Substituting the real solution of this expression in eq 1 and rearranging give

$$v = (V_{max}/[C]_0)\{\{[E]_0 + [C]_0 + K_d - [([C]_0 + [E]_0 + K_d)^2 - 4[E]_0[C]_0]^{1/2}\}/2\} \quad (2)$$

The corresponding expression for fixed [factor Xa] and varying [factor V] would be the same, except that $[C]_0 \equiv [Xa]_0$. Rate data were fitted to eq 2 to obtain the best fit values of $K_{0.5}$ and V_{max} by using a weighted nonlinear least-squares method and the concentration of $[C]_0$ (whether [V] or [Xa]) obtained by clotting assay. We also tested the validity of the latter by treating $[C]_0$ as a third unknown, having first tested various estimates in the range of the value obtained by clotting assay for the one that gave the lowest sum of squares. The solution of eq 2 for the condition where $v = 0.5V_{max}$ gives

$$K_d = K_{0.5} - [C]_0/2 \quad (3)$$

Results

Use of HFL-II in a Continuous Fluorescence Assay. The fluorescence response elicited from HFL-II by its complete activation was an increase in the fluorescence intensity at the peak of emission of fluorescein, 520 nm (λ_{ex} 480 nm), of between 30 and 40%. An example showing the effect of activation of 40 μg of HFL-II/mL is given as emission spectra in Figure 1 (panel A) and as replicate progress curves obtained by monitoring over time at the peak of emission in Figure 1 (panel B). The fractional increase in fluorescence (Methods) corresponding to the activation of a given batch of HFL-II judged complete by other criteria (such as gel electrophoresis or S-2238 assay) was found to be the same at all prothrombin concentrations, despite a slight inner filter effect evident at the higher HLF-II concentrations used here. It is noteworthy that the fluorescence increase is contingent upon the attachment of 4 mol of fluorescein/mol of prothrombin. Thus, the activation of prothrombin carrying only 1 mol of fluorescein

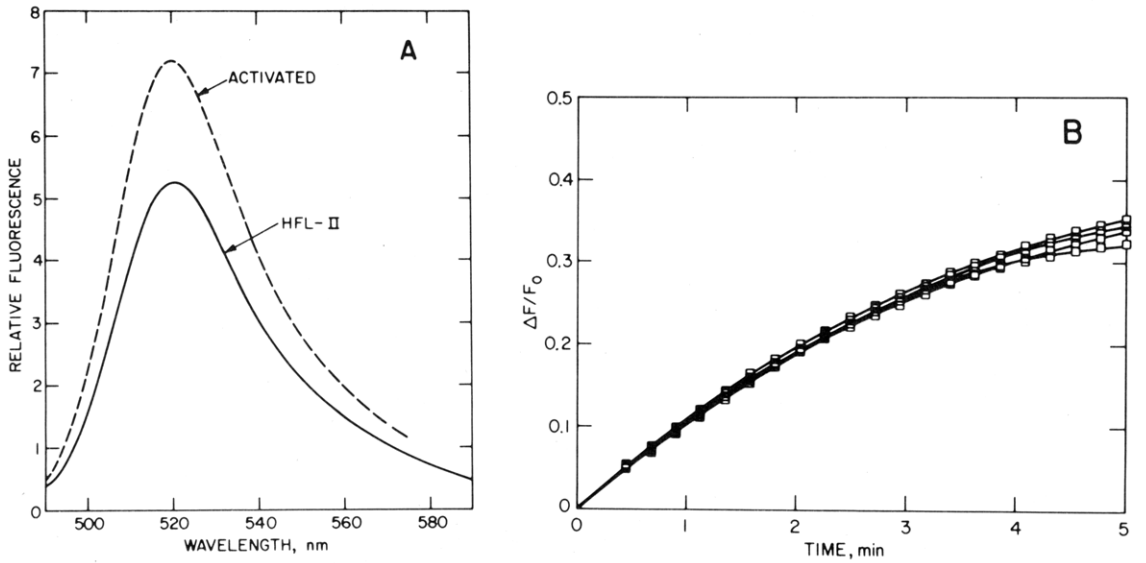

FIGURE 1: Increase of fluorescein fluorescence upon activation of HFL-II. (Panel A) Uncorrected spectra. (Solid line) HFL-II [40 μg/mL in NaCl/Tris/1% poly(ethylene glycol)] in the presence of 10 nM factor V, 5 mM Ca^{2+}, and 25 μM PS:PC (none of which affects the spectrum). (Dashed line) Same after incubation for 10 min with 0.2 nM factor Xa. λ_{ex} 480 nm with 100-nm half-bandwidth filter in place. (Panel B) Progress curves for the same activation, measured at 520 nm with both filters in place; replicate determinations. The points were read off each curve obtained with background fluorescence (F_0) partly subtracted to permit increased sensitivity. The curves were fitted by using a polynomial cubic function and are shown as elevation above predicted starting fluorescence.

[prepared either with DCTAF at pH 5.5, conditions unfavorable for the labeling of lysines, or with FITC by the method of Fass & Mann (1973)] is accompanied by a fluorescence increase of <5%.

It was important to establish that the fluorescence increase reflected proteolysis by factor Xa rather than cleavage of prothrombin to fragment 1 and prethrombin 1 by the thrombin being formed. Figure 2 shows the fluorescence response of HFL-II to incubation with 20 units of human thrombin/mL, in the presence of all the components of activation except factor Xa. Unlike activation, cleavage by thrombin per se (curve a) elicits a negligible fluorescence increase even at this almost stoichiometric amount of thrombin, equivalent to an instantaneous 30% activation. Also, the addition of 20 μg/mL soybean trypsin inhibitor, an inhibitor of factor Xa but not of thrombin (curve c), prevented the rapid fluorescence change during activation by a high prothrombinase concentration (curve b) and halted the change when added after activation had proceeded for 2 min (data not shown). These controls show that the fluorescence increase accompanying activation contains no significant contribution from the product-catalyzed removal of fragment 1 from prothrombin (Suttie & Jackson, 1977). The feasibility of monitoring the fluorescence change in plasma is shown by curves d–f. Curve d is an activation done in a mixture comprising 80% fibrinogen-deficient plasma to which HFL-II was added as a tracer at 11 μg/mL, together with 1 nM factor Xa. Soybean trypsin inhibitor at 20 μg/mL was less effective in plasma than in buffer at preventing activation by exogenous factor Xa (curve f) but was completely effective at higher concentrations (not shown).

The method used to assess the competence of HFL-II as a substrate for prothrombinase was to mix it in varying proportions with native prothrombin, a "silent" substrate in the fluorogenic assay. Monitoring the fluorescence increase gave results [Figure 3 (●)] which showed the rate of increase to be proportional to the mole fraction of labeled prothrombin at a fixed, overall concentration of prothrombin of 20 μg/mL. In contrast, the rate of appearance of activity toward S-2238 was independent of the proportion of HFL-II [Figure 3 (▲)]. These data establish that HFL-II is equivalent to native

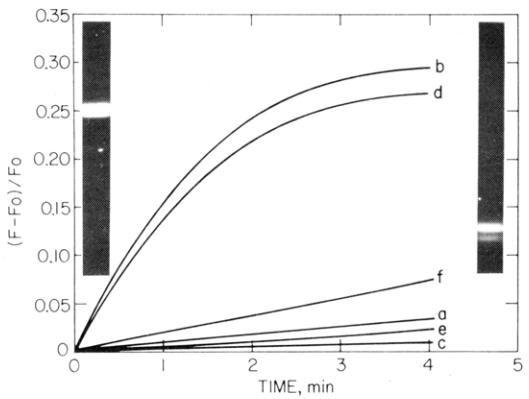

FIGURE 2: Controls for origin of fluorescence increase in buffer and plasma. (Curve a) Effect of thrombin (20 units/mL) rather than factor Xa (0.2 nM; curve b) on mixtures in NaCl/Tris/1% poly(ethylene glycol) containing 40 μg of HFL-II/mL, 10 nM factor V, 5 mM Ca^{2+}, and 25 μM PS:PC. (Curve c) As curve b but also containing 20 μg of soybean trypsin inhibitor/mL. (Curve d) Activation in fibrinogen-deficient citrated plasma (80% v/v) recalcified with 15 mM Ca^{2+} and also containing 11 μg of HFL-II/mL, 25 μM PS:PC, and 1 nM factor Xa. (Curve e) Same, omitting added factor Xa. (Curve f) Same, containing 1 nM factor Xa and 20 μg of STI/mL. All measurements are normalized to the starting fluorescence, F_0. Insets show gel electrophoresis in sodium dodecyl sulfate of samples corresponding to curve b, taken at 0 and 5 min after the addition of factor Xa. Gel photographed over near-UV light.

prothrombin in its kinetic behavior. However, HFL-II differs from native prothrombin in one respect, that the product of its activation has lost its activity toward fibrinogen. Thus, a two-stage assay of the progress of activations performed as in Figure 1A showed that the yield of thrombin activity from 20 μg of native prothrombin/mL was 38.2 ± 0.7 NIH units/mL (five determinations) by a rate assay with S-2238 as substrate (Silverberg, 1980) and 35 units/mL by a clotting assay. In contrast, the complete activation of HFL-II produced no detectable clotting activity (<0.5% normal) although the yield of activity toward S-2238 was 43.3 ± 1.1 units/mL (three determinations). This finding is consistent with reports that a lysine residue is important for the proteolytic activity of thrombin toward fibrinogen (Silverberg, 1980; Griffith, 1979).

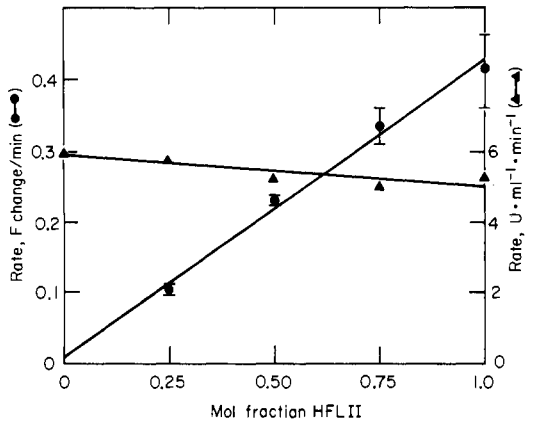

FIGURE 3: Relation of rate of activation to mole fraction of HFL-II in mixtures with unlabeled prothrombin of constant overall concentration. The concentration of prothrombin was 20 μg/mL and rate limiting. Other details as in Figure 1. (●) Rate by fluorescence change (mean of four determinations ± standard errors) shown in arbitrary units without normalization to starting fluorescence. (▲) Rate by S-2238 assay of 50-μL samples withdrawn from the same mixtures at 30-s intervals for 3 min, quenched in 0.45 mL of 0.1% serum albumin/0.1 M phosphate buffer, pH 7.0, and then assayed (after further dilution as necessary) with 0.1 mM S-2238. Points are means of two determinations. Lines were drawn by linear regression.

Conditions of Kinetic Measurements. Subsequent experiments using HFL-II involved a kinetic characterization of its activation in a purified system, through a systematic variation of the concentrations of some components of the reaction. The three variables studied were (1) factor Xa (human), (2) factor V(a) (bovine), and (3) HFL-II itself. The two remaining constituents of prothrombinase, Ca^{2+} and a membrane substitute (a PS:PC dispersion), were held invariant at 5 mM and 25 μM, respectively. The latter concentration was chosen in preliminary trials that indicated it to be "optimal". That is, at lower or higher levels of PS:PC and at a concentration of prothrombin that was close to the apparent K_m for activation, 40 μg/mL, the activation rates were somewhat less. This finding agrees with that of Rosing et al. (1980), who demonstrated a pronounced and complicated effect of phospholipid concentration on the activation of bovine prothrombin, such that the apparent K_m passed through a minimum at about the same concentration as will be used here. No further studies of the effects of varying [Ca^{2+}] or [PS:PC] were made.

[Factor Xa] as Variable. To investigate whether the rate of fluorescence change accurately reflects the proteolytic activity of prothrombinase, rates were determined for a fixed concentration of HFL-II (40 μg/mL or 0.56 μM) and high [factor V] (10 nM) as a function of factor Xa concentration between 0 and 0.3 nM. At Xa concentrations at or above 0.2 nM the progress curves showed pronounced nonlinearity (cf. Figure 1B) because of significant substrate depletion. These curves were fitted to determine initial slopes (Methods). Other, slower rates were obtained by inspection. Figure 4 (●) shows the secondary plot of the rate of activation obtained from the rate of fluorescence increase (Methods) vs. [factor Xa]. Its linearity indicates (1) that the fluorescence change is a true index of activation rate and (2) that factor Xa is saturated by this level of factor V at all the concentrations tested.

No fluorescence increase was observed in the absence of factor V over this factor Xa range. Equally, there was no loss of signal, indicating that no photolytic degradation of fluorescein occurred. At high levels of factor V there was a slow, factor V dependent fluorescence increase in the absence of factor Xa, indicating that Xa might be present in either the prothrombin or the factor V as a contaminant. The level of

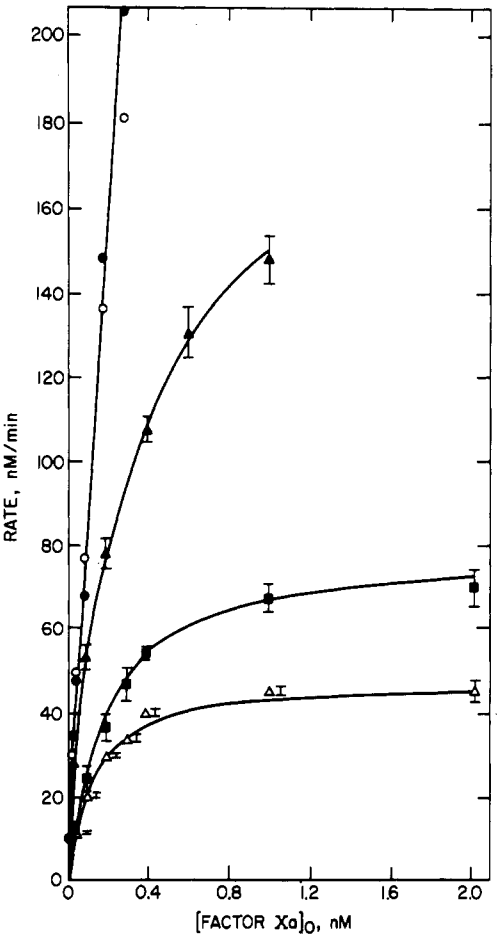

FIGURE 4: Dependence of rate of activation of HFL-II on factor Xa concentration at five concentrations of factor V. HFL-II (0.56 μM) and 25 μM PS:PC in 5 mM $CaCl_2$/1% poly(ethylene glycol)/NaCl/Tris, pH 7.5, were preincubated for 10 min at 37 °C with factor V, and factor Xa (abscissa) was then added. The factor V concentrations were 10 (●), 2 nM (○), 1 (▲), 0.2 (■), and 0.1 nM (△). Points are means of three or four rate determinations. λ_{ex} 480 nm, and λ_{em} 520 nm, with narrow band filters in each light path. Data for 0.2 and 0.1 nM factor V were fitted to a binding isotherm (Methods), allowing the concentration of factor V to "float" in the fitting procedure until the least sum of squares was reached.

contamination was evidently in the picomolar range and therefore unmeasurable by available assay methods.

These initial experiments show that the rate of the fluorescence increase accompanying the activation of HFL-II was proportional to the concentration of factor Xa over a subnanomolar range when factor V was supplied at a high level (10 nM). A more extensive investigation was then carried out in which the factor V was fixed at four lower concentrations between 2 and 0.1 nM and factor Xa was varied at concentrations between 0 and 2.0 nM. Secondary plots of rate vs. [factor Xa] at each factor V concentration (Figure 4) showed that as [factor V] was decreased, the relation of rate to total [factor Xa] began to show evidence of saturation. At 0.2 nM factor V (and below) this relation appeared truly hyperbolic, and accordingly, these data were treated as a binding isotherm. The form of the isotherm used (Methods) has the advantages that (1) it does not require a value for the asymptote of the isotherm (V_{max}) to be known a priori, (2) it takes into account that a significant proportion of the total variable component of prothrombinase (in this case factor Xa) is bound, i.e., $[Xa]_f < [Xa]_0$, and (3) it allows the value of the constant component, $[V]_0$, to be treated as an unknown if desired. When the third of these was utilized, the least sum of squares for the fit was

Table I: Kinetic Parameters[a] for HFL-II Activation as a Function of Factor V

[factor V] (nM)	10	2.0	0.4	0.08
[Xa·V][b] (nM)	0.20	0.19	0.14	0.039
V_{max} (nM/min) ± SE	226 ± 10	126 ± 8.3	81.4 ± 4.2	33.5 ± 3.1
K_m (μM) ± SE	0.28 ± 0.02	0.16 ± 0.02	0.21 ± 0.02	0.17 ± 0.03
k_{cat}[c] (s^{-1})	19.0	11.1	9.83	14.2
k_{cat}/K_m (s^{-1} M^{-1})	6.8×10^7	6.9×10^7	4.7×10^7	8.4×10^7

[a] Experimental details are given in the legend to Figure 6. [b] Calculated from measured value of K_d. [c] $k_{cat} = V_{max}/[Xa \cdot V]$.

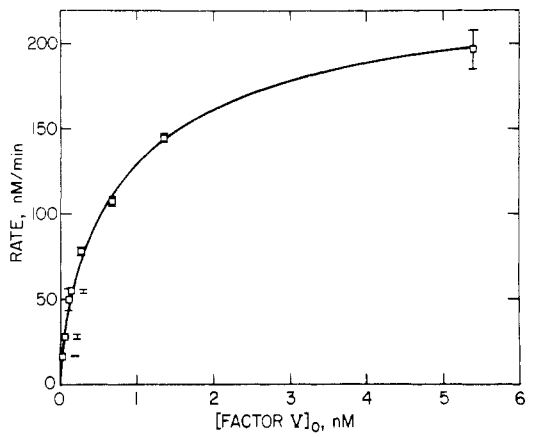

FIGURE 5: Dependence of activation rate on factor V concentration at fixed factor Xa. Factor V (abscissa) and factor Xa at 0.4 nM; details otherwise as in Figure 4. The points are means of four determinations, and the line is by inspection.

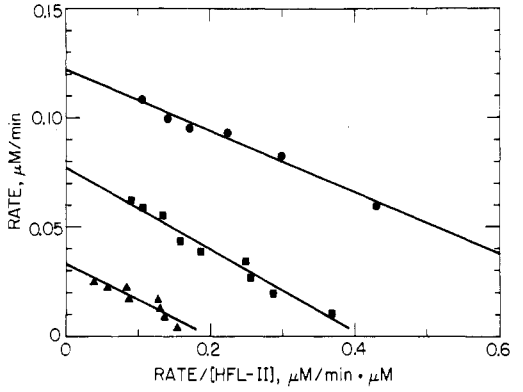

FIGURE 6: Eadie plots of prothrombin activation at three concentrations of factor V. Activations of HFL-II at concentrations between 0.03 and 1.04 μM were carried out in quadruplicate at 37 °C with 0.2 nM factor Xa, 25 μM PS:PC, and 5 mM $CaCl_2$ in 1% poly(ethylene glycol)/NaCl/Tris, pH 7.5, and 2.0 (●), 0.4 (■), and 0.08 nM (▲) factor V. Lines were drawn by unweighted linear regression of the mean rate on mean rate/[prothrombin].

obtained at a somewhat lower value for $[V]_0$ than the nominal one of 0.2 nM, namely, 0.134 ± 0.0004 nM. This is consistent with the fact that the nominal factor V concentration is based on specific activity, which is somewhat elevated compared to the best (i.e., least activated) preparations that have been reported (Nesheim et al., 1981b). Consequently, the molar concentration might be expected to be somewhat lower. The corresponding value for $K_{0.5}$ was 0.126 ± 0.0003 nM factor Xa, with a V_{max} of 76.3 ± 1.2 nM/min. This value of $K_{0.5}$ gives a K_d of 0.059 nM (Methods, eq 3) and a stoichiometry for binding of 1.14 mol of factor V/mol of factor Xa. The "binding curve" from which these parameters were derived is shown in Figure 4 (■); it should be noted that although the abscissa shows total factor Xa, the fitting of both lower curves took into account the binding of a varying proportion of it to factor V.

[Factor V] as Variable. In a converse experiment, the rate of activation was measured at eight factor V concentrations between 0.03 and 6.0 nM, with the level of factor Xa held constant at 0.4 nM. The results (Figure 5) showed the same type of saturable response to variation of the cofactor concentration as was seen when the enzyme concentration was changing. However, when the rate data were fitted to the binding isotherm as before, the rates appeared to be too high at low [factor V] and too low at high [factor V] for a fit to the model to be obtained. A possible reason for the former is that the simplifying assumption of the model that factor Xa has no activity in the absence of factor V is incorrect: factor Xa does cleave prothrombin in the absence of cofactor, at a rate about $^1/_{600}$ times the rate per mole of factor Xa in the presence of saturating factor V (Nesheim et al., 1979b; Kosow & Orthner, 1979). Underestimation of the activation rate at high factor V could be a consequence of, among other things, the inadequacy of a cubic model for the progress curve when activation is extremely rapid, or product inhibtion, or both. No data could be obtained to distinguish among these possibilities.

[Prothrombin] as Variable. The results above suggest that the activity of factor Xa in the system is governed by the availability of factor V, and vice versa. Therefore, in experiments to determine the apparent kinetic parameters for activation the activity of factor Xa was first augmented fully by providing factor V in the saturating amount of 10 nM. The concentration of factor Xa was chosen to be compatible with initial rate conditions over as much of the range of prothrombin concentration to be covered as possible. The level of factor Xa was therefore 0.2 nM, or $^1/_{150}$ of the lowest level of prothrombin in these experiments. The concentration of HFL-II was then varied over a 35-fold range between 2 and 75 μg/mL (0.03 and 1.04 μM). The results showed a hyperbolic dependence of rate upon prothrombin concentration, in accordance with a Michaelis–Menten model. The experiment was then repeated with factor V each time at a 5-fold lower concentration. It was found that the relationship between rate and concentration remained hyperbolic at levels of factor V down to 0.08 nM, the lowest studied. The results from three such experiments are shown as Eadie plots in Figure 6; the corresponding data from all the experiments (with their confidence limits) comprise Table I. A drop in [factor V] from the saturating level to a concentration which previous experiments had shown to be limiting was accompanied by a 7-fold decrease in the apparent V_{max} for activation. This variation reflects the amount of factor Xa able to bind to factor V; thus, calculation of k_{cat} ($=V_{max}$ per mole of bound factor Xa) gives values that vary in random fashion by less than 2-fold. Likewise, the apparent Michaelis constant (defined here only as that concentration of prothrombin at which the velocity was $0.5V_{max}$) varied by only about 60% over the same range of [factor V]. Thus, the Eadie plots are essentially parallel. The coefficient of proteolytic efficiency, k_{cat}/K_m, for the condition where factor Xa was fully saturated with factor V was 6.8×10^7 s^{-1} M^{-1}.

Modulation of Prothrombin Activation by the Feedback

Activation of Factor V by Thrombin. It is well-known that, to participate most effectively as a cofactor in thrombin formation, factor V must be activated by exposure to traces of thrombin itself. This effect of thrombin has been established as being concomitant with multiple cleavages of single chain factor V, relatively ineffective when in precursor form, and with some enhancement of the binding of factor Xa to it (Esmon, 1979; Nesheim & Mann, 1979; Nesheim et al., 1979b). The most pronounced enhancement that has been reported is 80-fold by coagulation assays of the purified bovine protein (Nesheim et al., 1981b). The elevations of activity obtained here were more modest, between 20- and 50-fold. The most probable reason for a lesser enhancement of activity is that factor V is somewhat activated already, before and/or during its isolation. Nevertheless, it remains sufficiently responsive to thrombin that one could expect a lag or slow initial phase in the progress of activations before factor V has had time to be activated by thrombin. During that lag phase, the thrombin that is beginning to accumulate must in turn increase the activity of the cofactor by cleaving it.

In the progress curves for HFL-II activation, at concentrations of factor V of 2 nM or above and under conditions of plentiful Xa, there was no delay before the region of maximum rate of fluorescence change. But at 0.4 and, more noticeably, at 0.08 nM factor V, some progress curves showed a phase of accelerating fluorescence change at the outset, as in the example shown in Figure 7 (curve a). Gel electrophoresis in sodium dodecyl sulfate of samples taken at different points during the lag phase showed that no proteolysis of HFL-II of any kind had occurred before the fluorescence increase began. The first appearance of thrombin on the gel corresponded quite closely with the point at which the fluorescence change indicated activation to have begun (Figure 7, insets).

On the general considerations given above it appeared that a cause of the lag might be the requirement for thrombin action on factor V. However, when a trace of thrombin (0.01 unit/mL) was included in the cuvette contents for the 10-min preincubation period before the addition of factor Xa to start activation, it was found to have no effect (Figure 7, curve b). Only when prothrombin was witheld during the preincubation with thrombin and was then added with factor Xa was the lag before activation shortened (Figure 7, curve c). A further, curious finding was that lags were most pronounced at a higher concentration of prothrombin, when thrombin generation should have been more rapid and extensive. The example shown in curve a of Figure 7 was obtained at 50 μg/mL or ~50% of the plasma concentration. In contrast, there was almost no lag preceding the activation of 3.5 μg of HFL-II/mL (Figure 7, curve d), and preincubation with thrombin in the presence or absence of prothrombin had no additional effect (data not shown). The restriction of lags to higher prothrombin concentrations and the lack of effect of exogenous thrombin in the presence of prothrombin both suggest that the minimal amount of factor V was protected from preactivation by thrombin when high levels of prothrombin were present.

One possible, spurious cause of the damping of activation at higher prothrombin levels was the presence of fluorescein on the substrate. Therefore, a control experiment was done in which unlabeled prothrombin at 47 μg/mL was added to an activation of HFL-II at 3 μg/mL. This was found to introduce a prolonged lag before the fluorescence increase began (Figure 7, curve e), which was essentially absent when the same concentration of HFL-II was activated by itself. This result shows that the lag is a function of the prothrombin concentration, not an artifact introduced by the fluorescent modification.

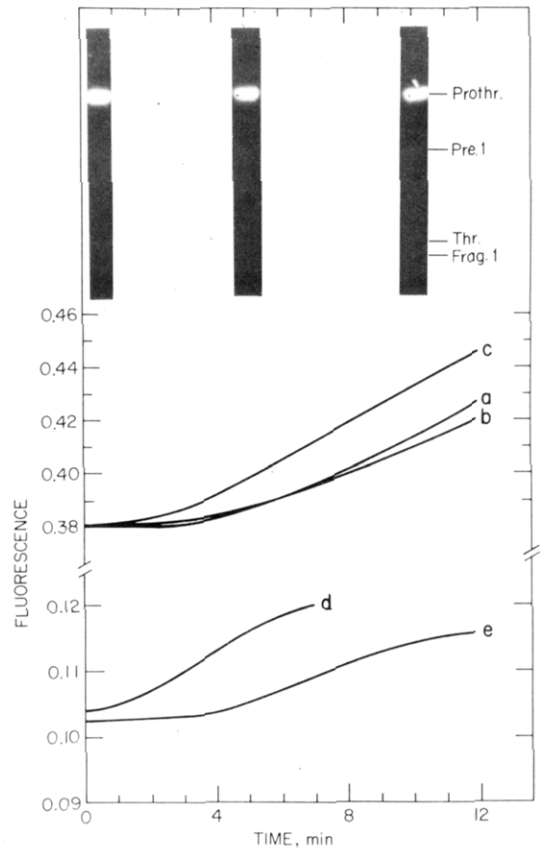

FIGURE 7: Progress curves for HFL-II activation showing effects of thrombin. (Curve a) HFL-II at 0.69 μM and 0.08 nM factor V activated with 0.2 nM factor Xa added at $t = 0$. (Curve b) Same, but preincubated for 10 min with 0.01 unit of thrombin/mL. (Curve c) Same, omitting HFL-II from preincubation. (Curve d) HFL-II at 0.049 μM and 0.08 nM factor V, with 0.2 nM factor Xa added at $t = 0$. (Curve e) HFL-II at 0.042 μM with unlabeled prothrombin at 0.65 μM, otherwise as curve d. Fluorescence measurements were made at different sensitivity settings for each set of curves and are shown without normalization.

Irrespective of whether the factor V was preactivated, the maximum (but slow) rates of activation reached at a strongly rate-limiting concentration of factor V were the same. When maximum slopes were measured over a 25-fold range of prothrombin as above, with 0.2 nM factor Xa and either unactivated factor V at 0.08 nM (taking the maximum slope to be the rate) or the same concentration of factor V activated beforehand with thrombin, the Michaelis–Menten curves obtained were essentially superimposable (Figure 8). In other words, the necessity for feedback activation did not affect the apparent kinetic constants when maximum slopes were used to obtain them.

Discussion

By use of a fluorescent derivative that permits determination of activation rates by direct observation, the effects of varying the concentrations of some components of human prothrombin activation have been determined. The assay is independent of the subsequent reactions of thrombin (although these appear, with one exception, to be normal) and from additional evidence[3] can be used in plasma. The results of varying the concentration of factor Xa in the presence of Ca^{2+}, a sonicated

[3] S. A. Morrison, unpublished observations.

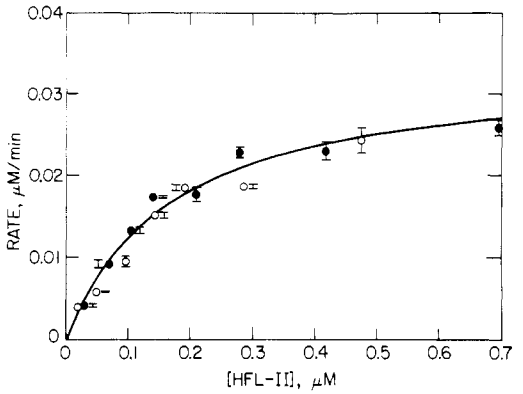

FIGURE 8: Dependence of activation rate on prothrombin concentration showing effects of limiting factor V or factor Va. Factor V (●) at 0.08 nM and HFL-II (abscissa) were preincubated for 10 min with 5 mM $CaCl_2$ and 25 μM PS:PC in NaCl/Tris/1% poly(ethylene glycol), and 0.2 nM factor Xa was added to start activation. (○) Same, except that factor V was activated beforehand by incubating 0.8 μM factor V with 1 unit of thrombin/mL for 10 min. Each point is the mean of four rate determinations; standard errors are shown as bars. The curve is the results for unactivated factor V (●) fitted to a rectangular hyperbola by a weighted nonlinear least-squares method.

phospholipid dispersion, and a fixed nonlimiting level of factor V indicated that the initial rate of the fluorescence change was proportional to [factor Xa]. Thus, the technique provides a measure of prothrombinase activity that is sensitive with respect to the concentrations of both factor Xa (2.25 ng/mL or below) and prothrombin (2 μg/mL or below) that can be employed. The technique is also convenient because the fluorescence change is monitored with activation in progress in the cuvette rather than after subsampling.

The use of a fluorescent probe of activation arose in part from earlier work (Silverberg, 1980) in which I showed a red shift of the intrinsic fluorescence spectrum of bovine prothrombin accompanying its activation. For various reasons, including its lack of sensitivity, lack of utility in a complex system such as plasma, and the possibility that the red shift was not due to proteolysis per se, the intrinsic fluorescence change was inferior as an index of activation to the possible use of a covalently attached probe. Evidence is presented here that the fluorescence increase with fluorescein requires multiple sites of attachment to prothrombin, while other, unpublished, data suggest that the change depends on the interaction between dye molecules on single polypeptide chains that become separated specifically on the products of proteolysis by factor Xa.

The concentration ranges for each variable covered in the experiments were somewhat arbitrary to remain as far as possible within the confines of initial rate conditions and permit a simple interpretation of the progress curves. They are not meant to reflect the situation in plasma. In particular, the amount of factor Xa to be expected during coagulation is unknown, and the availability of factor V(a) to bind it at the platelet surface is an additional variable not addressed in these experiments. This is strictly a model system, although it is fair to say that most investigators who have compared the kinetic behavior of platelet-bound factor V with purified plasma factor V on phospholipid micelles find only minor differences (Dahlback & Stenflo, 1980; Nesheim et al., 1979b).

It was first shown by Nesheim et al. (1979b) that the presence of factor V at a "limiting concentration" brings about a saturable response of the prothrombin activation rate to variation in the concentration of factor Xa. This enabled these workers to derive a value for K_d by kinetic measurements that was taken to represent the interaction of these two components of prothrombinase, independently of prothrombin. Their assumption was subsequently validated (Nesheim et al., 1981a) because the numerical value of this constant was very similar when the appearance of thrombin active sites was the basis of the assay and when a fluorescent probe of factor Xa was used to measure binding to factor V directly, by fluorescence titration. Lindhout and co-workers (1982) extended the kinetic aspect of this by showing that the apparent K_d of the Xa·Va complex is completely independent of the prothrombin concentration over a wide range. The present work uses the same assumption to arrive at an apparent dissociation constant for human factor Xa with bovine factor V. The value indicates approximately 10-fold tighter binding than Nesheim et al. (1979b) reported for the bovine proteins, at a stoichiometry of 1.14 mol of factor V/mol of factor Xa. The present K_d of 5.9×10^{-11} M is in very close agreement with the value of 3×10^{-11} M that Lindhout et al. (1982) obtained for the bovine proteins under optimal conditions of phospholipid concentration and composition.

The value of K_m for activation obtained here is somewhat lower (though not dramatically so) than that for the activation of bovine prothrombin reported by Nesheim et al. (1979b) and almost identical with the one reported by Rosing et al. (1980). Thus, the plasma concentration of 1.4 μM exceeds the K_m (0.2–0.3 μM) by enough to ensure that the normal plasma concentration is but poorly rate limiting in activation, since the reaction is approaching zero order at this level of substrate. Mann and co-workers (1981) present a convincing argument for the idea that K_m in this instance does not represent the interaction of prothrombin with the enzyme, factor Xa, as it would for a reaction in the solution phase, but rather the binding of the prothrombin to the phospholipid or platelet surface. This is thought to explain the invariance of the apparent K_m for activation with the concentration of factor V (presumed to serve as the anchor for factor Xa at the phospholipid or platelet surface) that these workers showed, and that is shown here to hold for human prothrombin. In contrast, the V_{max} for human prothrombin activation varies with [factor Va]. This reflects the requirement that factor Xa be bound to the cofactor, since the V_{max} per mole of Xa·V is invariant.

The use of a chemically modified substrate in such experiments is open to the objection that kinetic parameters derived with it will be peculiar to that form of the substrate, through its having been altered in some significant way by the modification. Evidence suggesting that this is not the case can be drawn in part from the fact that mixing the labeled substrate with native prothrombin to a constant, rate-limiting concentration resulted in activation rates that were identical when the rate of appearance of thrombin was measured but, by fluorescence, were proportional to the mole fraction of labeled protein. Therefore, the binding of HFL-II to prothrombinase is equivalent to that of native prothrombin, and both then generate product at the same rate.

The same result addresses the issue of the status of factor V during the activation of HFL-II. In the present experiments, factor V was usually added without prior activation because the effect of the feedback activation of the cofactor when occurring in parallel with prothrombin activation was of interest. However, since the product of HFL-II activation has lost its proteolytic activity toward fibrinogen, one might anticipate a similar loss of the ability to activate factor V. The results of the mixing experiment suggest that HFL-II is not at a gross disadvantage over normal prothrombin in this respect, and more detailed kinetic measurements confirm a

normal response of factor V in the activation mixture. Although I have shown that under some special conditions the feedback activation of factor V may be rate limiting at the start of HFL-II activation (especially when the factor V concentration is very low), the same holds when HFL-II is used as a tracer at a level of only 6% of the total prothrombin. Thus, the evidence suggests that this is a property shared by the activation of normal prothrombin, not a result of the generation of abnormal thrombin from HFL-II. The origin of the effect has not been established, but a possible candidate is the competition of prothrombin for the thrombin being formed. The reaction of thrombin with prothrombin in the presence of Ca^{2+} is essentially first order in this range of prothrombin concentration (Silverberg, 1979), making the inhibition of factor V activation especially sensitive to the progressive consumption of the prothrombin.

The present work also shows that the value of the apparent Michaelis constant for prothrombin activation is unaffected by whether factor V has been preactivated with thrombin or not. However, this should not be taken to mean that factor Xa binds equally well to factor V or factor Va; rather, it shows that the rates that were used to derive the constant were not constrained by the requirement for feedback activation of the cofactor. The indifference of the rates that could eventually be reached to whether or not factor V had been preactivated, combined with a pronounced effect of prothrombin concentration on the shape of the progress curve, in fact has potential significance as a regulator of prothrombin activation. When factor V is scarce and not preactivated, there is a delay of several minutes before the maximum rate of prothrombin activation is reached, though only when the prothrombin concentration is high, that is, in the plasma range. It is proposed that this reflects a period during which low levels of thrombin, and possibly factor Xa (Tracy et al., 1983), can potentiate the activity of factor V, since a similar result was obtained with a much higher concentration of factor V when dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide was used to block its feedback activation by thrombin (Nesheim et al., 1979b). However, in a system in which thrombin is irreversibly removed by other means, e.g., by antithrombin III, or is bound to other substrates, the protection of low factor V from activation by a prevailing high concentration of prothrombin could absorb the effects of low levels of proteases and suppress prothrombin activation altogether. Another way to look at the same phenomenon is that the prothrombin concentration and the thrombin concentration are reciprocally related in a synergistic way, rather than simply as precursor and product. Both the appearance of thrombin above a certain threshold level and the disappearance of prothrombin will encourage the activation of factor V, leading to a progressively brisker activation rate over time.

Acknowledgments

Debbie Aufiero's ever-excellent technical assistance is much appreciated. I also thank John Sachs of this Division for extensive help with fitting and interpreting curves and Barry Coller and James Hayward for their helpful criticism of the manuscript.

Registry No. Prothrombin, 9001-26-7; prothrombinase, 72162-96-0; factor V, 9001-24-5; factor Xa, 9002-05-5.

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