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Activation of Porcine Factor VIII:C by Thrombin and Factor Xa†

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ABSTRACT: The activation of porcine factor VIII:C by thrombin and by factor Xa was studied by a chromogenic substrate assay and by sodium dodecyl sulfate–polyacrylamide gel radioelectrophoresis of ^{125}I-labeled factor VIII:C activation products. In the chromogenic assay, the kinetics of factor VIII:C dependent activation of factor X by factor IXa in the presence of calcium and phosphatidylserine/phosphatidylcholine vesicles were measured with *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide (S2222) as substrate. Substrate dependence of initial rates of the reaction at fixed factor IXa, factor VIII:C, lipid, and calcium obeyed Michaelis–Menten kinetics. At fixed factor IXa, factor X, lipid, and calcium the initial rates of the reaction varied linearly with lower factor VIII:C concentrations and plateaued at higher concentrations. The linear initial rate dependence formed the basis of a rapid, plasma-free assay of activated factor VIII:C. The activation of factor VIII:C by thrombin or factor Xa and the enzyme-independent rate of spontaneous inactivation were studied under conditions of excess enzyme. A model of the activation kinetics was developed and fit to the data by a nonlinear least-squares technique. From the model, the catalytic efficiencies (k_{cat}/K_m) of factor VIII:C activation by thrombin and factor Xa were 5.0×10^6 M^{-1} s^{-1} and 1.1×10^6 M^{-1} s^{-1}, respectively. By comparison with published values of the catalytic efficiencies of several other coagulation enzymes for various substrates, both thrombin and factor Xa are efficient enzymes toward factor VIII:C. Additionally, the model allows calculation of the relative cofactor activities of thrombin-activated factor VIII:C (factor VIII:Ca_{IIa}) vs. factor Xa activated factor VIII:C (factor VIII:Ca_{Xa}). The ratio of cofactor activities (VIII:Ca_{IIa}/VIII:Ca_{Xa}) is 3.0. This indicates that significantly more activity is generated when factor VIII is fully activated by thrombin than when factor VIII is fully activated by factor Xa. The formation of cofactor activity by both enzymes is closely paralleled by proteolysis of factor VIII:C polypeptides although thrombin and factor Xa give distinctly different products.

Activated factor VIII:C (antihemophilic factor) is a protein that is a cofactor for the activation of factor X by factor IXa and is necessary for normal hemostasis in vivo. Factor VIII:C circulates as a procofactor and requires activation for coagulant activity. Activation of human, bovine, and porcine factor VIII:C occurs after thrombin-catalyzed limited proteolysis of the procofactor molecule (Vehar & Davie, 1980; Hoyer & Trabold, 1981; Knutson & Fass, 1982; Fulcher & Zimmerman, 1982; Weinstein & Chute, 1984). Enzymes that are known to activate factor VIII:C in addition to thrombin include factor Xa (Davie et al., 1975; Vehar & Davie, 1980; Griffith et al., 1982; Hultin, 1982; Hultin & Jesty, 1982) and factor IXa (Rick, 1982). The relative catalytic efficiencies of these enzymes have not been reported, and the physiological activator(s) of factor VIII:C remains (remain) unknown. In this report we show that thrombin is a more efficient activator than factor Xa in a synthetic lipid system. Factor IXa is without effect at the concentrations used. Additionally, the thrombin-activated cofactor has more activity than the factor Xa activated cofactor. Factor Xa, however, is a relatively efficient catalyst when compared to other coagulation enzyme–substrate reactions, and since significant amounts of factor Xa formation may precede thrombin formation, factor Xa may participate in factor VIII:C activation in vivo.

Experimental Procedures

Materials. *N*-Benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine *p*-nitroanilide (S2222) was purchased from Kabi Diagnostica, Stockholm, Sweden. L-α-Phosphatidylcholine (PC), type III-E, and L-α-phosphatidylserine (PS) were purchased

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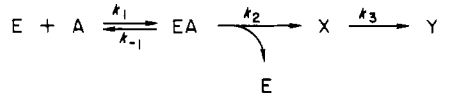
Scheme I



from Sigma Chemical Co., St. Louis, MO. *p*-Nitrophenyl *p*-guanidinobenzoate was purchased from ICN Pharmaceuticals, Cleveland, OH, and was stored at 2.5 mM in dimethylformamide/acetonitrile (1:4 v/v) at 4 °C before use. Other reagents are listed elsewhere in this paper or were reagent grade.

Phospholipid vesicles (25% PC/75% PS w/w) were prepared by a modification (Higgins & Mann, 1983) of the method of Barenholz et al. (1977). Molar concentrations of phospholipid were determined by assay for inorganic phosphorus (Gomori, 1942).

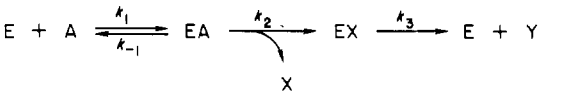
Proteins. Porcine factors II, IX, IXa, IXa_β, and $_\beta Xa$, thrombin, ^{125}I-labeled factor VIII:C (Lollar et al., 1984), and factor VIII:C (Fass et al., 1982) were prepared as described. Extinction coefficients ($E^{1\%}_{1cm}$) that were used for factor IXa, thrombin, and factor X were 15.2, 19.9, and 8.4, respectively (Lollar et al., 1984). Molar concentrations of factor VIII:C were estimated by using an average molecular weight estimated from sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of ~200000 and a specific activity of 6 units/μg (Fass et al., 1982). Factor VIII:C coagulant activity was measured as described (Owen et al., 1975). One unit of activity is defined as the amount of factor VIII:C present in 1 mL of normal citrated porcine plasma. The concentration of factor Xa was measured by active-site titration with *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) (Chase & Shaw, 1970). Dilutions of factor Xa in 0.15 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 0.1% poly(ethylene glycol) 8000 (TBS–PEG), and 50 mM ethylenediaminetetraacetic acid (EDTA) were frozen in small aliquots at −20 °C and were used to develop a factor Xa standard curve with S2222 as substrate.

Chromogenic Assay. Factor Xa production by an enzymatic complex consisting of factor IXa, (PC/PS) vesicles, thrombin or factor Xa activated factor VIII:C or unactivated factor VIII:C, and calcium was measured by adding 0.05-mL aliquots from the reaction mixtures (see Results) to 5 μL of 0.5 M EDTA, pH 7.4, in a sample well of an Abbott ABA bichromatic analyzer equipped with 380/450-nm filters. This concentration of EDTA stops the reaction within 4 s. The instrument subsequently adds 0.01 mL of the factor Xa/EDTA mixture to 0.25 mL of 0.4 mM S2222 in TBS–PEG and 50 mM EDTA, pH 7.5, and reads the change in absorbance between 0 and 5 min. A standard curve of absorbance change vs. porcine β-factor Xa was established each day. The curve was linear to greater than 40 mM factor Xa, at which concentration less than 5% of the S2222 had been hydrolyzed in 5 min. Factor X activation studies were performed at 22 °C.

Kinetics. Analysis of factor VIII:C activation is complicated by the spontaneous inactivation of the cofactor. We propose the model shown in Scheme I for factor VIII:C activation, where E represents enzyme, A is substrate (procofactor), EA is the enzyme–substrate complex, X is activated factor VIII:C (cofactor), and Y is inactive cofactor. All species are referred to in concentration terms. Reactions are carried out with enzyme in excess over procofactor instead of the more common situation of excess substrate. Additionally, we make the rapid-equilibrium assumption that the enzyme–substrate complex can be described at all times by the relationship:

$$[E][A]/[EA] = [E]_0[A]/[EA] = K_s \quad (1)$$

Scheme II



where $[E]_0$ is the nominal concentration of enzyme. Making the usual assumption that all reactions are first order with respect to reactants, we have

$$d[X]/dt = k_2[EA] - k_3[X] \quad (2)$$

$$d[Y]/dt = k_3[X] \quad (3)$$

Using the conservation equation for total substrate

$$[A]_0 = [A] + [EA] + [X] + [Y] \quad (4)$$

we can solve the resulting linear, second-order homogeneous differential equation (with constant, positive coefficients) for [X] (Simmons, 1972):

$$[X] = \frac{b_0 b_1}{b_1 - b_2}[\exp(-b_2 t) - \exp(-b_1 t)] \quad (5)$$

where

$$b_0 = [A]_0(1 + [E]_0/K_s) \quad (6)$$

$$b_1 = k_2\{[E]_0/([E]_0 + K_s)\} \quad (7)$$

$$b_2 = k_3 \quad (8)$$

$$[X] = 0 \text{ when } t = 0$$

and

$$\frac{d[X]}{dt} = k_2[EA] \text{ when } t = 0$$

Equation 5 predicts the time course for the concentration of factor VIII:C ([X]) as a function of nominal factor VIII:C concentration ($[A]_0$), nominal enzyme concentration ($[E]_0$), the catalytic rate constant k_2 ("k_{cat}"), and the spontaneous inactivation rate constant (k_3).

The time course of experimental determinations of factor VIII:C activity, which is proportional to [X], was fit to eq 5 by a nonlinear least-squares program. Parameters b_0, b_1, and b_2 were thus obtained. It will be argued under Results that $[E]_0 \ll K_s$ for the range of enzyme concentrations that were used in this study so that $b_0 = [A]_0$ and $b_1 = (k_2/K_s)[E]_0$. The units of b_0 are arbitrary since the measured cofactor activity is proportional to the cofactor concentration.

Another model that initially seemed feasible is shown in Scheme II. This model differs from Scheme I in that a stoichiometric complex of enzyme and a proteolytically modified factor VIII:C molecule is the active species. When the same reaction conditions that are imposed on Scheme I are used, Scheme II leads to an analytical solution that is identical in algebraic form with that for Scheme I.

Electrophoresis. Sample preparation of ^{125}I-labeled factor VIII:C, SDS–polyacrylamide gel electrophoresis, and calibration for molecular weight were performed as described by Lollar et al. (1984) with the exception of one analysis as noted in the figure legends. A nongradient 7% acrylamide gel was used to demonstrate the 82-kilodalton (kDa) and 76-kDa chains in a sample of ^{125}I-labeled unactivated porcine factor VIII.

Data Analysis. Statistics programs were run on an Apple IIe microcomputer. All graphics were performed with software written in the laboratory to drive a Hewlett-Packard 7470A plotter. Nonlinear least-squares fits to the data were done by using a Basic program (NLLSQ, CET Research, Norman, OK) based on Marquardt's algorithm (Bevington, 1969). Uncertainties in the fitted parameters are expressed as the standard

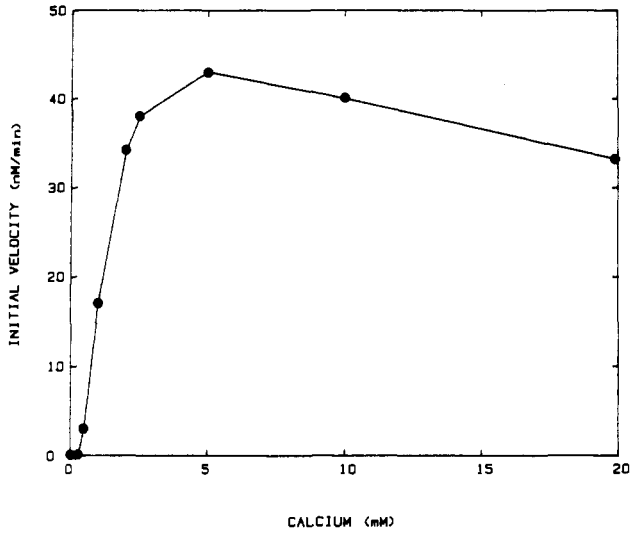


FIGURE 1: Calcium dependence of factor X activation. Factor X was added to a final concentration of 0.24 μM to a solution containing 15 μM PC/PS vesicles, 0.6 nM factor IXa, 7 nM thrombin, 0.53 unit/mL factor VIII:C, and variable $CaCl_2$. The solution was preincubated for 3 min to allow factor VIII:C activation. Aliquots were taken at 0.25-min intervals for 1 min for determination of factor Xa (Experimental Procedures), and the initial rate of factor X activation was measured by linear regression of a plot of factor Xa vs. time.

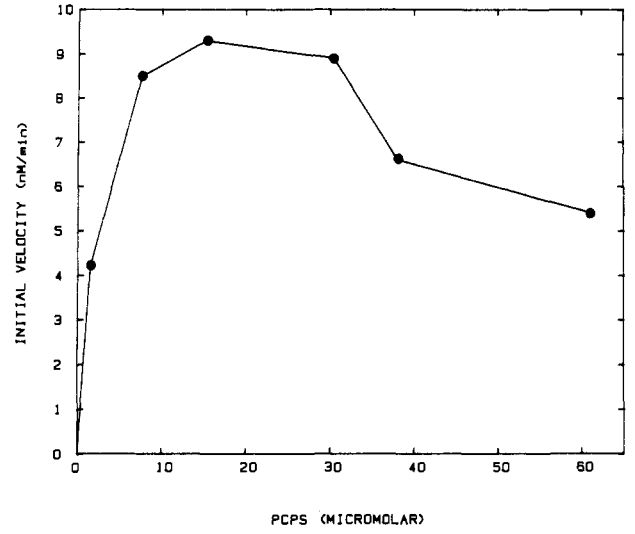


FIGURE 2: Lipid dependence of factor X activation. Initial rates of factor X activation were measured in Figure 1 except the $CaCl_2$ concentration was fixed at 5 mM and the lipid concentration was varied with factor VIII:C, factor IXa, and thrombin fixed at 0.16 unit/mL, 0.85 nM, and 7 nM, respectively.

deviation estimated from the goodness of fit.

RESULTS

Steady-State Kinetics of Factor X Activation. Preincubation of factor VIII:C with thrombin in the presence of factor IXa, phospholipid vesicles, and calcium followed by addition of factor X results in a linear initial velocity of formation of factor Xa (Lollar & Fass, 1984). Prior to the study of factor VIII:C activation, preliminary studies were performed to partially characterize the kinetic properties of the intrinsic pathway factor X activator. The initial velocity dependence on the calcium and phospholipid concentrations is shown in Figures 1 and 2, respectively. These results are very similar to those obtained in the prothrombinase system using the same phospholipid preparation (Nesheim et al., 1979). The substrate dependence of the initial velocity of factor Xa formation using

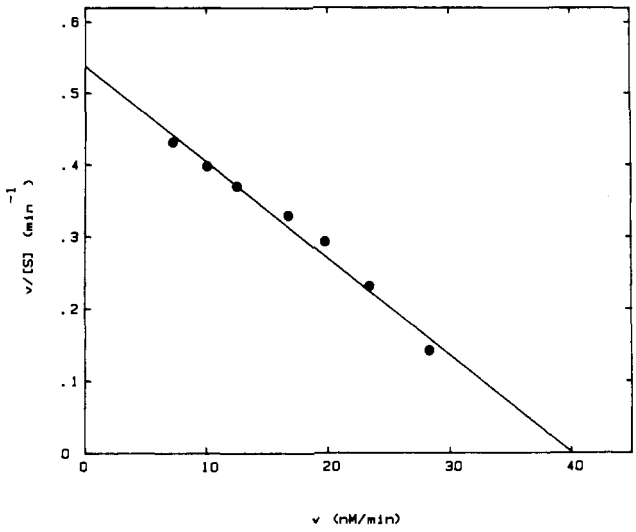


FIGURE 3: Eadie–Hofstee plot of steady-state kinetics of factor X activation. Initial rates of factor X activation were determined as described in Figure 1 at 5 mM $CaCl_2$, 24 μM PC/PS, 1.8 nM factor IXa, 1.2 nM thrombin, 0.05 unit/mL factor VIII:C, and factor X varying from 15 to 200 nM. The kinetic parameters were determined by a nonlinear least-squares fit to the equation for a rectangular hyperbola. The line is from linear regression analysis of the data.

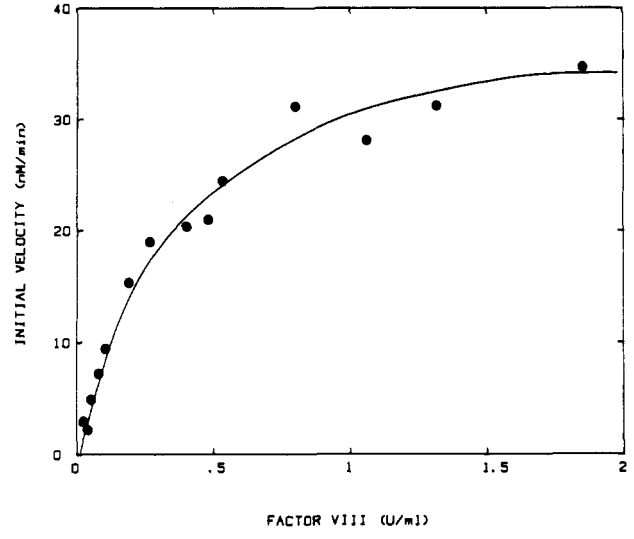


FIGURE 4: Factor VIII:C dependence of factor X activation. Initial rates of factor X activation were measured as in Figure 1 at 5 mM $CaCl_2$, 15 μM PC/PS, 0.24 nM factor IXa, 8 nM thrombin, and varying factor VIII:C. The factor X concentration was 0.16 μM. The curve is hand-drawn.

optimal calcium and phospholipid concentrations can be described by Michaelis–Menten kinetics (Figure 3). The K_m and V_{max} values under the conditions used are 40 ± 3 nM and 75 ± 5 nM/(min·units of factor VIII:C), respectively. When the factor IXa concentration is fixed and the factor VIII:C concentration is increased, at optimal concentrations of phospholipid and calcium there is a linear dependence of initial velocity and nominal factor VIII:C concentrations, and then the initial velocity saturates with respect to the concentration of the cofactor (Figure 4). The linear dependence of initial velocity at factor VIII:C concentrations below approximately 0.1–0.2 unit/mL is the basis for the assay of activated factor VIII:C used in this study.

Activation of Factor X in the Absence of Thrombin. When factor X is incubated with factor IXa, phospholipid, calcium, and factor VIII:C, the initial velocity of the reaction is less than 0.001 nM/min. After a 2-min lag phase, however, there

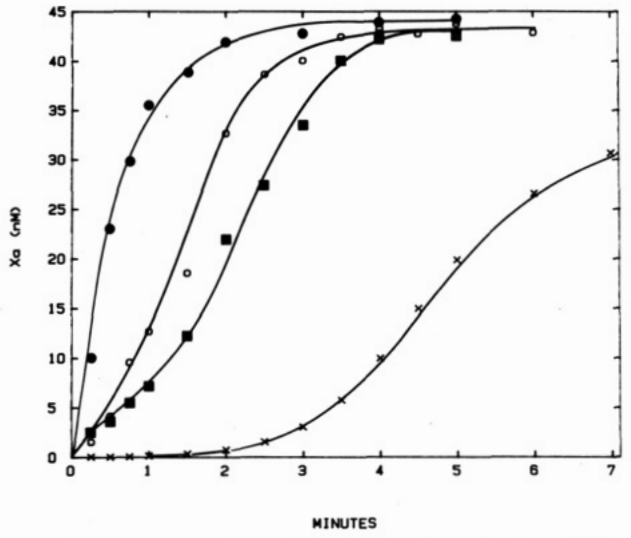


FIGURE 5: Time courses of factor X activation. The following additions were made to a solution containing TBS–PEG, 5 mM $CaCl_2$, 17 μM PC/PS, 2 nM factor IXa, 0.42 unit/mL unlabeled factor VIII:C, and trace ^{125}I-labeled factor VIII:C, 6.5×10^4 cpm/mL: none (×), 10 nM thrombin (●), 0.5 nM thrombin (○), or 2.6 nM factor Xa (■). Within 10 s factor X was added to a final concentration of 40 nm ($t = 0$). At various times samples were taken both for determination of factor Xa concentration and for SDS–PAGRE. The curves are hand-drawn.

is a progressive increase in factor X formation (Figure 5). Analysis by sodium dodecyl sulfate–polyacrylamide gel radioelectrophoresis (SDS–PAGRE) of samples taken from this reaction reveals that there is proteolysis of all ^{125}I-labeled factor VIII:C related peptides concomitant with formation of the factor X activator (Figure 6A). Structural studies of Factor VIII:C isolated from porcine plasma suggest that the starting material consists of three species consisting of a 76-kDa polypeptide chain that is associated with 166-, 130-, and 82-kDa polypeptide chains via a calcium-dependent linkage (Figure 6E) (Fass et al., 1982; Toole et al., 1984). This structure is preserved in ^{125}I-labeled factor VIII:C preparations with the exception of occasional additional heterogeneity in the 130–166-kDa region. Thrombin treatment of ^{125}I-labeled factor VIII:C in the absence of factor X or in the presence of factor X and soybean trypsin inhibitor, which inhibits factor Xa (Figure 6F), results in a cleavage pattern identical with that resulting from unlabeled porcine factor VIII:C (Fass et al., 1982). However, the products shown in Figure 6A are distinctly different from those for activation of factor VIII:C by thrombin.

This experiment suggests possible feedback activation of factor VIII:C by factor Xa. This is supported by the fact that no proteolysis of factor VIII:C is observed when the experiment is done in the presence of soybean trypsin inhibitor or factor Xa (Figure 6E). Factor Xa catalyzed activation of factor VIII:C is further supported by an experiment in which 2.5 nM factor Xa is added to the system. There is faster formation of factor Xa (Figure 5) coincident with formation of proteolysis products (Figure 6B) identical with those obtained in the absence of the exogenous addition of factor Xa (Figure 6A).

The rate of activation of factor VIII:C by 2.5 nM factor Xa is roughly comparable to the rate of activation by 0.55 nM thrombin (Figure 5). Analysis of the proteolysis products of factor VIII:C activated in the presence of 0.55 nM thrombin shows a pattern (Figure 6C) different from that for the activation of factor VIII:C by thrombin in the absence of ongoing factor Xa formation (Figure 6F). This is also seen when factor VIII:C is rapidly activated by 10 nM thrombin (Figures 5 and

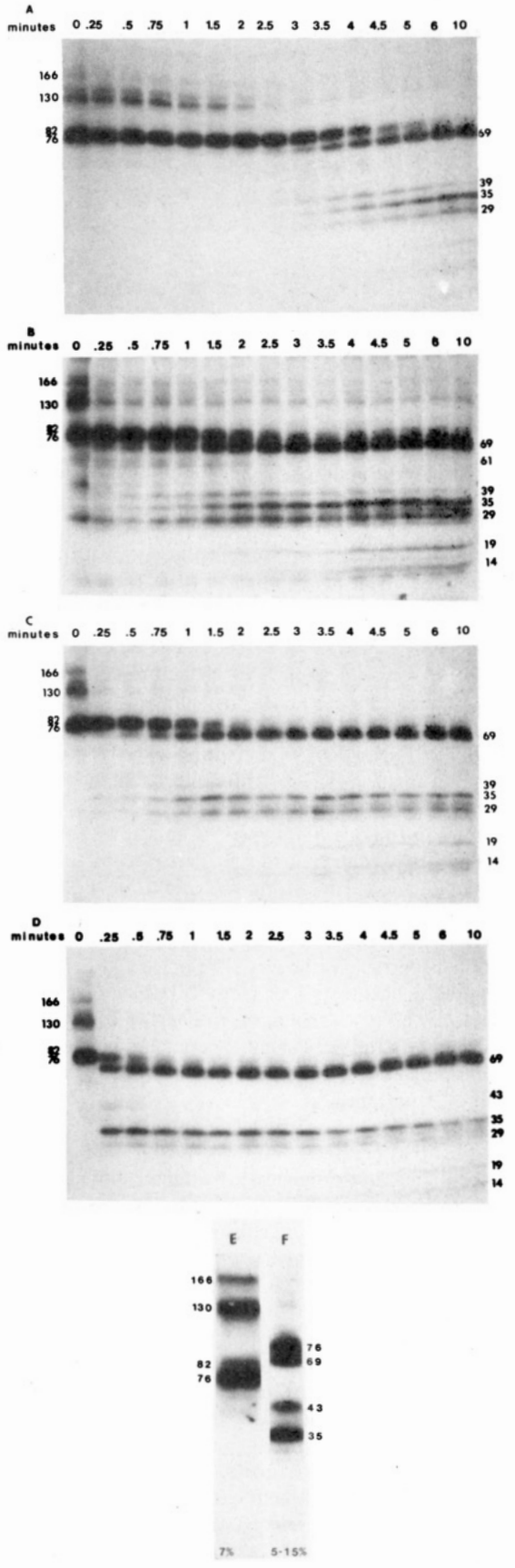


FIGURE 6: Time courses of ^{125}I-labeled factor VIII:C activation. SDS–PAGRE of samples from the experiment depicted in Figure 5 was done as described under Experimental Procedures. Exogenous protein addition consisted of the following: (A) no addition; (B) 2.6 nM factor Xa; (C) 0.55 nM thrombin; (D) 10 nM thrombin; (E) unactivated factor VIII analyzed by SDS–7% PAGE instead of the 5–15% gradient gels; (F) 10 nM thrombin and 50 μM soybean trypsin inhibitor for 5 min analyzed by SDS–5–15% PAGE.

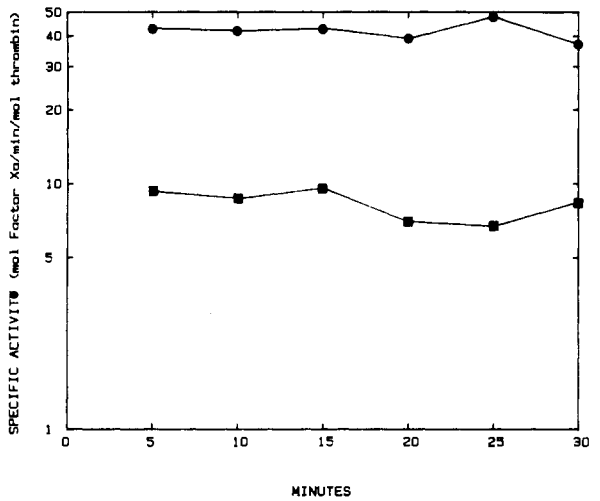


FIGURE 7: Activation of excess factor VIII:C by thrombin. Factor VIII:C, 20 units/mL ($\sim 2 \times 10^{-8}$ M), was activated by 0.2 (●) or 2 nM (■) thrombin in TBS–PEG, 15 μM PC/PS, and 50 nM factor IXa for various times. The mixture was then diluted 300-fold into TBS–PEG, 15 μM PC/PS, and 50 nM factor IXa to dilute the factor VIII:C to a measurable concentration. After a 2-min reequilibration period factor X was added to 300 nM, and the initial velocity was measured.

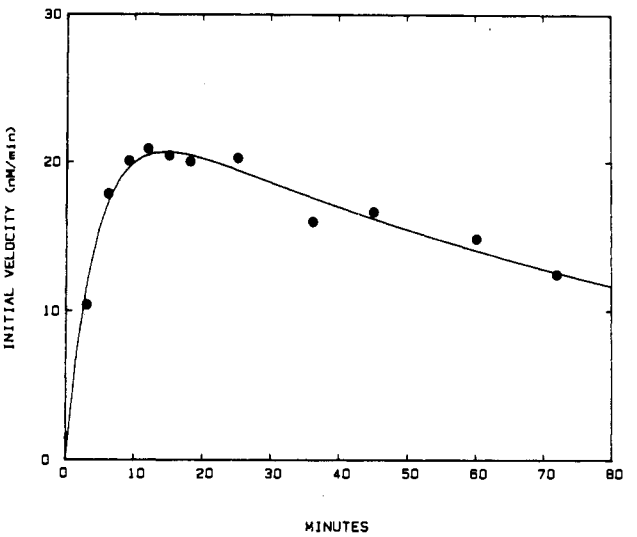


FIGURE 8: Activation of factor VIII:C by thrombin. Thrombin (0.55 nM) was mixed in TBS–PEG with 5 mM $CaCl_2$, 20 μM PC/PS, 1.5 nM factor IXa, and 0.06 unit/mL ($\sim$0.06 nM) factor VIII:C. At the indicated times the initial velocity of factor X activation by the solution was determined by adding one-tenth volume of factor X to a final concentration of 300 nM to an aliquot of the solution. The curve represents a computer-directed nonlinear least-squares fit of eq 5 to the data.

6D). These results suggest that thrombin-activated factor VIII:C is susceptible to further proteolysis by factor Xa and that the factor Xa activated product retains cofactor activity.

Kinetics of Factor VIII:C Activation. The relative rates of activation of factor VIII:C by thrombin vs. factor Xa and the relative activities of the activated cofactors were investigated by incubating the procofactor (0.06 unit/mL) with various concentrations of enzyme in the presence of factor IXa, phospholipid, and calcium for a variable time. Factor X was then added, and the initial velocity was measured over a 45-s interval. Under these conditions, with factor VIII:C at a limiting concentration, the initial velocity is proportional to the nominal concentration of factor VIII:C. Therefore, the initial velocity becomes an indirect measure of cofactor activity in the system. In the subsequent experiments, cofactor activity was determined under conditions in which factor VIII:C was less than 0.1 unit/mL.

We have developed a kinetic model (Scheme I, Experimental Procedures) that predicts simultaneous first-order production of cofactor and first-order loss of cofactor activity. In this model, a Michaelis complex (EA) is formed between enzyme (thrombin or factor Xa) and substrate (factor VIII:C). The enzyme then turns over during a proteolytic event in which factor VIII:C is modified to become an active species (X). This step is assumed to be equivalent to a typical acylation/deacylation event in the course of a reaction catalyzed by a serine protease and is kinetically evident as a single step governed by the catalytic rate constant k_2. Following activation, factor VIII:C undergoes spontaneous first-order inactivation to species Y.

Another description (Scheme II, Experimental Procedures) was also considered. In this model, thrombin forms a stoichiometric complex with proteolytically modified factor VIII:C (EX) which is the active species. This species then undergoes inactivation by dissociation or some other first-order process (e.g., a conformational change of either thrombin or modified factor VIII:C). This model is consistent with the observation (Figure 6) that generation of cofactor activity is paralleled by proteolysis of the factor VIII:C molecule. It also yields the same biexponential form as Scheme I. However, the stoichiometric nature of the active species proposed in Scheme II results in predictions that are not supported by experiment. Figure 7 depicts the results of an experiment in which factor VIII:C is activated by thrombin under conditions of excess substrate. In this figure the specific activity, defined as the ratio of the initial velocity (or cofactor activity) to thrombin concentration, is plotted vs. time. According to Scheme II, the initial velocity should peak at a level dictated by the limiting thrombin concentration. However, by dividing through by the thrombin concentration, the specific activity of the putative stoichiometric complex should not change. Figure 7 shows, however, that the specific activity increases approximately 5-fold with a 10-fold reduction in thrombin concentration, which contradicts Scheme II. Another way of stating this result is that factor VIII:C has similar activatability at the two different thrombin concentrations. This is consistent with (but does not prove) Scheme I, in which there is catalysis and turnover by thrombin.

Figure 8 shows an example of a theoretical fit according to Scheme I to a time course of activation of procofactor by 0.55 nM thrombin by obtaining the optimal estimates of the parameters in eq 5 (Experimental Procedures) with a nonlinear least-squares method. In all experiments there was no evidence of correlation between parameters. Under identical conditions factor VIII:C was activated by 40 nM thrombin (a 70-fold higher concentration than in Figure 8). This allows very rapid activation of factor VIII:C so that the time course of inactivation can be followed without the complication of ongoing activation (Figure 9). The inactivation rate constant (k_3) in this experiment is 0.022 ± 0.004 min^{-1}, which is similar to the value of 0.017 ± 0.001 min^{-1} in Figure 8. This result is consistent with the kinetic model in which loss of cofactor activity is independent of thrombin concentration. Shown in Figure 10 is a theoretical fit to the activation of factor VIII:C by 1.1 nM factor Xa. The curve suggests that activation by factor Xa is slower and that the maximum obtainable cofactor activity is lower.

In order to assess this suspicion more quantitatively, further interpretation of the kinetic parameters is necessary. Equation 5 (Experimental Procedures) predicts that, under conditions in which the nominal enzyme concentration is far below the

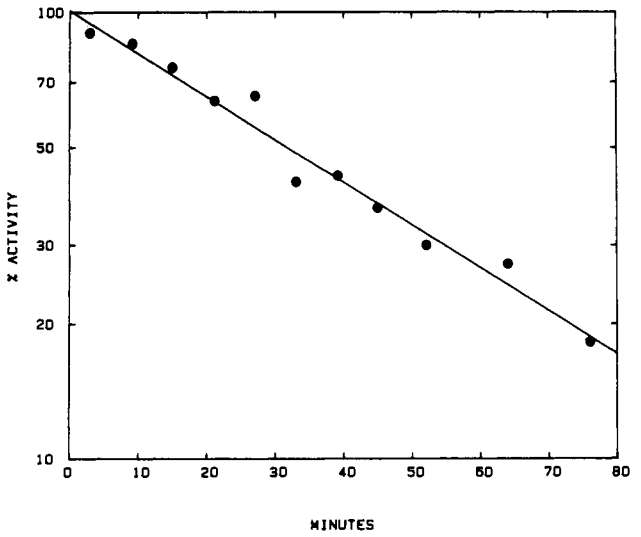


FIGURE 9: Measurement of the inactivation rate constant, k_3. The experiment was carried out as in Figure 8 except that 40 nM thrombin was used. The line represents a nonlinear least-squares fit to eq 5 at infinite k_2.

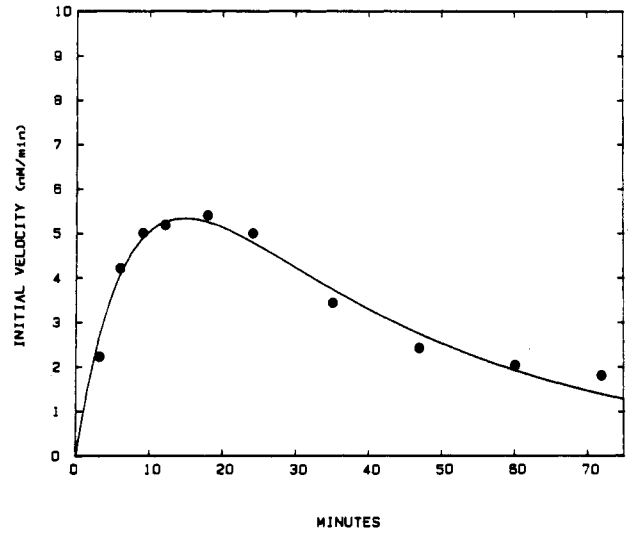


FIGURE 10: Activation of factor VIII:C by factor Xa. The experiment was carried out as in Figure 8 except that 1.1 nM factor Xa was substituted for thrombin.

dissociation constant (K_s) for the enzyme–substrate complex, there will be no change in parameter b_0 and a linear increase in parameter b_1 with enzyme concentration. Table I lists values of b_0, b_1, and b_2 as a function of enzyme concentration for one experiment. There is little, if any, change in b_0 over a 4–6-fold increase in enzyme concentration, but b_1 increases 3–4-fold. Therefore, if the enzyme concentrations used are assumed to be well below the dissociation constant for the enzyme–substrate complex, the slope of a linear regression of b_1 vs. enzyme concentration is a measurement of the catalytic efficiency (k_{cat}/K_s) of the enzyme. The results of this calculation from the data in Table I are shown in Table II (experiment 3). Additionally, calculations from three other experiments using two other factor VIII:C preparations are shown. From these calculations it is estimated that the catalytic efficiency of thrombin toward factor VIII:C is approximately 5-fold greater than that of factor Xa toward factor VIII:C.

Similarly, under conditions of enzyme concentration below the dissociation constant for the enzyme–substrate complex, b_0 becomes a measurement of the maximum obtainable cofactor activity (i.e., the cofactor activity that would be obtained if no inactivation occurred). Table II lists the relative cofactor

Table I[a]

	b_0 (arbitrary units)	b_1 (min^{-1})	b_2 (min^{-1})
thrombin (nM)			
0.55	29.2 ± 2.1	0.25 ± 0.05	0.012 ± 0.003
0.75	36.1 ± 1.1	0.31 ± 0.03	0.012 ± 0.001
1.1	37.9 ± 1.5	0.70 ± 0.18	0.012 ± 0.001
2.2	37.4 ± 1.0	0.61 ± 0.10	0.012 ± 0.001
	35.2 ± 4.2[b]		
factor Xa (nM)			
1.1	12.1 ± 3.0	0.092 ± 0.038	0.015 ± 0.007
2.2	13.1 ± 0.4	0.22 ± 0.02	0.015 ± 0.001
4.4	16.3 ± 1.3	0.21 ± 0.05	0.015 ± 0.003
6.6	16.4 ± 0.9	0.34 ± 0.06	0.015 ± 0.002
	14.4 ± 2.2[b]		

[a] Experiment 3, Table II. Errors represent standard deviations estimated from least-squares fits. [b] Mean and sample standard deviation.

Table II

expt	factor VIII preparation	rel cofactor act. ($b_{0,IIa}/b_{0,Xa}$) IIa	Xa	catalytic efficiency $k_2/K_s \times 10^{-6}$ (M^{-1} s^{-1})[a] IIa	Xa
1	1	3.4		6.7 ± 0.58	1.4 ± 0.5
2	1	3.1		5.5 ± 0.15	0.92 ± 0.07
3	2	2.4		4.8 ± 1.8	0.75 ± 0.2
4	3	2.9		3.2 ± 0.5	1.4 ± 0.2
		3.0 ± 0.4[b]		5.0 ± 1.5[b]	1.1 ± 0.3[b]

[a] Slope and standard deviation of a linear regression plot of b_1 vs. enzyme concentration. [b] Mean and sample standard deviation, $n = 4$.

activity ($b_{0,IIa}/b_{0,Xa}$) of the two species of factor VIII:C and indicates that factor VIII:C is 3 times more activatable by thrombin than by factor Xa.

When porcine factor IXa is incubated with factor VIII:C at a concentration of 2 nM for 10 min, there is no evidence of factor VIII:C activation in the chromogenic substrate assay or by SDS–PAGRE. This result indicates that the catalytic efficiency of factor IXa toward factor VIII:C is less than 1 × 10^4 M^{-1} s^{-1}.

Discussion

In this study we have examined, in a model phospholipid vesicle system, the relative potency of thrombin and factor Xa as activators of porcine factor VIII:C and the relative activities of the two cofactors (factor VIII:Ca_{Xa} and factor VIII:Ca_{IIa}). To do this, a rapid, plasma-free assay using an automated spectrophotometer was developed. Preliminary studies were performed to partially characterize the kinetic properties of the porcine intrinsic pathway factor X activator in order to define the experimental conditions necessary for optimal study of factor VIII:C activation. Figure 1 shows that, at the given experimental conditions, the sigmoidal calcium dependence of the factor X activator is similar to that shown in studies involving prothrombinase (Nesheim et al., 1979; Rosing et al., 1980) and the bovine factor X activator (additionally containing Willebrand factor; van Dieijen et al., 1981) and underscores the similarity of the two enzymatic systems. The phospholipid dependence of the factor X activator, under the conditions of the experiment, has an optimum at approximately 20 μM and decreases at higher concentrations, which is also similar to results reported for prothrombinase and the bovine factor X activator. Steady-state kinetics of the substrate dependence of the reaction result in a hyperbolic dependence

of the initial velocity on substrate concentration and are represented as an Eadie–Hofstee plot in Figure 3. The kinetic parameters, K_m and V_{max}, are 40 nM and 75 nM/(min·units of factor VIII:C), respectively. These values are obviously dependent on phospholipid, factor IXa, factor VIII:C, and calcium but emphasize that the intrinsic pathway factor X activator, at fixed concentrations, can be considered to have functional properties similar to a classic enzyme without allosterism, hysteresis, or substrate/product inhibition. The dependence of the reaction rates on individual components (e.g., factor VIII:C) can be exploited to produce convenient and accurate assays. For example, in the experiment summarized in Figure 4 the steady-state initial rate of factor X activation is plotted as a function of factor VIII:C. The rate is linear under the conditions used at factor VIII:C concentrations below 0.1–0.2 unit/mL and then saturates with respect to the cofactor concentration. This phenomenon has been explored in the prothrombinase system (Nesheim et al., 1979) and factor X activation systems (Griffith et al., 1982; van Dieijen et al., 1983) to infer binding interactions of enzyme and cofactor on the lipid surface and to measure cofactor activities. For our purposes we have studied activation of factor VIII:C below 0.1 unit/mL and used the steady-state determinations as a measure of cofactor activity.

Using this technique, we have evaluated the activation of factor VIII:C by thrombin and factor Xa quantitatively. It has been suggested that the activation by factor Xa occurs by a similar mechanism as activation by thrombin on the basis of enzyme inhibitor effects on the kinetics of activation (Hultin & Jesty, 1982). In Figures 5 and 6 data are represented that summarize several observations regarding the activation of factor VIII:C. When factor X is added to a system containing lipid, calcium, factor IXa, and factor VIII:C but no exogenous enzyme, factor X activation is unmeasurable for approximately 2 min under the conditions used. This is followed by rapid development of functional factor X activator, which is temporally related to proteolysis of the factor VIII:C molecule. The results in Figure 5 are similar to those reported by other investigators using factor VIII preparations with (Griffith et al., 1982; Hultin, 1982) and without (Vehar & Davie, 1980) Willebrand factor. These findings are not consistent with findings of van Dieijan et al. (1981), who did not find that factor Xa activated factor VIII.

By use of ^{125}I-labeled factor VIII:C, it is possible to observe changes in the factor VIII:C molecule during the activation process. In the absence of exogeneous enzyme, the cleavage products (Figure 6A) are different from those previously reported for the thrombin-catalyzed reaction in the porcine system (Fass et al., 1982; Lollar et al., 1984) and reproduced in this study by studying thrombin-catalyzed activation of factor VIII:C in the presence of factor X and soybean trypsin inhibitor (Figure 6F) or in the absence of factor X (Lollar et al., 1984). The cleavage products in Figure 6A are identical with those that result from the activation of factor VIII:C by exogenous factor Xa (Figure 6B). These results differ from the observations of Vehar and Davie, who reported identical cleavage patterns of bovine factor VIII:C by thrombin and factor Xa. Coupled with the observation that no proteolysis of the factor VIII:C molecule is seen when soybean trypsin inhibitor is added to the system in the absence of exogenous factor Xa (Figure 6E), this strongly suggests that the activation of factor VIII:C in the absence of exogenous enzyme results from feedback activation by factor Xa. Although factor IXa is included in the system in this study, it does not appear to catalyze factor VIII:C activation since it does not shorten the lag phase seen in Figure 5 despite prolonged incubation (10 min) with factor VIII:C. This is consistent with earlier work involving the activation of factor VIII:C by factor IXa in which much higher concentrations of factor IXa then were used in this study were required to obtain measurable factor VIII:C activation (Rick, 1982). Interestingly, when factor VIII:C is reacted with thrombin in the presence of factor X, a new cleavage pattern appears (Figure 6C,D). Generation of these novel peptides is prevented by soybean trypsin inhibition (Figure 6F). These results suggest that thrombin-activated factor VIII:C can undergo further proteolysis by factor Xa. At this point the properties of this modified factor VIII:C have not been explored. Conceivably, the modification could be a control mechanism in view of the observation that factor Xa and thrombin appear to produce functionally different cofactors (see below).

Currently, the initiation of the feedback activation of factor VIII:C in the absence of exogenous enzyme is poorly understood. Similar questions exist regarding initial activation of factor V in the prothrombinase complex (Nesheim et al., 1979) and initial activation of factor VII in the extrinsic pathway factor X activator (Radcliffe & Nemerson, 1976; Zur & Nemerson, 1980; Zur et al., 1982). From these studies it has been proposed that "unactivated" factor VII and factor V possess intrinsic activity that can initiate events that lead to amplification by positive feedback. A similar situation may exist for factor VIII:C; however, trace contamination with activated factor VIII:Ca or factor VIII:C activating proteases would give similar results. Alternatively, factor V initially may be activated by factor Xa, which then leads to positive feedback by thrombin (Foster et al., 1983). Considering an analogous situation, factor VIII:C, however, does not appear to be activated by factor IXa under the conditions of the experiment in Figures 5 and 6 since preincubation of factor VIII:C with factor IXa, lipid, and calcium does not shorten the lag phase (data not shown).

The data in Figure 5 indicate that factor Xa activates factor VIII:C more slowly than thrombin. The activation kinetics were studied more quantitatively by developing a kinetic model. Two similar models were considered. The model shown in Scheme II previously has been suggested as a possibility for factor VIII:C activation (Switzer & McKee, 1978; Hultin & Jesty, 1982). The model shown in Scheme I was used for further study of this system since Scheme II was found to be inconsistent with data summarized by Figure 7 (Results).

Also included in Scheme I is the spontaneous inactivation of factor VIII:Ca. This labile nature of the cofactor has been observed in many systems (Cooper et al., 1975; Rick & Hoyer, 1977; Vehar & Davie, 1980; Hoyer & Trabold, 1981; Hultin & Jesty, 1982). It does not appear to be further proteolysis of the molecule since inhibitors of thrombin and factor Xa do not stabilize the molecule (Hultin & Jesty, 1982). Additionally, ongoing proteolysis is not seen on SDS–PAGRE of the ^{125}I-labeled factor VIII:C activation products (Lollar et al., 1984). Therefore, the inactivation step is modeled as a first-order process. Factor VIII:Ca is markedly stabilized by lipid and factor IXa, presumably because incorporation of cofactor into the enzymatic assembly results in a stable conformation (Lollar et al., 1984, Lollar & Fass, 1984). In this study the activation of factor VIII:C by Factor Xa and thrombin was done under conditions that stabilized the cofactor. This faciliated the analysis of the system by expanding the independent variable (time) of the reaction.

The experiments were conducted under conditions in which the enzyme concentration was at least 10 times the estimated

Table III

substrate	catalytic efficiency $k_{cat}/K_m \times 10^{-6}$ (M^{-1} s^{-1})	reference[a]
	Thrombin	
fibrinogen Aα:Bβ	11.6	Higgins et al., 1983
factor V	nr[b]	
factor VIII	5.0	Table II
protein C (+Ca^{2+})	0.003	Esmon et al., 1983
protein C (+Ca^{2+}, soluble thrombomodulin)	0.51	Esmon et al., 1983
antithrombin III	0.007	Jordan et al., 1980
antithrombin III (+heparin)	5.0	Jordan et al., 1980
heparin cofactor II	0.08	Tollefson et al., 1982
heparin cofactor II (+heparin)	7.5	Tollefson et al., 1982
α-2 macroglobulin	0.0001	Downing et al., 1978
S2238	60	Lottenberg et al., 1981
	Factor Xa	
prothrombin (+lipid, factor Va, Ca^{2+})	35	Nesheim & Mann, 1983
antithrombin III	0.003	Jordan et al., 1980
antithrombin III (+heparin)	4.0	Jordan et al., 1980
factor V	nr[b]	
factor VIII	1.1	Table II
S2222	0.9	Lottenberg et al., 1981

[a] Values are in some cases calculated from the data. Examples chosen are not meant to include all studies. [b] nr, not reported.

concentration of the procofactor. This facilitates the analysis of the model so that an analytical solution to the problem may be obtained. This approach may be physiologically relevant since enzyme concentrations in vivo may exceed the trace amounts of factor VIII:C that apparently exist. Additionally, by use of excess enzyme the entire course of the activation/inactivation process can be analyzed. Enzyme kinetics under conditions of excess enzyme have been studied extensively, and models very similar to those considered here can be found (Laidler & Bunting, 1973).

Theroetical fits of eq 5 to the data for activation of factor VIII:C by 0.55 nM thrombin and 1.1 nM factor Xa are shown in Figures 8 and 10, respectively. Intuitively, it appears that thrombin activates factor VIII:C more rapidly and to a greater extent than does factor Xa. This is supported by interpretation of the kinetic parameters b_0 and b_1 of eq 5 as outlined under Results. By varying the enzyme concentration (Table I), we calculated the catalytic efficiency of the enzyme (Table II). From calculations involving four experiments the catalytic efficiency of thrombin is seen to be 5 times greater than that of factor Xa in this system. Similarly, by averaging b_0 values from the results with different enzyme concentrations (Table I) and taking the ratio of values obtained with thrombin vs. factor Xa (Table II), we determined the relative cofactor activity of the different factor VIII:Ca molecules. From this calculation, it is estimated that factor VIII:C is 3 times more activatable by thrombin than factor Xa.

It is interesting to compare the catalytic efficiencies of thrombin and factor Xa toward factor VIII:C with literature values of coagulation enzyme catalyzed reactions (Table III). From the data it appears that the activation of factor VIII:C by factor Xa is relatively fast. Since factor Xa formation precedes thrombin formation during coagulation, it is possible that it is the predominant activator of factor VIII:C in vivo.

It should be emphasized that these results are obtained in a model lipid system. The platelet may support qualitatively and/or quantitatively different reactions. Additionally, the role of factor Va in the factor Xa catalyzed activation of factor VIII:C has not been evaluated. Conceivably, it could sequester factor Xa from the factor VIII:C molecule. Alternatively, formation of the complete prothrombinase complex could result in a more potent activator of factor VIII:C.

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Registry No. Ca, 7440-70-2; factor VIII, 9001-27-8; factor Xa, 9002-05-5; factor X, 9001-29-0; thrombin, 9002-04-4.

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Comparison of Lipid Binding and Kinetic Properties of Normal, Variant, and γ-Carboxyglutamic Acid Modified Human Factor IX and Factor IX_a†

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ABSTRACT: The abilities of normal and three abnormal factor IX_a molecules to activate factor X and to bind to phospholipid membranes have been compared to define the contributions of protein–lipid interactions and factor IX_a light chain–heavy chain interactions to the functioning of this protein. The abnormal proteins studied had altered amino acid residues in their light chains. The heavy-chain regions, containing the active site serine and histidine residues, were normal in the abnormal proteins on the basis of titration by antithrombin III. The binding constants (K_d) for normal (N), variant [Chapel Hill (CH) and Alabama (AL)], and γ-carboxyglutamic acid (Gla) modified (MOD) factors IX and IX_a to phosphatidylserine (PS)/phosphatidylcholine (PC) small, unilamellar vesicles (SUV) were measured by 90° light scattering. The K_d values for factor IX_N binding were quite sensitive to the PS content of the membrane but less sensitive to Ca^{2+} concentrations between 0.5 and 10 mM. The zymogen and activated forms of both normal and abnormal factor IX bound with similar affinities to PS/PC (30/70) SUV. In the cases of factor IX_{aN} and factor IX_{aAL}, but not factor IX_{aCH} or factor IX_{aMOD}, irreversible changes in scattering intensity suggested protein-induced vesicle fusion. Since the activation peptide is not released from factor IX_{aCH}, the normal interaction of factor IX_a with a membrane must require the release of the activation peptide and the presence of intact Gla residues. The rate of factor X activation by normal and abnormal factor IX_a was obtained by using a chromogenic substrate for factor X_a in the presence of PS/PC (30/70) SUV and 5 mM Ca^{2+}. A comparison of the relative amount of surface-bound factor IX_a (calculated with the measured K_d values) and the relative rates of factor X activation indicated that only in the case of factor IX_{aMOD} did decreased lipid binding account for decreased activity of the abnormal proteins. Because the structural alterations of the variant proteins are in the light chain and because decreased lipid affinity did not account for decreased activity, results suggest that the proper functioning of factor IX_a must entail interactions between the light and heavy chains on the phospholipid surface.

Human factor IX is a vitamin K dependent glycoprotein that, during the process of blood coagulation, is activated in the presence of Ca^{2+} by factor XI_a and/or factor VII_a and tissue factor (Bajaj et al., 1983). In turn, activated factor IX (factor IX_a)[1] catalyzes the activation of factor X, with Ca^{2+}, factor $VIII_a$, and a negatively charged phospholipid surface serving as cofactors (van Dieijen et al., 1981). The complete activation of human factor IX to factor IX_a involves the cleavage of two specific peptide bonds (see Figure 1). These two proteolytic events result in the release of an activation peptide and in the appearance of light and heavy peptide chains linked by a disulfide bridge (Fujikawa et al., 1974; Østerud

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[1] Abbreviations: factor IX_a, activated factor IX; factor IX_{CH}, factor $IX_{Chapel\ Hill}$; factor IX_{AL}, factor $IX_{Alabama}$; factor IX_{MOD}, Gla-modified factor IX; factor IX_N, normal factor IX; PS, bovine brain phosphatidylserine; PC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; Gla, γ-carboxyglutamic acid; SUV, small unilamellar vesicles; TES, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PEG, poly(ethylene glycol); TEA, triethanolamine; Tos-Gly-Pro-Arg-NA, N^{α}-*p*-tosylglycyl-L-prolyl-L-arginine-*p*-nitroanilide.

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