Assembly of the intrinsic factor X activating complex

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Assembly of the Intrinsic Factor X Activating Complex – Interactions between Factor IXa, Factor VIIIa and Phospholipid

Gerbrand van Dieijen, Jan L. M. L. van Rijn, José W. P. Govers-Riemslag, H. Coenraad Hemker and Jan Rosing

From the Department of Biochemistry, University of Limburg, Maastricht, The Netherlands

Key words

Intrinsic factor X activation – Factor VIII – Kinetics and mechanism – Binding parameters

Summary

The activation of blood coagulation factor X by factor IXa is strongly stimulated by the non-enzymatic cofactors phospholipid, Ca^{2+} and activated factor VIII. In this paper we present a method by which we were able to determine binding affinities of factor IXa for phospholipids (either in the absence or presence of factor VIIIa) from kinetic measurements of factor X activation. It is shown that rates of factor X activation in the presence of phospholipids can be saturated with an excess factor VIIIa at limiting amounts of factor IXa and vice versa. Our data indicate that the enzymatic unit in the intrinsic factor X activator is a 1:1 stoichiometrical complex of factor IXa and factor VIIIa bound to phospholipid. Titrations with factor IXa at fixed concentrations of phospholipid and factor X show that the apparent dissociation constant of factor IXa for phospholipid is lowered from 10^{-6} M to 10^{-8} M by the presence of factor VIIIa. We conclude, that in analogy with the role of factor Va in prothrombin activation, phospholipid-bound factor VIIIa functions as a high-affinity binding site (»receptor«) for factor IXa in the intrinsic factor X activating complex. Therefore, factor VIIIa increases the observed Vmax of factor X activation by 1) enhancing the k_{cat} of the reaction and 2) increasing the amount of phospholipid-bound factor IXa that participates in factor X activation.

Introduction

In the intrinsic pathway of the blood coagulation cascade the zymogen factor X is converted into factor Xa by the serine protease factor IXa (For a review see ref. 1). The rate of factor Xa formation is strongly increased by the presence of negatively charged phospholipids, Ca^{2+} ions and activated factor VIII (For a review on factor VIII see ref. 2). It is thought that during factor X activation, factor IXa and VIIIa are associated in a complex adsorbed at the phospholipid surface. This complex is called the intrinsic factor X activator or the intrinsic factor X activating complex (3–7). A detailed understanding of the mode of action of the intrinsic factor X activator ultimately requires quantification of the interactions between the individual components. Binding parameters, i. e. dissociation constants and number of binding sites, have been determined for interaction of factors IXa and X with phospholipid vesicles of varying composition (8, 9). Until now no data are available, however, on interactions that involve factor VIII.

Correspondence to: Dr. J. Rosing, Department of Biochemistry, Biomedical Centre, Beeldsnijdersdreef 101, 6216 EA Maastricht, The Netherlands

The organisation of the components of the factor X activating complex as postulated above strongly resembles the structure of the prothrombinase complex (3–10). Data on the interactions between the components of the latter complex have been obtained by several methods. One technique was to infer binding parameters from the kinetics of prothrombin activation (11, 12).

The binding data presented in this paper, which were derived from kinetic studies of factor X activation, provide evidence that a factor IXa-factor VIIIa-phospholipid complex is the catalytic unit in factor X activation and further indicate that the incorporation of factor IXa in this enzyme complex is strongly promoted by activated factor VIII.

Materials and Methods

Materials

S2337 (Bz-Ile-Glu[Piperidyl]-Gly-Arg-p-nitroanilide) was purchased from Kabi AB, Sweden; Sephacryl-S300 was from Pharmacia Sweden. Phosphatidylcholine ($18:1_{cis}/18:1_{cis}$-phosphatidylcholine) was from Sigma Chemicals, St. Louis, USA; Sphingomyelin was from Koch-Light, England. All reagents were of highest grade available supplied by Merck, Darmstadt, Germany and by Sigma Chemicals, St. Louis, USA.

Phospholipids and Phospholipid Vesicles

Phosphatidylethanolamine and phosphatidylserine (both $18:1_{cis}/18:1_{cis}$) were prepared by enzymatic synthesis (13). Phospholipid vesicles were prepared by sonication in a buffer containing 50 mM TrisHCl and 175 mM NaCl pH 7.9 as described before (10). The composition of the vesicles was: 25% phosphatidylserine, 28% phosphatidylethanolamine, 19% sphingomyelin, 28% phosphatidylcholine and cholesterol in a 1:1 molar ratio with respect to the total phospholipid concentration.

Proteins

Bovine factors X_2, IXa and thrombin were prepared as described previously (6). Factor VIII:C was purified from bovine blood using the method of Vehar and Davie (14), modified as follows. After chromatography on sulfatesepharose we omitted the factor X-sepharose step because factor VIII failed to bind to this column. The final purification step for factor VIII consisted of gelfiltration on Sephacryl-S300. Factor VIII eluted as a broad peak corresponding to the elution position of bovine catalase (M_r 232,000). Analysis of the column fractions by SDS-polyacrylamide gelelectrophoresis followed by silver staining showed two predominant bands at 250,000 and 74,000. Factor VIII stock solutions were dialyzed against a buffer containing 10 mM morpholinoethane sulfonic acid, 175 mM NaCl, 10 mM $CaCl_2$, 10% glycerol, adjusted to pH 6.5 with 1 M Tris. Factor VIII was diluted in the same buffer containing 10 mg/ml human albumin.

Activation of Factor VIII

Factor VIII was activated by thrombin. Optimal conditions were determined as described before by direct measurement of the effect of factor VIIIa on the rate of factor X activation (6). In a typical experiment factor VIII was activated for 1 min at 37° C and pH 7.9 at a thrombin concentration of 3.65 nM (130 ng/ml, 0.36 NIH-units/ml).

Assay System for Measurement of Factor X Activation

Factor IXa and phospholipid vesicles were prewarmed at 37° C in 0.3 ml of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 10 mM $CaCl_2$ (pH 7.9). Factor X activation in the absence of factor VIIIa was started by addition of 0.2 ml prewarmed factor X. After 1 min and 2 min samples were taken and assayed for factor Xa using the chromogenic substrate S2337 as described before (6). When factor VIIIa was present the activation of factor X was started by the addition of 0.1 ml factor X and 0.1 ml activated factor VIII. Samples were taken 45 s and 90 s after starting the reaction. Both in the presence and absence of factor VIIIc the formation of factor Xa was linear with time for at least 3 min.

Quantification of Factor VIII

The concentration of factor VIII was determined by a one-stage clotting assay using factor VIII deficient plasma and was expressed in units/ml, assuming the presence of 1 unit/ml of factor VIII in plasma pooled from 10 cows. The purified factor VIII preparation had a specific activity of 1600–3000 units/mg in a clotting assay, assuming that $A^{1\%}_{280\ nm} = 10.0$. It is uncertain which of the bands in the factor VIII:C preparation as seen on SDS-gels are associated with the procoagulant activity. Therefore the concentration of active factor VIII:C cannot be determined from the absorbance at 280 nm. The results presented in this paper show that the intrinsic factor X activator consists of a 1:1 stoichiometrical complex of phospholipid-bound factor IXa with phospholipid-bound factor VIIIa. This enables the determination of the molar concentration of factor VIIIa on the basis of its activity in factor X activation. The method involves saturation of a limiting known amount of factor IXa with factor VIIIa, followed by saturation of a limiting unknown amount of factor VIIIa with factor IXa. When υ (VIIIa) and υ (IXa) are the rates of factor X activation determined for a known concentration of factor IXa saturated with factor VIIIa and for an unknown amount of factor VIIIa saturated with factor IXa, respectively, the molar factor VIIIa concentration can be calculated from the equation:

$$\frac{\upsilon\ (IXa)}{[VIIIa]} = \frac{\upsilon\ (VIIIa)}{[IXa]}$$

Results

Derivation of Binding Parameters from Kinetic Measurements

In previous studies (6) we have demonstrated that the rate of activation of factor X by factor IXa is increased in 3 orders of magnitude by negatively charged phospholipids and that the presence of the non-enzymatic protein cofactor VIIIa causes an additional 200,000-fold rate enhancement. Kinetic analysis (6) revealed that factor VIIIa increases the Vmax of factor X activation whereas the presence of a phospholipid surface decreases the Km for factor X. These effects of accessory components on the kinetic parameters of factor X activation do explain the above mentioned rate enhancements. The drop of the Km in the presence of phospholipids is explained in a model in which phospholipids promote the formation of the enzyme-substrate complex by binding both factor IXa and factor X. The factor X and factor IXa concentrations in the presence of phospholipids can be chosen such that only factor IXa molecules which are incorporated in either factor IXa-phospholipid- or factor IXa-factor VIIIa-phospholipid complexes (factor IXa_{bound}) contribute to the rate of factor X activation (V). In that case

$$V = c.[IXa]_{bound} \qquad (I)$$

where c is a proportionality factor determined by the kinetic parameters of factor X activation. The dissociation constant Kd for dissociation of factor IXa from kinetically functional binding sites is given by

$$Kd = \frac{[IXa]_{free} \cdot ([sites]_{total}-[IXa]_{bound})}{[IXa]_{bound}} \qquad (II)$$

Table 1 Assembly of the intrinsic factor X activating complex in absence and presence of factor VIIIa at varying phospholipid and factor X concentrations

Components in reaction mixture		Absence of factor VIIIa		Presence of factor VIIIa	
Factor X (μM)	Phospholipid (μM)	Kd^{app} factor IXa (nM)	υ (IXa) (nM Xa/min)	Kd^{app} factor IXa (nM)	υ (IXa) (nM Xa/min)
0.5	10	370	2.8	6.7	9.1
0.5	25	770	10.4	10.0	11.4
0.5	50	1110	23.2	16.1	12.3
0.2	50	830	14.3	5.1	7.2
0.05	50	480	4.2	3.0	4.1

The reaction conditions were as described in Fig. 1. The concentration of factor VIIIa, when present, was 0.017 nM. υ (IXa) is the rate of factor X activation at infinitely high concentration of factor IXa. Kd^{app} is the factor IXa concentration required to obtain 0.5 υ (IXa).

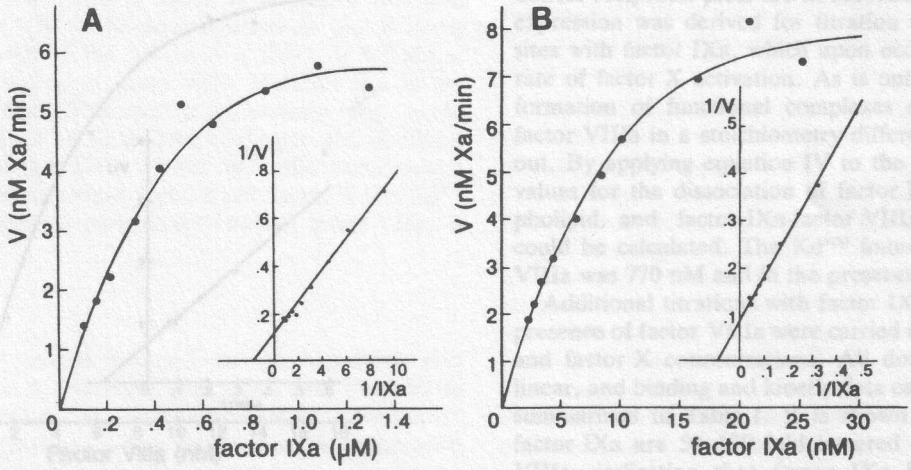

Fig. 1. Effect of factor IXa on the rate of factor X activation in the absence and presence of factor VIIIa. Factor IXa and phospholipid vesicles were prewarmed at 37° C in 0.3 ml of a buffer containing 50 mM Tris-HCl, 175 mM NaCl, 10 mM $CaCl_2$, pH 7.9. Factor X activation was started by addition of 0.2 ml prewarmed factor X (panel A) or 0.1 ml of activated factor VIII plus 0.1 ml of factor X (panel B). After 60 s and 120 s (A) or 45 s and 90 s (B) samples were taken and assayed for factor Xa as described in Experimental Procedures. The final composition of the reaction mixtures was: factor IXa as indicated, 25 μM phospholipid, 0.5 μM factor X, 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 6 mM $CaCl_2$, 0.5 mg/ml human albumin. The composition of the reaction mixtures of the experiments shown in panel B were identical to panel A, except for the presence in B of thrombin-activated factor VIII in a final concentration of 0.012 nM (0.011 units/ml). The insets depict double-reciprocal plots of the titration curves.

Table 2 Factor IXa titrations at four different factor VIIIa concentrations

Factor VIIIa	Kd^{app}	υ (IXa)	υ (IXa)/[VIIIa]
(nM)	(nM)	(NM Xa/min)	(nM Xa/min per nM VIIIa)
0.0125	16.7	12.3	984
0.033	17.8	28.2	855
0.0375	14.0	35.2	939
0.1	16.0	92.6	926

The Kd^{app} and υ (IXa) values were calculated from the double reciprocal plots shown in Fig. 2.

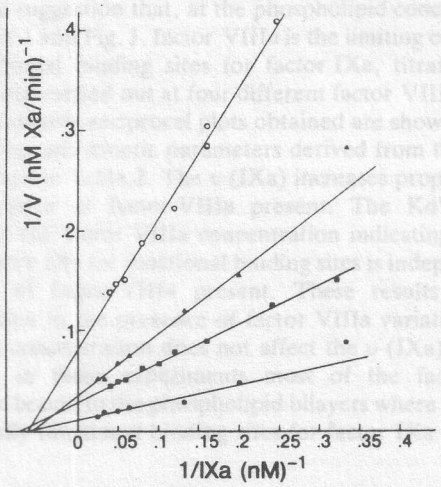

Fig. 2 Double reciprocal plots of the effect of factor IXa on the rate of factor X activation at varying concentration of activated factor VIII. Factor IXa titration curves were made at four different factor VIIIa concentrations as described in the legend of Fig. 1. The final composition of the reaction mixtures (0.5 ml) was: 50 μM phospholipid, 0.5 μM factor X, 3–50 nM factor IXa, 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 6 mM $CaCl_2$, 0.5 mg/ml human albumin and (○—○), 0.0125 nM factor VIIIa; (▲—▲), 0.033 nM factor VIIIa; (■—■), 0.0375 nM factor VIIIa (●—●), 0.1 nM factor VIIIa.

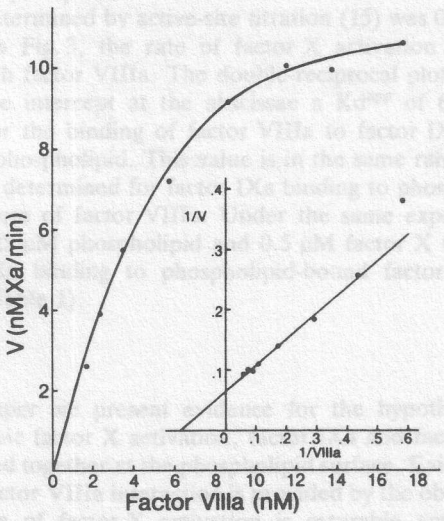

Fig. 3 Effect of varying amounts of activated factor VIII on the rate of factor X activation in the presence of a limiting amount of factor IXa. Factor X activation was measured as described in the legend to Fig. 1B. Factor VIII was activated at a constant concentration of 70 Units/ml whith 3.65 nM thrombin. The final composition of the reaction mixture for factor X activation was: 50 mM Tris-HCl (pH 7.9), 175 mM NaCl, 6 mM $CaCl_2$, 2.5 mg/ml ovalbumin, factor VIIIa as indicated in the Fig., 0.017 nM factor IXa, 25 μM phospholipid and 0.5 μM factor X_2.

in which $[IXa]_{free}$ is the concentration factor IXa free in solution and $[sites]_{total}$ is the total concentration of factor IXa binding sites (phospholipid or factor VIIIa-phospholipid). Rearrangement of equation II yields:

$$\frac{1}{[IXa]_{bound}} = \frac{Kd}{[IXa]_{free} \cdot [sites]_{total}} + \frac{1}{[sites]_{total}} \qquad (III)$$

Substitution of equation I in III results in:

$$\frac{1}{V} \cdot = \frac{1}{c}\left\{\frac{Kd}{[IXa]_{free} \cdot [sites]_{total}} + \frac{1}{[sites]_{total}}\right\} \qquad (IV)$$

A plot of V^{-1}vs. $[IXa]^{-1}_{free}$ is linear, with the intercept at the ordinate being $c^{-1}[sites]^{-1}_{total}$ and the intercept at the abcissa equal to $-Kd^{-1}$. The Kd is the concentration $[IXa]_{free}$ required to occupy 50% of the $sites_{total}$, which will result in half-maximal factor X activation rates. When $[IXa]_{bound} \ll [IXa]_{free}$ (a condition met in the experiments presented in this paper), the free factor IXa concentration equals the total added concentration of factor IXa, which allows the estimation of the binding parameters of factor IXa for phospholipid from intercepts in plots of V^{-1}vs $[IXa]^{-1}_{total}$.

Assembly of the Intrinsic Factor X Activating Complex, Studied by Varying the Factor IXa Concentration.

The relationship between the rate of factor X activation and the concentration of factor IXa, in the presence of phospholipids, was examined to determine whether binding parameters for factor IXa could be derived from kinetic experiments. In the two experiments shown in Fig. 1, initial rates of factor X activation were determined as a function of the concentration factor IXa at fixed concentrations of phospholipids vesicles (25 μM) and factor X (0.5 μM). In panel A no factor VIIIa was added and in panel B thrombin activated factor VIII was present at a concentration of 0.012 nM (0.011 units/ml). In both experiments the rate of factor X activation reached a plateau value and could, therefore, be saturated with respect to factor IXa. However the factor IXa concentration required for saturation was considerably lower in the presence of factor VIIIa (B) than in its absence (A). Double-reciprocal plots of the data of Fig. 1 show a linear relationship between the plotted variables. Straight lines in double-reciprocal plots are in accordance with equation IV. This expression was derived for titration of single identical binding sites with factor IXa, which upon occupation, contribute to the rate of factor X activation. As is outlined in the appendix, the formation of functional complexes containing factor IXa and factor VIIIa in a stoichiometry different from 1:1 can be ruled out. By applying equation IV to the kinetic data, apparent Kd values for the dissociation of factor IXa from factor IXa-phospholipid and factor IXa-factor VIIIa-phospholipid complexes could be calculated. The Kd^{app} found in the absence of factor VIIIa was 770 nM and in the presence of factor VIIIa 10 nM.

Additional titrations with factor IXa both in the absence and presence of factor VIIIa were carried out at varying phospholipid and factor X concentrations. All double-reciprocal plots were linear, and binding and kinetic data calculated from the plots are summarized in Table 1. It is shown that all Kd^{app} values for factor IXa are 50–100 fold lowered by the presence of factor VIIIa, indicating that factor IXa has a higher affinity for factor VIIIa-phospholipid complexes than for phospholipid alone. The small effects of varying phospholipid and factor X concentrations on Kd^{app} will be further considered in the discussion.

In the absence of factor VIIIa, the υ (IXa) values (rates of factor X activation obtained by extrapolation to infinitely high concentration of factor IXa) increased at increasing phospholipid

concentrations. The proportional increase of υ (IXa) with the phospholipid concentration is consistent with an increased number of binding sites for factor IXa available at the phospholipid surface. In the presence of factor VIIIa, υ (IXa) was hardly affected by the phospholipid concentration, suggesting that under these conditions factor VIIIa rather than phospholipid limits the total amount of factor IXa-factor VIIIa-phospholipid complexes which can be formed. Both in the absence and presence of factor VIIIa, decrease of the factor X concentration resulted in a decrease of υ (IXa), which is due to the fact that rates of factor X activation were measured at a factor X concentration around the Km. The Km for factor X at 50 μM phospholipid was 0.15 μM in the absence of factor VIIIa and was 0.053 μM with factor VIIIa (data not shown).

To test the suggestion that, at the phospholipid concentrations given in Table 1 and Fig. 1, factor VIIIa is the limiting component for the functional binding sites for factor IXa, titrations with factor IXa were carried out at four different factor VIIIa concentrations. The double-reciprocal plots obtained are shown in Fig. 2 and the binding and kinetic parameters derived from these plots are summarized in Table 2. The υ (IXa) increases proportionally with the amount of factor VIIIa present. The Kd^{app} is not influenced by the factor VIIIa concentration indicating that the affinity of factor IXa for functional binding sites is independent of the amount of factor VIIIa present. These results and the observation that in the presence of factor VIIIa variation of the phospholipid concentration does not affect the υ (IXa), strongly suggest that in these experiments most of the factor VIIIa molecules are bound to the phospholipid bilayers where they form the catalytically functional binding sites for factor IXa.

Assembly of the Factor X Activating Complex at Increasing Factor VIIIa Concentrations

Having shown that the rate of factor Xa formation at limiting factor VIIIa concentrations is saturable with respect to factor IXa, it was of interest to examine whether with limiting amounts of factor IXa, the rate of factor Xa formation could also be saturated with factor VIIIa. Varying amounts of activated factor VIII were added to reaction mixtures containing factor IXa, phospholipid, Ca^{2+} and factor X. The factor IXa concentration determined by active-site titration (15) was 0.017 nM. As shown in Fig. 3, the rate of factor X activation was also saturable with factor VIIIa. The double-reciprocal plot is linear and from the intercept at the abscissae a Kd^{app} of 6.7 nM is calculated for the binding of factor VIIIa to factor IXa in the presence of phospholipid. This value is in the same range as the Kd^{app} values determined for factor IXa binding to phospholipids in the presence of factor VIIIa. Under the same experimental conditions (25 μM phospholipid and 0.5 μM factor X the Kd^{app} for factor IXa binding to phospholipid-bound factor VIIIa is 10 nM (see Table 1).

Discussion

In this paper we present evidence for the hypothesis that during intrinsic factor X activation, factor IXa and factor VIIIa are complexed together at the phospholipid surface. Evidence for factor IXa-factor VIIIa interaction is provided by the observation that the rate of factor X activation is saturable with excess factor IXa in the presence of limiting amounts of factor VIIIa and vice versa. The amount of complex that is formed was proportional with the amount of limiting component present. The saturation curves were hyperbolic suggesting that single identical sites were titrated in each case. By titration of a limited amount of factor VIIIa with factor IXa in the presence of phospholipid a Kd^{app} for factor IXa between 3–16 nM was found. Titration of a limited amount of factor IXa with factor VIIIa in the presence of phospholipid resulted in a Kd^{app} of 6.7 nM for factor VIIIa.

We further carried out factor X activation experiments in the absence of factor VIIIa. The Kd^{app} for factor IXa binding to phospholipids obtained from kinetic experiments varied between 0.4–1.1 μM. These values are comparable to binding parameters determined by Nelsestuen et al. (8) in direct binding studies. At a suboptimal Ca^{2+} concentration they observed a Kd of 2 μM for dissociation of factor IX or RVV-activated factor IX from phospholipid vesicles.

Comparison of factor X activation experiments in the absence and presence of factor VIIIa indicates that the apparent Kd for factor IXa binding is lowered 50–100 fold in the presence of factor VIIIa. We conclude that factor VIIIa functions as a high affinity binding site for factor IXa at the phospholipid surface. It is likely that the combination of factor IXa-phospholipid and factor IXa-factor VIIIa interactions promote the incorporation of factor IXa into the factor X activating complex. In that respect factor VIIIa has a similar function as factor Va in prothrombin activation. Nesheim et al. (11) and Lindhout et al. (12) reported that factor Va promotes the binding of factor Xa to negatively charged phospholipid surfaces.

The dependence of Kd^{app} from the phospholipid and the factor X concentration is not completely understood. The following factors may contribute to the observed dependence: a) Up to 15% of added factor IXa is bound, which at the higher phospholipid concentration results in somewhat higher Kd^{app} values b) Factor X binds to phospholipids with a Kd of 50 nM (9) and may compete with factor IXa for binding to the phospholipid surface. To appreciate the interaction of factor VIIIa with factor IXa and phospholipids it would be important to study factor VIIIa binding using a direct binding assay. Presently, however, the instability of activated factor VIII preparations does not allow such an approach.

In our previous experiments on the intrinsic factor X activator we used factor VIII complex consisting of factor VIII:C and von Willebrand factor (6). In the present work we used a factor VIII:C preparation (14) that contains little or no von Willebrand factor. Using factor VIII complex, a Vmax of 500 moles of factor Xa min^{-1} per mol of factor IXa was reported, whereas in this study the Vmax value is 900. This result suggests that the von Willebrand factor does not strongly influence the Vmax of factor Xa formation.

The physiological implications of the high-affinity binding sites for factor IXa, formed by factor VIIIa at a lipid- or activated platelet surface appear important. The concentration of factor VIII in human plasma is 0.25 nM (unpublished observations). The concentration of factor IX, which is at least 200-fold higher, is approximately 60 nM (16). During the coagulation process only small amounts of factor IX are converted into factor IXa and the concentrations of factors VIIIa and IXa will remain in the nanomolar range. The 50–100 fold lowering of the Kd for factor IXa binding (to the nM range) by factor VIIIa therefore allows more efficient factor IXa-factor VIIIa complex formation. Previously we found that factor VIIIa caused a 200,000 fold enhancement of the Vmax of factor Xa formation (6). It now appears that the overall enhancement is explained by two major effects of factor VIIIa: a) greatly enhanced catalytic power of factor IXa-VIIIa-phospholipid, compared to factor IXa-phospholipid; b) enhanced factor IXa affinity for the lipid surface in the presence of factor VIIIa, which increases the amount of phospholipid-bound factor IXa that participates in factor X activation.

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Appendix

A general equation can be derived, which describes the relation between the reciprocal rate of factor X activation and the reciprocal free ligand concentration when the intrinsic factor X activator consists of a complex of factor IXa and factor VIIIa with a stoichiometry different from 1:1. When protein A at a fixed concentration ([A]) is titrated with protein B and when AB_n is the enzymatically functional unit, the following binding equilibria are involved:

$$A + B \rightleftarrows AB \qquad K_1 = \frac{[A]\,[B]}{[B]}$$

$$AB + B \rightleftarrows AB_2 \qquad K_2 = \frac{[A]\,[B]}{[AB_2]}$$

$$AB_{n-1} + B \rightleftarrows AB_n \qquad K_n = \frac{[AB_{n-1}]\,[B]}{[AB_n]}$$

Since $[A_t] = [AB_n] + [AB_{n-1}] + \ldots\ldots + [AB] + [A]$ and the reaction rate $V = c.[AB_n]$ (c is a proportionality factor determined by the kinetic parameters of the reaction catalyzed by AB_n), the following relation between 1/V and 1/[B] is obtained:

$$1/V = \frac{1}{c.[A_t]}\left\{ \prod_{j=1}^{n} K_j \frac{1}{[B]^n} + \prod_{j=2}^{n} K_j \frac{1}{[B]^{n-1}} + \ldots\ldots + K_n \cdot \frac{1}{[B]} + 1 \right\}$$

From this equation it is obvious that only if n = 1 (1:1 stoichiometry) 1/V will be linearly proportional with 1/[B].