Analysis of the Generation and Inhibition of Factor Xa

AREA UNDER GENERATION CURVES IS INDEPENDENT OF ENZYME GENERATION RATE*

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The activation of factor X in the presence of antithrombin has been studied in order to determine the parameters that control the area under the resulting factor Xa generation curve. Generation curves were analyzed using a model containing three parameters: the total generation of factor Xa, $E_{\text{max}}$; the rate of factor Xa generation, expressed as a first-order rate constant, $k_1$; and the rate of inhibition, expressed as another first-order rate constant, $k_2$. Using factor IXa-VIIIa to activate factor X, we found the area under the generation curve to be proportional to $E_{\text{max}}$, which was varied by varying the factor IXa concentration, and inversely proportional to $k_2$, which was varied by varying the antithrombin concentration. With this activator, however, $k_1$ varied in parallel with $E_{\text{max}}$, resulting in a correlation between integrated area and $k_1$. In order to determine whether $E_{\text{max}}$ or $k_1$, or both, was a controlling parameter, similar activations were done with varying concentrations of the factor X-activating enzyme of Russell's viper venom. With this activator it was possible to vary $E_{\text{max}}$ and $k_1$ independently, again at varying antithrombin concentrations. These results showed the integrated area to be proportional to $E_{\text{max}}$ and inversely proportional to $k_2$, as before, but independent of the activation rate, $k_1$. In this system, therefore, the area under the factor Xa generation curve is controlled by the amount of factor Xa generated and its rate of inhibition but is independent of the rate of factor Xa generation.

One of the major controls of the clotting system in vivo is the inhibition of clotting proteases by plasma protease inhibitors. The importance of antithrombin, in particular, is borne out by the high incidence of thrombosis in individuals who have levels of this inhibitor lower than normal. However, unlike deficiencies of clotting factors, where major hemostatic disorders are generally not seen until the level of a factor falls below about 25% of normal, deficiency of antithrombin can cause significant problems at levels as high as 50% of normal.

At first sight it may appear that the sensitivity of the clotting system to antithrombin concentration is a defect in inhibitory capacity, but this is probably not the case. Even at 50% of its normal plasma level of 4–5 μM, antithrombin is still in about 2-fold molar excess over the sum of all the clotting zymogens. Under normal conditions of hemostasis, where the extent of zymogen activation is relatively small, there is ample inhibitory capacity in terms of the amount of antithrombin available.

In contrast to the ample mass capacity, there is a clear kinetic defect when the antithrombin concentration is reduced. Like all common protease inhibitors, except perhaps α2-macroglobulin, antithrombin inhibits its target enzymes in simple second-order fashion (Downing et al., 1978; Jesty 1978, 1979, Ellis et al., 1982). In plasma, where its concentration is well in excess of the levels of target enzyme that will normally be generated, this means that inhibition will be pseudo-first order (Jesty, 1986b). In other words, although antithrombin may be in large excess over the target enzyme, the rate of inhibition is proportional to antithrombin concentration. Thus, half-normal levels of inhibitor cause a half-normal rate of inhibition, etc.

The question addressed in this paper is what effect this has on the overall kinetics of the generation and inhibition of a target enzyme, i.e. on the enzyme generation curve. Intuitively, it seems likely that an important parameter in the control of clotting processes is the integrated area under these generation curves. This can be obtained from first principles as follows.

Since inhibition is a second-order process, $d[IE]/dt = k[I][E]$, where [IE] is the concentration of enzyme-inhibitor complex, [I] is inhibitor concentration, and [E] is enzyme concentration. If inhibitor is in large excess over the total enzyme generated ([E]_{\text{tot}}), then $k[I]$ is constant. Integrating, we may then obtain $[IE] = k[I][E]dt$. At $t = \infty$, all enzyme generated has been inhibited, so that $[IE] = [E]_{\text{tot}}$. The actual form of the integral term may be complex, depending on the kinetics of enzyme generation, but regardless of this, the integrated area under any enzyme generation curve at $t = \infty$, $\int [IE]dt$, should equal $[E]_{\text{tot}}/k[I].$

In a previous paper (Jesty, 1986a) I described the course of generation and inhibition of factor Xa in both pure systems and plasma and demonstrated that generation curves can be empirically described by a simple two exponential model that describes the sum of two first-order processes, corresponding to generation and inhibition (see "Materials and Methods," Equation 2). Integration of this equation indeed confirms that the area under a generation curve should be proportional to $[E]_{\text{max}}$, inversely proportional to the rate of inhibition ($k[I]$), and independent of the rate of enzyme generation. The possibility of control by the extent of enzyme generation is particularly significant in the light of reports, going back many years, showing that in some clotting reactions the level of activating enzyme can control not only the rate, but also the extent, of zymogen activation. Such reactions have been termed self-damping (Nemerson et al., 1974).

In this paper I describe the experimental test of these predictions, using as a test system the generation of factor Xa by the factor IXa-VIIa complex and by the factor X-

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activating enzyme of Russell's viper venom (RVV-X). 1 Anti-thrombin is the factor Xa inhibitor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin (fatty acid-free), trypsin, aprotinin, and Russell's viper venom were obtained from Sigma. Phosphatidylserine and phosphatidylcholine were from Supelco and were prepared as a sonicated equimolar stock mixture (PS:PC) at 2 mg/ml in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5 (Tris-buffered saline (TBS)). Bio-Gel A-15m-agarose is a product of Bio-Rad. Celite was obtained from Fisher. The chromatographic substrate for factor Xa, N-methoxycarbonyl-D-NorLeu-Gly-Arg-p-nitroanilide (where NorLeu is norleucine) (Chromozym X) is a product of Boehringer Mannheim. Other chemicals were reagent grade products of various suppliers.

Factors IX and X were isolated from non-heparin-treated concentrates, very kindly donated by Alpha Therapeutics, by the method of Morrison and Jesty (1984). Factor Xa-von Willebrand complex was prepared from cryoprecipitate as described by Neuenhawer and Jesty (1988). RVV-X was prepared essentially by the method of Kisiel et al. (1976). Factor Xa was prepared from pure factor X by the method Jesty and Nemerson (1976) described for the bovine enzyme. Human α-thrombin was prepared by a modification of the method of Fenton et al. (1977), using chromatography on CM-Sepharose. Factor Xa was purified from fresh human plasma by chromatography on heparin-agarose and DEAE-Sephadex (Jesty, 1970). By comparison of the rates of thrombin inhibition in the presence and absence of protamine sulfate, this material contains no heparin.

Factor Xa was prepared from pure factor IX as follows. A Celite eluate was prepared from 1 liter of citrated human plasma by stirring the plasma with 20 g of Celite for 30 min, centrifuging, washing with TBS, and then eluting the Celite-bound protein with 1 M NaCl. The eluate was dialyzed against 0.1 M NaHCO₃ and then treated with a 0.1 volume of trypsin-agarose (2 mg/ml of trypsin/ml of beads, prepared with CNBr-activated agarose) until the factor Xa activity (by clotting assay) reached a maximum. The trypsin-agarose beads were removed by filtration through siliconized glass wool, and 1 μg/ml of aprotinin was added to inactivate any trace of soluble trypsin leached from the beads. This material, which is crude factor Xa, was coupled to 10 ml of CNBr-activated Bio-Gel A-15m-agarose. Factor IX (0.5 mg/ml) was activated immediately before each incubation in the presence of factor IXa (variable concentration), 5 mM CaCl₂, followed by filtration through siliconized glass wool.

Examination by gel electrophoresis showed >95% conversion of the factor IXa generated was observed to suffer slow decay, possibly through autolysis, at a rate of 10.01 min⁻¹. To determine Eₙ₉ in this situation, it was therefore necessary to fit data to the same function was used to describe enzyme generation and inhibition (Equation 2).

**Fitting Generation Curves**—Factor Xa generation curves that featured any decay of enzyme, in either the presence or absence of inhibitors, were fitted to a two exponential function,

$$E_t = E_{max} \left(1 - e^{-k_1 t}\right),$$

where $E_t$ is the concentration of factor Xa at time $t$, and $E_{max}$ and $k_1$ are as defined above. Equation 1 was used to analyze the generation of factor Xa in the presence of inhibitors, but only if the activity of the generated enzyme was stable. This was true of factor Xa generated by RVV-X in the absence of phospholipid, but when factor Xa was activated by factors IXa-VIIIa in the presence of phospholipid, the factor Xa generated was observed to suffer slow decay, possibly through autolysis, at a rate of $0.01 \text{ min}^{-1}$. To determine $E_{max}$ in this situation, it was therefore necessary to fit data to the same function was used to describe enzyme generation and inhibition (Equation 2).

**Measurement of Factor Xa**—Factor Xa generation was followed by discontinuous chromogenic assay, as described by Neuenhawer and Jesty (1988). In experiments concerning factor X activation by factors IXa-VIIIa, factor VIII (0.5 unit/ml) was activated immediately, data were thereby integrated to

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**Determination of $k_1$ and Initial Rate**—In all experiments concerning factor Xa generation curves in the presence of inhibitor (anti-thrombin), data were fitted to Equation 2 (or its derivative including a lag phase term) with $E_{max}$ held constant at its known value, which was determined from generation curves in the absence of antithrombin. Only one parameter significant to this study was derived from these fits of generation curves: the initial rate constant, $k_1$. The initial rate of enzyme generation, $(dR/dt)_{0}$, can be obtained from the fitted parameters as $u = E_{max} k_1$.
RESULTS

The study was based on three predictions concerning the regulation of the area under factor Xa generation curves, viz. the integrated area should be 1) proportional to $E_{\text{max}}$, 2) inversely proportional to the rate of inhibition, $k_i$, and 3) independent of the rate of enzyme generation, $k$. While it was easy to vary inhibition rate ($k_i$) independently of $E_{\text{max}}$ and $k$, in this study by simply varying antithrombin concentration, independent variation of $E_{\text{max}}$ and $k_i$ was more difficult. Indeed, in the activation of factor X by the factor IXa-VIIIa complex, these two are tightly linked; increases in the rate of factor X activation are almost invariably coupled to increases in the extent of activation. Reasons for this are considered under "Discussion."

The Activation of Factor X by Factor IXa-VIIIa—Fig. 1 shows a series of activations of factor X (fixed concentration, 18 nM) at varying factor IXa concentrations (between panels) and antithrombin concentrations (within panels). Subsequent derivative plots (Fig. 2) were derived from this data set and another identical one. The method of analysis necessarily uses the values of $E_{\text{max}}$ obtained without inhibitor to derive the rates of activation in the presence of inhibitor (see "Experimental Procedures"), and this requires that factor IXa is not subject to significant inhibition by antithrombin under the conditions studied.

We therefore determined the kinetics of inhibition of factor IXa by antithrombin. Factor IXa (90 nM) was incubated with antithrombin (5.5 and 9.0 nM) + PS:PC + Ca++. Timed samples (0-30 min) were diluted 50-fold to stop inhibition and were then assayed by a non-activated partial thromboplastin time in factor IX-deficient plasma. The assay was standardized with known concentrations of factor IXa. First-order decay was observed in both cases, giving a second-order rate constant (for the inhibition of factor IXa by antithrombin) of 4700 M$^{-1}$ min$^{-1}$. At the highest concentration of antithrombin used in the measurement of generation curves in Fig. 1 (5.5 nM), this corresponds to a first-order inhibition rate of factor IXa of 0.026 min$^{-1}$ or a half-life of 27 min. Since the great bulk of factor X activation in these incubations occurs well before 10 min, it is unlikely that the inhibition of factor IXa by antithrombin causes significant changes in $E_{\text{max}}$ and, therefore, no corrections were made. Two observations are clear from the data of Fig. 1.

1) From the set of generation curves within each panel, it is plain that the area under each curve is inversely related to antithrombin concentration. This relationship is shown in quantitative fashion in Fig. 2A, where we see that at each level of factor IXa or extent of factor X activation, the integrated area is in exact proportion to reciprocal inhibitor concentration.

2) Fig. 1 also shows that the yield of factor Xa, $E_{\text{max}}$, is a function of the concentration of factor IXa even in the absence of inhibitor, particularly as factor IXa drops below 10 ng/ml. For example, factor Xa yield at 1 ng/ml factor IXa is less than one-fifth that at 10 ng/ml factor IXa. Using the values of $E_{\text{max}}$ obtained in each panel in the absence of inhibitor, we can then plot integrated area, determined from the data from each incubation, against $E_{\text{max}}$ in each case (Fig. 2B). It is clear that at each antithrombin concentration the area is almost exactly proportional to $E_{\text{max}}$.

These results confirm the initial predictions concerning the dependence of the integrated area on $E_{\text{max}}$ and inhibition rate ($k_i$). The test of the final prediction, however, is more difficult. In order to show that the integrated area is independent of the activation rate, it is necessary to vary that rate independently of $E_{\text{max}}$. When factor IXa-VIIIa is the activator this cannot easily be done. To illustrate the problem, I show in Fig. 2C the relation of integrated generation curve area to $k_i$. While the proportionality is not as good as that for the dependence of area on $E_{\text{max}}$ (Fig. 2B), area is definitely correlated with $k_i$ and, we therefore have no evidence that area is independent of rate.

Attempts were made to vary the rate of activation by factors IXa-VIIIa while maintaining constant $E_{\text{max}}$. In particular, we tried to study activation at sufficiently high levels of factor IXa to activate all the factor X present. Under these conditions, however, activation was too fast to enable adequately accurate determination of the rate. For this phase of the study we therefore turned to RVV-X as the activator.
The Activation of Factor X by RVV-X—Unlike the physiological activators of factor X, RVV-X behaves as an enzyme in a textbook manner; regardless of the concentration of activator or the rate of activation, factor X activation goes to completion (Fig. 3A). Fig. 3B shows in addition the linear relation between activation rate (k1) and RVV-X concentration. In this system, then, we have means of varying k1 while holding Emax constant. In addition we have confirmed, using clotting assays, that antithrombin does not significantly inhibit RVV-X under the conditions used here.

With this system we first confirmed that the integrated area is (as in activation with factors IXa-VIIIa) inversely proportional to antithrombin concentration and proportional to Emax. Fig. 4A shows one typical set of data at a single concentration of factor X (i.e. fixed Emax) and varying antithrombin concentration. In Fig. 4B we show a typical example of the reverse: the effect of varying Emax at a single concentration of antithrombin, done by varying the factor X level. In all, duplicate experiments were done at four antithrombin concentrations and four factor X concentrations. The derivative results of these are shown in Fig. 4C and demonstrate the proportional relationship between integrated area and Emax at each antithrombin concentration. Analysis also confirmed a side benefit of activating with RVV-X; varying the factor X concentration has little effect on k1 (data not shown). In these experiments, therefore, we have good evidence that integrated area depends on Emax.

Finally, we wished to demonstrate unequivocally that the integrated area actually is unaffected by changes in the rate of factor X activation. In order to vary k1 while holding Emax constant, activations were done at fixed concentrations of factor X and varying concentrations of RVV-X. Under these conditions we know that factor X activation always goes to completion (Fig. 3A). One typical set of data is shown in Fig. 5A. These raw data are included because they show qualitatively how it is that the area under a generation curve can be independent of k1. As the rate of activation drops, the peak activity is reduced, but, in addition, factor Xa generation continues for a longer period causing trailing of the generation curve. The combined results from duplicate experiments at four RVV-X and three antithrombin concentrations are shown in Fig. 5B, and it is plain that at each antithrombin concentration, or rate of inhibition, the area under the generation curves varies by less than 20% over a 10-fold range of factor X activation rates.

With RVV-X as activator, therefore, we have proven that the area under the factor Xa generation curve is inversely proportional to inhibition rate (k1), directly proportional to the total enzyme generated (Emax), and independent of the rate of enzyme generation (k1).

DISCUSSION

Generation curves, particularly those of thrombin, have been the subject of generally qualitative study over many years, and Denson and Biggs (1976) have made a brief analysis of their control. Although these authors did not take their analysis beyond a qualitative demonstration of behavior and comparison with a few observed thrombin generation curves, they predicted the essential postulates of the present study. As I showed in an earlier report (Jesty, 1986a), factor Xa generation curves observed in the presence of antithrombin (a factor Xa inhibitor) can be empirically described by just three parameters: 1) the total amount of enzyme generated or amount of factor X activated, Emax; 2) the rate of activation or attainment of Emax, expressed as a first-order rate constant, k1; and 3) the rate of factor Xa inhibition, expressed as another...
first-order rate constant, \(k_1\). If one assumes no lag phase, integration of the appropriate differential equations leads to Equation 2 (see "Experimental Procedures"). Having the equation, we may then integrate it from \(t = 0\) to \(t = \infty\) to obtain the area under the generation curve, \(E_{\text{max}}/k_2\). Fig. 6 shows the effect on such (theoretical) curves of varying the three parameters separately, each over a 16-fold range, and it clarifies (panel B) why it is that varying \(k_1\) has no effect on the area under the curve.

**Area Is Inversely Proportional to Inhibition Rate**—This was the easiest part of both the study and the analysis. With both factor X activators used, generation curves were made over a range of antithrombin concentrations. Since we already know that the kinetics of factor Xa inhibition by antithrombin are second order (Jesty, 1978; Ellis et al., 1982), we can safely assume that under the conditions in this study (with a large excess of antithrombin over factor Xa) inhibition is pseudo-first order and that the inhibition rate is proportional to inhibitor concentration. The results with both factors IXa-VIIIa (Fig. 2A) and RVV-X (Fig. 5B) confirm that the integrated area is, within the experimental limits, in exact inverse proportion to antithrombin concentration; and this is true over a wide range of activation extents (\(E_{\text{max}}\)) and activation rates (\(k_1\)).

**Area Is Proportional to \(E_{\text{max}}\)**—The data shown in Figs. 2B and 4C from generation curves produced by factors IXa-VIIIa and RVV-X, respectively, show that the integrated area is directly proportional to the extent of factor X activation, \(E_{\text{max}}\). Although the final plots are not so nicely linear as the relation of area to inhibition rate, the fit is reasonable when one considers that each area determination derives from the mean of two generation curves, each of 13 or 14 factor Xa activity measurements.

Despite this apparently satisfactory result, a significant problem arose with the results obtained from activation by factors IXa-VIIIa. While one can see in Fig. 2B that there is a satisfactory correlation between area and \(E_{\text{max}}\), area is also correlated with \(k_1\) (Fig. 2C). In other words, with this activator we could not prove the area to be both dependent on \(E_{\text{max}}\) and independent of \(k_1\). This, it should be noted, affects not only the amount of enzyme generated (\(E_{\text{max}}\)) but also must affect estimates of \(k_1\). Indeed it is quite possible that the correlation between \(E_{\text{max}}\) and \(k_1\) is caused solely by the self-damping behavior.

Although such mechanisms as product inhibition could be invoked, the most likely cause of self-damping is the spontaneous first-order decay of factor VIIIa (Hultin and Jesty, 1981). If, however, this were the only cause of incomplete activation, \(k_1\) in the damped reactions (Fig. 1, panels D-F) would be the rate constant for factor VIIIa decay. (If we differentiate Equation 1, we obtain an expression for the rate of factor Xa generation as a function of time: \(v = v_0 \exp(-k_1t)\), where \(k_1\) is the rate constant for enzyme (factor VIIIa) decay, this is equal to the value of \(k_1\) obtained by fitting factor Xa generation data to Equation 1.) The relevant data are shown in Fig. 2, B and C. There is a definite tendency toward a minimum value of \(k_1\) at the lower \(E_{\text{max}}\) levels, equal to about 0.2 min \(^{-1}\). This is confirmed by the values of \(k_1\) obtained from the incubations lacking antithrombin (Fig. 1, open circles; data not shown in Fig. 2); although as high as 0.94 min \(^{-1}\) at high levels of factor IXa, \(k_1\) falls to the 0.2 min \(^{-1}\) range at factor IXa levels below 100 nM. We may provisionally conclude that self-damping is caused by decay of the activating complex, at a rate of about 0.2 min \(^{-1}\). This, one may note, is much faster than Lollar et al. (1984) have reported for the decay of porcine factor VIIIa in the presence (as in this study) of factor IXa, phospholipid, and Ca\(^{2+}\), but the difference may reflect species variations only.

**Area Is Independent of \(k_1\)**—The inability to hold \(k_1\) constant in activations with factors IXa-VIIIa led us to study activation of factor X with RVV-X. With this activator the two parameters can be independently controlled, and it was possible to prove directly that area is essentially unaffected over a 10-fold range of factor X activation rate. At this point I should discuss a possible source of confusion concerning \(k_1\). In this study we have seen that generation curves can be described by Equation 2, and \(k_1\) in that equation is defined as a first-order rate constant, i.e. it is a measure of the rate of attainment of \(E_{\text{max}}\). Since its units are (time) \(^{-1}\), \(k_1\) does not tell us the initial rate of factor Xa formation; this is \(E_{\text{max}}k_1\). Only when \(E_{\text{max}}\) is constant, as in Fig. 5, is \(k_1\) a direct measure of initial rate; and only in that experiment is it therefore possible to conclude that the integrated area is independent of activation rate.

**Self-damped Factor X Activation by Factors IXa-VIIa**—As was pointed out some years ago (Nemerson et al., 1974), a number of reactions in coagulation are self-damped. Meriz et al. (1939) were the first to demonstrate self-damping in prothrombin activation in the absence of inhibitors, an observation confirmed by Silverberg and Nemerson (1975). Self-damping behavior has also been demonstrated in factor X activation by tissue factor + factor VIIa (Jesty et al., 1974). In this study, we see that factor X activation via the intrinsic pathway is also self-damped in the absence of inhibitors; not only the rate, but the yield, of factor Xa is a function of the activator concentration. The present study suggests that of these two it may be the yield that is the major control.

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**REFERENCES**


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**Fig. 6. The effect of independent variation of \(E_{\text{max}}, k_1,\) and \(k_2\) in a three-parameter model (Equation 2).** A, \(E_{\text{max}}\) was varied over the concentrations shown (nM); \(k_1\) and \(k_2\) were both fixed at 0.2 min \(^{-1}\). B, \(k_1\) was varied as shown, \(E_{\text{max}}\) was 20 nM, and \(k_2\) was 0.2 min \(^{-1}\). C, \(k_2\) was varied as shown; \(E_{\text{max}}\) was 20 nM, and \(k_1\) was 0.2 min \(^{-1}\).


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