Titration of Activated Bovine Factor X*

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SUMMARY

p-Nitrophenyl-p'-guanidinobenzoate is a suitable substrate for the titration of purified bovine Factor X which has been activated by a coagulant protein from Russell’s viper venom. Reaction of Factor Xa with p-nitrophenyl-p'-guanidinobenzoate occurs at the site which is responsible for the clotting activity of Factor Xa. Activation of bovine Factor X by the Russell’s viper venom coagulant protein yields a single active site per 53,000 daltons of zymogen.

Advances in the field of blood coagulation have been rapid and numerous in recent years. The application of new and powerful analytical tools, especially those of protein chemistry and enzymology, is currently providing detailed knowledge of the structures of several of the molecular principals of blood coagulation and their functions in this complex process. Factor X is a key component in the sequence of reactions leading to clot formation. Activation of Factor X produces the proteolytic enzyme which is directly responsible for the conversion of prothrombin to thrombin (6, 7, 9).

Concentrations of enzyme solutions are most often determined by specific rate assays. However, active site titrations, or “all-or-none” enzyme assays have several advantages over such rate assays when studying the effects of structural modifications on enzyme activity. These advantages have been emphasized by Bender et al. (10). The suitability of the reagent p-nitrophenyl-p'-guanidinobenzoate as a titrant of bovine thrombin, human plasmin, and both bovine and human thrombin has recently been demonstrated (11).

In this report we have adapted the use of p-NPGB for use as a titrant of purified bovine blood clotting Factor X which has been activated with the coagulant protein from Russell’s viper venom. Under suitable conditions, p-NPGB reacts essentially stoichiometrically with activated Factor X in a reaction in which the active site of the enzyme is acylated by the p-guanidinobenzoate group, and an amount of p-nitrophenol equivalent to the concentration of active sites present is simultaneously released. Deacylation occurs in a much slower step, completing the reaction.

A comparison of kinetic constants for the reaction of p-NPGB with Factor Xa and with trypsin reveals large differences in the values for $K_a$ and for $k_3$, the acylation rate constant. However the rates of deacylation by the guanidinobenzoate ion are similar for both trypsin and Factor Xa. Acylation of Factor Xa by p-NPGB inhibits clotting activity, and deacylation is accompanied at the same rate by the return of clotting activity. Activation of Factor X with the venom coagulant protein generates a single active site per mole of zymogen.

THEORY OF TITRATION

Kinetic expressions for the titration of enzymes which catalyze reactions according to a three-step sequence have been developed by Bender et al. (10, 12).

Here, $E$ is enzyme, $NPGB$ is the titrant substrate, $NPGB - E$ is the adsorptive enzyme-substrate complex, $GB - E - NP$ is the observed burst, and $NP$ is the product of the stoichiometric reaction of the enzyme with substrate which liberates 1 eq of nitrophenol (NP), and GB is the p-guanidinobenzoate ion which is released upon deacylation of GB-E.

As shown by Bender et al. (10), if $k_2 \gg k_3$ and $[p-NPGB]_o \gg [E]_o$ the observed burst, $\pi$, is equal to the concentration of enzyme active sites multiplied by a factor as shown:

$$\pi = [E]_0 \left[ \frac{k_2}{1 + K_{m_{app}}/[p-NPGB]_0} \right]$$  

where $K_{m_{app}} = K_{m}[k_3/(k_2 + k_3)]$. If, in addition, $[p-NPGB]_o \gg K_{m_{app}}$, $\pi$ will not be affected significantly by titrant concentration and is approximately equal to $[E]_0$. Therefore, it is desirable to determine $k_2$, $k_3$, and $K_{m_{app}}$ in order to show that both $k_2 \gg k_3$ and $[p-NPGB]_o \gg K_{m_{app}}$ hold, so that titration at a single substrate concentration is a valid measurement of $[E]_0$. If only the first assumption holds, $\pi$ is dependent upon concentration of the titrant as described in Equation 2, and must be determined at several titrant concentrations to provide a plot of $1/\sqrt{\pi}$ versus $1/[p-NPGB]_o$, the $1/[p-NPGB]_o$ intercept of which is $1/\sqrt{[E]_0}$.

This follows from the rearrangement of Equation 2.
Both $k_2$ and $K_1$ were determined by observing the pre-steady state reaction at varying concentrations of $p$-NPGU, and calculating $(E_0 - [E-GB])/[E_0]$, the fraction of enzyme not yet acylated, at various times. Plots of $\ln [(E_0 - [E-GB])/[E_0]]$ versus time have as their slope $-b$, $b$ being the first order rate constant of the pre-steady state reaction at a given titrant concentration. A plot of the reciprocal of the operational first order rate constant so obtained versus $1/(p$-NPGU)$_0$ yields $1/k_2$ as the intercept of the ordinate, and $K_1/k_2$ as the slope (12).

To determine $K_m$, it is necessary to know $k_3$, the rate constant of deacylation. This constant is best determined by actual isolation of the acyl-enzyme, incubation under the conditions used for titration, and observation of the recovery of enzymic activity. A plot of $\ln [([E_0 - [E-GB]])/[E_0]]$ versus time will have a slope $-k_1$.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

Bovine Factor X was purified from large volumes of oxalated bovine blood by the method of Jackson et al. (13). The product so obtained was essentially homogeneous upon disc gel electrophoresis, except for minor contaminants which were judged to comprise less than 5% of the total protein stainable by Amido black.

Factor X was assayed by the technique of Bachmann et al. (4). The only modification made in this procedure was the substitution of partially purified Russell's viper venom coagulant protein for the crude venom in the venom-ceilamin mixture. Factor X-deficient plasma for the assay was obtained from Sigma. A commercial preparation of celamin (Platelet, Warner-Chilcott Laboratories) was used. A standard dilution curve was prepared using pooled human plasma (Coagulation Control, Ortho Pharmaceutical). One unit of Factor X activity was defined as that amount present in 1 ml of this pooled human plasma. A linear dilution curve is obtained by plotting the logarithm of the number of units of Factor X present in dilutions of the reference plasma versus the logarithm of the clotting time.

Clotting activity of Factor Xa was assayed by a modification of the Bachmann technique in which no Factor X activator (Russell's viper venom or coagulant protein) was added to the celamin suspension. The "standards" used in this assay were highly purified samples of Factor X which had been fully activated according to the procedure described below. Plots of log of clotting time versus log of Factor Xa concentration were linear and highly consistent between assays performed on activation mixtures from the same Factor X preparation as well as between different preparations of Factor X (Fig. 1).

Solutions to be assayed for clotting activities were diluted with Michaelis buffer (3.6 $\times$ 10$^{-3}$ M sodium acetate, 3.6 $\times$ 10$^{-3}$ M sodium Veronal, and 1.45 $\times$ 10$^{-4}$ M NaCl adjusted with HCl to pH 7.35) containing 0.1 mg of bovine serum albumin per ml. A BRL Fibrometer with a 0.4-ml probe was used in all clotting assays.

Russell's viper venom was obtained from the Miami Serpentarium Laboratories, Miami, Florida. Partial purification of the Factor X-activating coagulant protein was performed by chromatography of crude venom on DEAE-cellulose (Schleicher and Schuell, type 20) with 0.01 M pH 8.5 Tris-phosphate, followed by a linear gradient from 0 to 0.4 M NaCl in 0.01 M, pH 6.0 Tris-phosphate (14). Coagulant protein obtained by this procedure was essentially free of esterase activity toward Tos-Arg-OMe and possessed clotting specific activity about 12 times higher than that of crude venom. Clotting activity of venom fractions was assayed on normal oxalated human plasma by a one-stage method (14). $p$-Nitrophenyl-$p'$-guanidinobenzoate hydrochloride was synthesized as described by Chase and Shaw (15).

Bio-Gel P-2 was a product of Bio-Rad Laboratories, Richmond, California. Water was passed through a Barnstead demineralizer, then distilled in a Corning all glass still. Concentration of total protein in solutions was determined by absorbance at 280 nm; $E_{1%}$ for Factor X = 12.4 (13), for Russell's viper venom coagulant protein $E_{1%}$ = 14.7 (14).

**Activation of Factor X**—Activation was carried out in volumes of 1 to 3 ml containing 1.5 mg per ml of purified Factor X, 0.5 M NaCl, 0.005 M CaCl$_2$, 0.01 M Tris-phosphate, pH 7.5 to 7.8. 3.3 $\mu$L (0.7 $\mu$g) of coagulant protein was added per ml of activation mixture, and the mixture was incubated at 37°. The ratio of Factor X to coagulant protein was approximately 2000:1 (w/w). Activation was considered to be complete when Factor Xa concentration as determined by the specific clotting assay had reached a maximum value (70 to 80 min). By the criteria of clotting activity and $p$-NPGU titration, Factor Xa is stable in the activation mixture for more than 24 hours at 37°.

**Titration of Factor Xa**—Reaction of Factor Xa with $p$-NPGU was followed at 402 nm in a Gilford model 240 spectrophotometer at 25°. The spectrophotometer was equipped with a model 244 cuvette positioner and a model 242 recorder. In a typical titration, a 1-ml cuvette containing 300 $\mu$L of Factor Xa in 0.95 ml of 0.1 M sodium veronal buffer, pH 8.3, with 0.02 M CaCl$_2$, was balanced with a blank cell containing the same volume of buffer solution alone (nucleophilic buffers such as Tris should be avoided or kept to a minimum concentration in the titration mixture because they catalyze the nonspecific hydrolysis of $p$-NPGU (11); when Tris was present in the buffer in which Factor Xa was dissolved, its concentration was carefully matched in the blank cuvette). Using matched micropipettes, 50 $\mu$L of a 0.01 M solution of $p$-NPGU in dimethylformamide were simultaneously added to each cuvette, to give a final concentration of 5 $\times$ 10$^{-4}$ M $p$-NPGU, the cell contents were mixed simultaneously at time zero by inverting several times, and the cuvettes were then returned to the instrument. The pre-steady state reaction is complete in approximately 2 min under these conditions. Factor Xa at comparable concentrations does not give a "burst" of $p$-nitrophenol under the same conditions. To determine the effect
of the presence of Russell's viper venom coagulant protein, a titration was performed in which Factor X was omitted and coagulant protein was present at a concentration of 0.14 µg per ml. The resulting nitrophenol production was indistinguishable from that in the blank cuvette containing only buffer and p-NPGB.

The concentration of p-nitrophenol is calculated from the zero time intercept of the extrapolated steady state absorbance minus that of the blank cell. In the Gilford spectrophotometer, ε400 for p-nitrophenol at pH 8.3 was 16,400.

**Determination of Kinetic Constants**—K₁ and K₄ were determined by titrating Factor Xa with various concentrations of p-NPGB. The presteady state reaction occurs slowly enough that continuous recording of this part of the reaction, beginning about 10 s after mixing (time zero) is easily accomplished. [E]₀ − [E-GB] is determined as the difference between the concentration of p-nitrophenol indicated by extrapolation of the steady state line back to time zero, and the actual concentration of p-nitrophenol which has been released at various times during the presteady state reaction. An excess of p-NPGB at least 40-fold over enzyme was used in these experiments. The operational first order rate constant k₁ was determined as described under “Theory of Titration” for the reaction at each concentration of p-NPGB by least squares analysis. A plot of 1/b versus 1/[p-NPGB] then yields the constants k₂ and K₃ as described under “Theory of Titration.”

The constant k₁ was determined as previously described (11) by the addition of 30 µl of 0.01 M p-NPGB in dimethylformamide to 2.3 ml of a solution of Factor Xa in 0.01 M Tris-phosphate, pH 7.5, containing 0.5 µl NaCl. Final concentration of p-NPGB was 1.3 × 10⁻⁴ M; the normality of Factor Xa was 2.0 × 10⁻⁵ M. Acylation was allowed to proceed for 15 min at 25°, then a 2-ml aliquot was applied to a column (0.9 × 28 cm) of Bio-Gel P-2 which was equilibrated with 0.1 M sodium Veronal buffer, pH 8.3, containing 0.02 M CaCl₂. After the void volume (5.4 ml), 4.8 ml of eluate were collected and incubated at 25°. At intervals, aliquots of the deacylation mixture were titrated with p-NPGB. The time at which the reaction mixture had sunk into the column was taken as zero time. The concentration of potentially active enzyme, [E]₀, was the normality measured by p-NPGB titration after 70 hours incubation of the deacylation mixture. This represented 93% of the original activity. The fraction of the acyl-enzyme remaining at each time, [GB-El]/[E]₀, was calculated and the slope, −k₃, of the plot of ln ([GB-El]/[E]₀) versus time was determined.

"Postburst" p-nitrophenol production, due to the combined turnover of p-NPGB at the active site plus the apparently nonspecific catalysis of p-NPGB hydrolysis by other parts of the protein (15), was measured from the observed steady state rate of p-nitrophenol production by different amounts of enzyme at various p-NPGB concentrations, and divided by the total concentration of enzyme. The operational rate constants so obtained, Kₚ₄, were plotted versus p-NPGB concentration. They should describe a straight line, of which the intercept is the first order (in enzyme) rate constant, K₃, of specific nitrophenol production, and the slope is the second order rate constant, K₄, of nonspecific nitrophenol production.

Dependence of burst size on p-NPGB concentration was determined by performing the titration as already described at constant enzyme concentration and various titrant concentrations, and plotting 1/√π versus 1/[p-NPGB].

**RESULTS**

**Determination of Kinetic Constants**—The linear nature of the plots of 1/b versus 1/[p-NPGB], shown in Fig. 2, and of ln ([GB-El]/[E]₀) versus time, shown in Fig. 3, show that the reaction of Factor Xa with p-NPGB adheres to the titration theory developed by Bender et al. (10). Table I summarizes the determined and derived kinetic constants. Of particular importance in confirming the utility of p-NPGB titration of Factor Xa is the large value of k₂/k₃. However, the magnitude of K₄ app is greater than those values observed in the reactions of trypsin, thrombin, and plasmin with p-NPGB (11). Therefore, the concentration of Factor Xa in the reaction is large value of k₂/k₃.

![Fig. 2. Plot of operational first order rate constants of the acylation of Factor Xa versus reciprocal of p-NPGB concentration to determine k₁ and K₄. Concentric circles represent identical duplicate values.](http://www.jbc.org/)

![Fig. 3. Deacylation of guanidinobenzoyl-Factor Xa. Closed circles represent the fraction of Factor Xa remaining acylated at the indicated times, as determined by p-NPGB titration of the deacylation mixture. k₃ was determined as the negative slope of the least squares line for these points. Coincidence of the rate of return of clotting activity with the rate of deacylation is shown by superimposing the percent of original clotting activity (X) at given times on the deacylation line.](http://www.jbc.org/)
Comparison of kinetic constants of reaction of bovine Factor Xa and bovine \( \beta \)-trypsin with \( p \)-NPGB

<table>
<thead>
<tr>
<th>Constant</th>
<th>Factor Xa</th>
<th>( \beta )-Trypsin$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_b )</td>
<td>( 17,000 \times 10^{-3} ) M</td>
<td>( 6.1 \times 10^{-7} ) M</td>
</tr>
<tr>
<td>( k_c )</td>
<td>( 0.085 ) s(^{-1} )</td>
<td>( 1.95 ) s(^{-1} )</td>
</tr>
<tr>
<td>( k_2 )</td>
<td>( 4.3 \times 10^{-4} ) s(^{-1} )</td>
<td>( 3.4 \times 10^{-5} ) s(^{-1} )</td>
</tr>
<tr>
<td>( k_2/k_1 )</td>
<td>( 2 \times 10^3 )</td>
<td>( 57 \times 10^5 )</td>
</tr>
<tr>
<td>( K_{\text{app}} )</td>
<td>( 510 \times 10^{-4} ) M</td>
<td>( 4.7 \times 10^{-8} ) M</td>
</tr>
<tr>
<td>( K_{ph} )</td>
<td>( 6.2 \times 10^{-4} ) s(^{-1} )</td>
<td>( 0.48 \times 10^{-4} ) s(^{-1} )</td>
</tr>
</tbody>
</table>

$^a$ Values for bovine trypsin are the data of Chase and Shaw (3).

$^b$ \( K_{ph} \) is the first order rate constant of observed postburst nitrophenol production at the \( p \)-NPGB concentration most useful for active site titration for each enzyme (5\( \times \) 10\(^{-4} \) M for factor Xa; 5\( \times \) 10\(^{-4} \) M for trypsin).

Concentration. The relatively high concentration of \( p \)-NPGB needed for valid titrations of Factor Xa at a single titrant concentration and the resulting rapid development of background color due to spontaneous hydrolysis of the titrant place additional constraints on the titration technique, i.e. that (a) the titrant solution have a low initial absorbance at 402 nm, and (b) that pipetting of titrant be performed simultaneously with matched micropipettes. Although spontaneous decomposition of stock solutions of \( p \)-NPGB in dimethylformamide occurs fairly rapidly at room temperature, they are sufficiently stable at 0-4°C to remain usable for several days. \( p \)-NPGB solutions giving initial blank values higher than 0.03 absorbance unit at a concentration of 5\( \times \) 10\(^{-4} \) M may seriously impair the accuracy of titrations at the lower enzyme concentrations (1 to 5\( \times \) 10\(^{-4} \) M). A plot of postburst \( p \)-nitrophenol production by Factor Xa as a function of \( p \)-NPGB concentration is shown in Fig. 5. The intercept, \( K_{1+b} \), should be equal to \( k_b \), the first order rate constant for deacylation. The fact that the observed value of \( K_{1+b} \) 1.0 \( \times \) 10\(^{-4} \) s\(^{-1} \), is considerably less than \( k_b \) probably reflects the low level of precision in determining \( K_{1+b} \). At 5\( \times \) 10\(^{-4} \) M \( p \)-NPGB, \( k_{ph} \) is 6.2 \( \times \) 10\(^{-4} \) s\(^{-1} \), more than 10 times \( k_b \), the rate constant for deacylation. This apparently nonspecific nitrophenol production is not great enough to seriously decrease the accuracy of Factor Xa titrations, although it is higher than that reported for trypsin (Table I) (11).

**Titration of Factor Xa**—A sample of bovine Factor Xa was titrated by the procedure given and found to be 2.49 \( \times \) 10\(^{-3} \) M in active sites. The actual protein concentration, assuming a zymogen molecular weight of 53,000 (16), was 2.83 \( \times \) 10\(^{-4} \) M, therefore, this sample was 88% active (Fig. 6). Several other preparations of bovine Factor X which were homogeneous by disc gel electrophoresis were found, after activation, to contain 95 to 100% of the active sites expected, using the above molecular weight and assuming the presence of a single active site per molecule. On the basis of these data it is proposed that activation of bovine Factor X by the Russell’s viper venom coagulant protein generates one active site per molecule of zymogen.

**Clotting Activity and Acylation-Decylation**—The reaction of Factor Xa with \( p \)-NPGB results in greater than 95% inhibition of Factor Xa clotting activity. However, when Factor Xa was separated from titrant and allowed to undergo deacylation, clotting activity was observed to return at the same rate as the \( p \)-NPGB esterase activity (Fig. 2). These data strongly suggest that the site of reaction of \( p \)-NPGB with bovine Factor Xa is the same as, or closely associated with, a site which is essential for its specific clotting activity.

**Potential \( p \)-NPGB-active Sites and Specific Clotting Activity**—
Table II

Number of p-NPGB-active sites and clotting activity in different preparations of Factor X

One unit of Factor Xa is defined as the maximum clotting activity derived from the complete activation of 1 mg of purified Factor X by Russell's viper venom coagulant protein under the conditions described (see "Activation of Factor X" under "Materials and Methods").

<table>
<thead>
<tr>
<th>Factor Xa preparation</th>
<th>Bachmann assay specific clotting activity</th>
<th>Active site per mole of Factor Xa</th>
<th>Factor Xa specific clotting activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-71</td>
<td>33</td>
<td>0.94</td>
<td>1.0</td>
</tr>
<tr>
<td>D-71</td>
<td>169</td>
<td>1.02</td>
<td>1.0</td>
</tr>
<tr>
<td>M-72</td>
<td>94</td>
<td>0.98</td>
<td>0.9</td>
</tr>
</tbody>
</table>

As described previously in Titration of Factor Xa, several different preparations of Factor X were found to possess one potential p-NPGB active site per molecular weight of 53,000. However, specific clotting activity as defined by the Bachmann assay was observed to vary considerably from one preparation to the next. Three different preparations of Factor X were observed to vary over a 5-fold range in their Bachmann specific activities. However, upon complete activation by the Russell's viper venom coagulant protein, all three preparations were found by p-NPGB titration to contain 1 mole of active sites per mole of protein (Table II). Furthermore, these samples of activated Factor X varied little in the clotting assay modified for Factor Xa. The Bachmann procedure is a rate assay, and is dependent upon, among other things, the rate of activation of Factor X by the coagulant protein. The differences in Bachmann specific activity might then be attributed to differences in the three preparations which affected the rate of activation. The existence of multiple forms of Factor X in preparations from bovine plasma has been recently demonstrated (16-18). It is possible that these forms have different characteristics with respect to activation by Russell's viper venom coagulant protein, and that their presence in differing ratios in the different preparations of Factor X result in variations in Bachmann specific activity. However, we have been unable to consistently demonstrate any appreciable differences in activation rate among the various Factor X preparations in separate experiments. At the present time this question remains unresolved, and is currently under investigation.

Discussion

It is apparent that, under the proper conditions, p-NPGB is an excellent titrant for bovine Factor X which has been activated by Russell's viper venom coagulant protein. The single most important feature of the reaction is the slow deacylation of the acyl-enzyme. The resulting low value for K_{app} allows titrations at a single substrate concentration. Titration of Factor Xa at 5 \times 10^{-4} M p-NPGB gives accurate results at active enzyme concentrations of 1 \times 10^{-4} M (53 \mu g/ml) or higher. Roberts et al. (19) have recently described the titration of Factor Xa using the fluorescent substrate 4-methylumbelliferyl p-guanidinobenzoate. Use of this substrate allowed molarity determination at enzyme concentrations more than 10 times lower than is possible with p-NPGB. However, the greater availability of sensitive spectrophotometers over spectrophuorimeters probably renders the p-nitrophenyl ester more generally useful.

Titration of Factor Xa with p-NPGB is limited to purified enzyme preparations, as has been noted for trypsin, thrombin, and plasmin (11), since the presence of other esterases with reactivity toward p-NPGB would tend to invalidate the results. However, the failure of the Russell's viper venom coagulant protein to react with p-NPGB, as described in the section on "Materials and Methods," indicates that, at the levels at which it was present in the solutions of Factor Xa assayed here, it does not interfere with the titration.

Active site titration of Factor Xa should prove useful in studying different naturally occurring or artificially modified forms of the enzyme. Whereas rate assays cannot discriminate between modification and destruction of active sites, a titration measures all functional species. The preparations of Factor X which differed in Bachmann clotting activity, yet contained the same amount of potential active sites is an excellent case in point.

The same active site present in Factor Xa activated by Russell's viper venom coagulant protein is responsible for both p-NPGB esterase activity and clotting activity, as shown by the identical rates of return of both of these activities upon deacetylation of the isolated guanidinobenzoyl-enzyme. Thus, the presence at the Factor Xa clotting site of an essential serine residue, functioning in a manner analogous to that seen in the common serine esterases such as trypsin and chymotrypsin, is indicated by the studies presented here. Earlier evidence for the serine esterase nature of Factor Xa was provided when it was demonstrated that both its clotting and Tos-Arg-OMe esterase activities were irreversibly inhibited by diisopropylfluorophosphate and phenylmethane sulfon fluoride (20, 21).

Factor Xa exhibits a limited specificity for low molecular weight substrates. In contrast to trypsin, thrombin, and plasmin, Factor Xa hydrolyzes arginine esters much more readily than similar lysine derivatives (22, 23). The present work extends the known specificity of Factor Xa to an ester of p-guanidinobenzoic acid. A comparison of the kinetic constants of the reaction of p-NPGB with bovine Factor Xa and bovine trypsin (Table I) reveals large differences in K_{m} and k_{cat}, however k_{cat} the deacylation rate constant, is quite similar for both enzymes. Furthermore, in deacylation rate of the guanidinobenzoyl enzyme, Factor Xa resembles trypsin more closely than two other enzymes involved in blood coagulation, bovine thrombin (k_{cat} = 98 \times 10^{-3} s^{-1}), and streptokinase-activated human plasmin (k_{cat} = 0.53 \times 10^{-3} s^{-1}) (15).

Finally, it has been suggested that 1 molecule of zymogen Factor X yields, upon activation, 2 molecules of Factor Xa (24). It is obvious from the results presented here that activation of bovine Factor Xa by the Russell's viper venom coagulant protein yields only 1 mole of active sites per mole of zymogen. The activation of Factor Xa by the intrinsic and extrinsic physiological systems, and the resulting p-NPGB reactivity is currently under investigation.

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