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Inhibition of Factor IX_a and Factor X_a by Antithrombin III/Heparin during Factor X Activation*

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We investigated the kinetics of the inhibitory action of antithrombin III and antithrombin III plus heparin during the activation of factor X by factor IX_a. Generation and inactivation curves were fitted to a three-parameter two-exponentional model to determine the pseudo first-order rate constants of inhibition of factor IX_a and factor X_a by antithrombin III/heparin. In the absence of heparin, the second-order rate constant of inhibition of factor X_a generated by factor IX_a was 2.5-fold lower than the rate constant of inhibition of exogenous factor X_a. It appeared that phospholipid-bound factor X protected factor X_a from inactivation by antithrombin III. It is, as yet, unclear whether an active site or a nonactive site interaction between factor X_a and factor X at the phospholipid surface is involved. The inactivation of factor IX_a by antithrombin III was found to be very slow and was not affected by phospholipid, calcium, and/or factor X. With unfractionated heparin above 40 ng/ml and antithrombin III at 200 nM, the apparent second-order rate constant of inhibition of exogenous and generated factor X_a were the same. Thus, in this case phospholipid-bound factor X did not protect factor X_a from inhibition. In the presence of synthetic pentasaccharide heparin, however, phospholipid-bound factor X reduced the rate constant about 5-fold. Pentasaccharide had no effect on the factor IX_a/antithrombin III reaction. Unfractionated heparin (1 μg/ml) stimulated the antithrombin III-dependent inhibition of factor IX_a during factor X activation 400-fold. In the absence of reaction components this stimulation was 65-fold. We established that calcium stimulated the heparin-dependent inhibition of factor IX_a.

Antithrombin III (ATIII)[1] is a plasma protein that plays an important role in the regulation of a series of activation reactions leading to clot formation (1). From studies with purified serine proteases and inhibitor, it is well known that the main target of ATIII is thrombin, whereas the activity toward activated factor X and activated factor IX is less pronounced (2, 3).

Heparin accelerates the rate at which ATIII inactivates the serine proteases of the blood coagulation system. Dependent on the molecular weight of the heparin preparation used, the rate enhancing effect of heparin on the inhibition of factor IX_a and factor X_a seems to vary (4). Inactivation of factor IX_a was found to decrease with decreasing molecular weight, whereas the inhibition of factor X_a was potentiated by low molecular weight heparin fractions that have virtually no effect on the inactivation of factor IX_a.

Factor X_a is the common product of the intrinsic and extrinsic coagulation pathway. Its formation has been postulated to be an important target for the control of thrombin formation (5, 6). Furthermore, factor X_a may play a role in the activation of factors V, VIII, and VII (7–9). Although the effect of ATIII and ATIII plus heparin on isolated factor X_a and factor IX_a has been studied (10–13), little is known about the kinetics of inactivation of factor IX_a and factor X_a during factor X activation.

Previous studies reported the effect of ATIII on factor X_a generated by simple activators of factor X, such as the factor X-activating protein from Russell's viper venom (14). Recently Jesty (15) described the inhibition of *in situ* generated factor X_a by ATIII. When using factor IX_a as an activator of factor X, however, he could not accurately analyze overall factor X_a generation and inactivation. This author also did not present kinetic parameters on the inactivation of factor X_a generated by factor IX_a.

In this paper we present a method which allows the simultaneous analysis of the kinetics of both factor IX_a and factor X_a inactivation by ATIII or ATIII plus heparin during factor X activation. By this approach we were able to assess the relative importance of factor IX_a inhibition and factor X_a inhibition to the overall effect of unfractionated heparin (UFH) and synthetic pentasaccharide heparin on factor X_a generation. We report some marked differences between the kinetics of the inhibition reactions during factor X activation and those observed in the absence of factor X, phospholipid, and/or calcium.

EXPERIMENTAL PROCEDURES

Materials—The synthetic peptide substrates Bz-Ile-Glu-(Piperidyl)-Gly-Arg-*p*-nitro-anilide hydrochloride (S-2337) and H-D-Phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2238) were purchased from Kabi. Fatty acid-free ovalbumin was a product of Sigma. All reagents used were of the highest grade commercially available.

Unfractionated heparin (168 USP units/mg) was from Pharmuka Laboratories, France, and synthetic pentasaccharide heparin (800 anti-factor X_a units/mg and no anti-thrombin activity) was a kind gift from Institut Choay, France.

Proteins—Bovine ATIII was purified by the method of Thaler and Schmer (16). The ATIII was subsequently freed of heparin contamination by ion-exchange chromatography (17). All other bovine clotting factors were prepared and quantitated as previously described (18–20).

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[1] The abbreviations used are: ATIII, antithrombin III; UFH, unfractionated heparin; PS, phosphatidylserine; PC, phosphatidylcholine.

Phospholipid Vesicles—Phospholipid vesicles were made from a mixture of phosphatidylserine (PS) and phosphatidylcholine (PC) as previously described (20). Vesicles composed of 25 mol % PS and 75 mol % PC were used throughout the experiments unless stated otherwise.

Determination of Inhibition Rate of Purified Protease—The inactivation of factor X_a by ATIII was investigated under pseudo first-order conditions essentially as previously described (3). The inactivation of factor IX_a was followed by incubations by factor IX_a with ATIII/heparin in the presence or absence of accessory components, and at several time points residual factor IX_a activity was assayed as described previously (21) with the following modifications. Factor IX_a-containing samples were diluted 1:1000 into buffer containing 50 mM Tris-HCl, pH 7.9, 175 mM NaCl, 0.5 mg of ovalbumin/ml, 5 mM $CaCl_2$, 20 μM phospholipid, and 100 nM thrombin, prewarmed at 37 °C for 4 min. Factor VIII:C (10 units/ml) was added and allowed to be activated for 15 s, after which the reaction was started by the addition of factor X (0.5 μM). The rate of factor X activation was determined by measuring the amount of factor X_a formed after 0.5 and 1.5 min using the chromogenic substrate S2337 as described earlier (3). Factor X_a formation was linear in time for at least 2 min. From the observed rate of factor X activation, the amount of factor IX_a present in the sample was calculated using a calibration curve made with known amounts of active site-titrated factor IX_a.

Inactivation of Factor IX_a and Factor X_a by ATIII/Heparin during Factor X Activation—Factor X (1 μM) was activated by 80 nM factor IX_a, in the presence of 100 μM phospholipid, 10 mM $CaCl_2$ and heparin when indicated in 50 mM Tris-HCl, 175 mM NaCl and 0.5 mg/ml ovalbumin, pH 7.9, at 37 °C. At timed intervals after the addition of factor IX_a, aliquots (7 μl) were removed and assayed for factor X_a activity. Factor X_a formation was linear in time for at least 30 min. After 10 min an aliquot (200 μl) was taken from the reaction mixture and added to a solution (14 μl) containing ATIII and rabbit anti-factor IX IgG. At the same time another aliquot (200 μl) was taken from the reaction mixture and added to a solution (14 μl) containing ATIII alone. Addition of anti-factor IX resulted in instantaneous and complete inhibition of factor X_a formation but had no effect on factor X_a activity or on the ATIII/factor X_a reaction. After the addition of ATIII or ATIII plus anti-factor IX, timed samples (7 μl) were taken and assayed for factor X_a activity.

Because the rabbit anti-factor IX IgG neutralized unfractionated heparin, anti-factor IX IgG could not be used to block further factor X_a formation to measure the effects of UFH on X_a inhibition. Therefore, we modified the conditions for the determination of the effect of UFH on the inactivation of factor IX_a and factor X_a during factor X activation. Factor X was activated in the presence of UFH under the same conditions as described before. Factor X_a formation was monitored by removing aliquots (7 μl) and assayed for factor X_a activity. After 10 min, 200 μl was removed and added to a solution of ATIII (14 μl). From this reaction mixture, timed samples (10 μl) were taken, and assayed for factor IX_a activity as already described. From the same mixture, aliquots (7 μl) were taken and assayed for factor X_a activity. The pseudo first-order rate constants of inhibition of factor X_a and factor IX_a were calculated as described in the following section.

Kinetic Analysis of the Inhibition of Factor IX_a and factor X_a during Factor X Activation—Under the experimental conditions of our studies, factor X_a formation was linear during the time period of the experiment when ATIII was absent. This was obtained by using factor X concentrations well above the K_m for factor X and less than 20% of factor X consumed during the experiment. Secondly, the ATIII concentrations were in excess over the factor X_a and factor IX_a concentrations, resulting in pseudo first-order kinetics of inhibition.

When ATIII or ATIII plus heparin was present during the activation of factor X by factor IX_a we assumed that the amount of amidolytic active factor X_a present at time t is a function of the rate of inactivation of factor IX_a and the rate of inactivation of factor X_a. In formula this reads:

$$\frac{d}{dt}[X_a] = v\, e^{-k_{IXa}t} - k_{Xa}[X_a] \quad (1)$$

where v is the rate of factor X_a formation in the absence of ATIII or ATIII plus heparin, k_{IX_a} and k_{X_a} are the pseudo first-order rate constants of inhibition of factor IX_a and factor X_a, respectively.

Integration of Equation 1 by Laplace transformation gives the following equation for the generation of factor X_a concomitant with its decay.

$$[X_a]_t = \frac{v}{k_{Xa} - k_{IXa}}\left(e^{-k_{IXa}t} - e^{-k_{Xa}t}\right) \quad (2)$$

The parameters, v, k_{IX_a} and k_{X_a}, could be determined by fitting Equation 2 to the experimental data points. However, we preferred an analysis of our data which also allows independent determinations of the parameters v and k_{X_a}. To this end, the activation of factor X in the absence of ATIII was performed for t_0 min, followed by the addition of ATIII or ATIII plus anti-factor IX IgG.

When ATIII is added at time t_0, the factor X_a concentration then obtained

$$[X_a]_{t=t_o} = vt_o \quad (3)$$

has to be included into Equation 2:

$$[Xa]_t = \frac{v}{k_{Xa} - k_{IXa}}\left(e^{-k_{IXa}(t-t_o)} - e^{-k_{Xa}(t-t_o)}\right) + [Xa]_{t=t_o}\, e^{-k_{Xa}(t-t_o)} \quad (4)$$

When ATIII plus anti-factor IX IgG are added at time t_0, factor X_a formation is completely blocked but not the decay of factor X_a and reduces Equation 4 to

$$[Xa]_t = [Xa]_{t=t_o}\, e^{-k_{Xa}(t-t_o)} \quad (5)$$

If $t \leq t_0$ then

$$[X_a]_t = vt \quad (6)$$

Equations 4–6 were simultaneously fitted to experimental data points, obtained in the absence or presence of ATIII/heparin or in the presence of ATIII/heparin plus anti-factor IX IgG. The fit procedure, based upon a nonlinear least squares regression method which uses a reiterative Gauss-Newton algorithm (22), produced v, k_{IX_a} and k_{X_a}. In addition, k_{X_a} and v were separately calculated using Equations 5 and 6, respectively, to estimate the validity of the parameter values as calculated by the fit procedure. The reported errors represent the 95% confidence interval, about twice the S.D. of the estimate.

RESULTS

Determination of Factor IX_a and Factor X_a Inactivation by ATIII during Factor X Activation—Factor X was activated by factor IX_a in the presence of phospholipid and calcium. After 10 min, to a part of the reaction mixture a solution of ATIII and rabbit anti-factor IX IgG was added, and at the same time ATIII alone was added to another part of the reaction mixture. The result of such a typical experiment is shown in Fig. 1. *Curve A* shows linear factor X_a generation and addition of both anti-factor IX IgG and ATIII at $t = 10$ min resulted in the exponential decay of the formed factor X_a (*curve C*), whereas addition of ATIII alone resulted in significantly higher residual factor X_a activities because of ongoing factor X_a generation (*curve B*).

Equations 4–6 (see "Experimental Procedures") were fitted to the experimental data and the factor X_a generation and inactivation curves, indicated by the *solid lines*, are the results of the best fit to the data points. The pseudo first-order rate constants of inactivation of factor X_a and factor IX_a by ATIII as well as the velocity of factor X_a formation in the absence of ATIII were obtained by the computer fit procedure. In this typical experiment the velocity of factor X_a formation (v) was found to be 2.1 ± 0.1 nM min^{-1}, whereas the pseudo first-order rate constants (k) of inactivation of factor X_a by ATIII (1 μM) was 0.21 ± 0.01 min^{-1}. However, the pseudo first-order rate constant of inactivation of factor IX_a by ATIII was too small to measure accurately (<0.01 min^{-1}).

The calculated parameters v and k can also be determined directly from part A and part C of the curves, respectively. The rate of factor X_a formation was 2.1 nM/min (part A). The pseudo first-order rate constant of inactivation of factor X_a by ATIII was determined from the semilogarithmic plot of

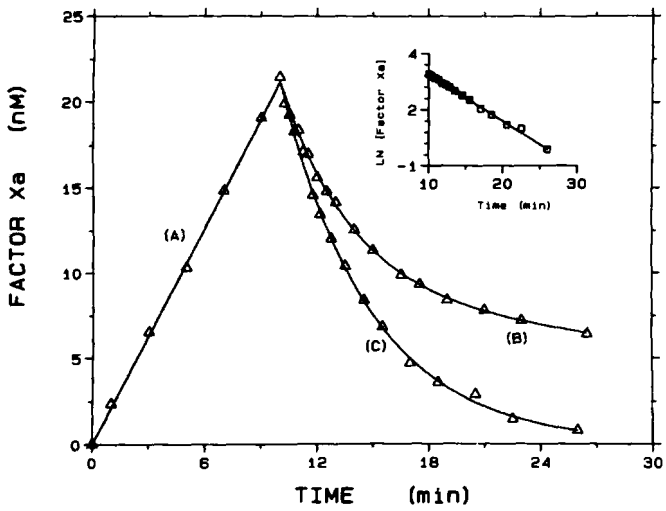

FIG. 1. **Factor X_a generation in the presence of ATIII.** Factor X (1 μM) was activated by 80 nM factor IX_a in the presence of 100 μM phospholipid and 10 mM calcium (*curve A*). At 10 min, to 200 μl of the reaction mixture, 1 μM ATIII was added (*curve B*). At the same time, to another 200 μl of the reaction mixture 1 μM ATIII and anti-factor IX IgG was added (*curve C*). Factor X_a activity was assayed as described under "Experimental Procedures." The *solid lines* represent factor X_a generation and inactivation as calculated by the fit procedure. *Inset*, semi-logarithmic replot of *curve C*.

part C of the curve *versus* time, $k_{k_a} = 0.21$ min^{-1} (Fig. 1, *inset*). Thus, the independently determined values for the rate of factor X activation and the rate constant of factor X_a inactivation were the very same as the values from the fit-procedure, indicating the validity of our mathematical model.

This procedure was repeated in the presence of various ATIII concentrations (0–1.5 μM). A linear relationship between the pseudo first-order rate constants of inhibition of factor X_a and the ATIII concentration was found. The second-order rate constant was $2.1 \pm 0.1 \times 10^5$ M^{-1} min^{-1}. The inactivation of factor IX_a during factor X activation at the ATIII concentrations used was too small ($k_{IX_a} < 0.010$ min^{-1}) to be measured accurately. Higher ATIII concentrations, however, could not be used under these conditions because then the factor X_a generated by factor IX_a was too rapidly inactivated and resulted in very low residual factor X_a activities.

In order to determine the inactivation of factor IX_a at higher ATIII concentrations we made use of a factor IX_a assay in which functional factor IX_a activity was determined via the factor $VIII_a$-enhanced activation of factor X (see "Experimental Procedures"). The pseudo first-order rate constants of factor IX_a inactivation by ATIII in the presence of factor X, phospholipid, and calcium were plotted *versus* the ATIII concentration (0–10 μM). A second order-rate constant of 8.2×10^3 M^{-1} min^{-1} was calculated. In the absence of the reaction components (factor X, phospholipid, and Ca^{2+}) the second-order rate constant of factor IX_a inactivation was found to be the same as in their presence ($k_2 = 8.2 \times 10^3$ M^{-1} min^{-1}).

Effect of Accessory Components on Factor X_a Inactivation by ATIII—As several investigators reported contradictory effects of phospholipid or calcium (23–27) on the inactivation of factor X_a by ATIII, we examined whether our decay constants, determined by the fit-procedure during factor X activation, differed from the decay constants of factor X_a in free solution.

To this end, we incubated purified factor X_a (10 nM) with ATIII (0–1.5 μM), either in the absence or presence of phospholipid (100 μM), calcium (10 mM), or both. The second-order rate constant of inhibition of factor X_a by ATIII in free solution was 5.0×10^5 M^{-1} min^{-1}, and neither the addition of phospholipid or calcium or phospholipid plus calcium protected factor X_a from being inactivated by ATIII. Under all conditions the rate constants were $5.0 \pm 0.1 \times 10^5$ M^{-1} min^{-1}. However, these rates of inactivation of factor X_a appeared to be still about 2.5-fold faster when compared to the rates of inactivation under the conditions of factor X activation (see previous section). In order to find an explanation for this discrepancy, we added, besides phospholipid and calcium, factor X (1 μM) to the inactivation mixture. The second-order rate constant of 2.1×10^5 M^{-1} min^{-1} for factor X_a inactivation by ATIII thus obtained, was the same as determined during factor X activation.

These results suggest that the decrease of the second-order rate constant of the factor X_a-ATIII reaction during factor X activation is due to the presence of phospholipid-bound factor X. Indeed, the pseudo first-order rate constant of inhibition of factor X_a in the presence of a fixed phospholipid concentration decreased with increasing factor X concentration (Fig. 2*A*). In addition, an increase of the phospholipid concentration at a fixed factor X concentration resulted in a decrease of the rate constant (Fig. 2*B*). In both cases the rate constant progressively decreased to an apparent end value of 0.2 min^{-1}.

It has been reported that bovine factor X can be converted by factor X_a into β-factor X, factor X_a, and/or β-factor X_a (28–30). In the presence of phospholipid the major reaction product appeared to be β-factor X (30). An exponentional increase in factor X_a generation as a result of the autocatalytic activation of factor X by factor X_a might explain the apparent lower pseudo first-order rate constant of inactivation of factor X_a in the presence of phospholipid-bound factor X. However, under none of our experimental conditions could an increase in amidolytic factor X_a activity be detected. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples taken from a mixture containing 10 nM factor X_a, 200 μM phospholipid, 10 mM Ca^{2+}, and 5 μM of factor X revealed the formation of β-factor X at a very slow rate. Under the same conditions but in the absence of phospholipid, no cleavage of factor X was observed over a 1-h period.

In order to explore the effect of phospholipid-bound factor X in more detail the following experiment was performed. Pseudo first-order rate constants of inactivation of factor X_a (10 nM) by ATIII (1 μM) were determined in the presence of factor X (10 μM), Ca^{2+} (10 mM) and varying concentrations of phospholipid composed of 10 mol % PS, 90 mol % PC, or

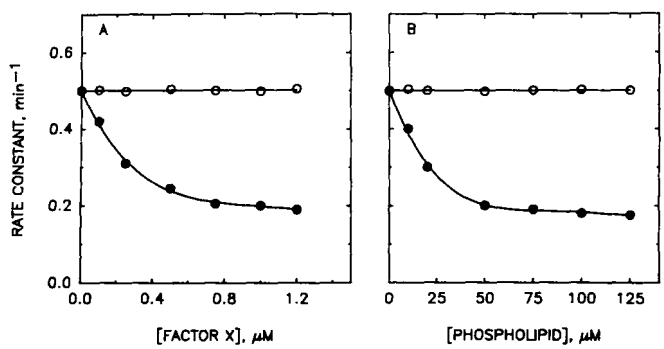

FIG. 2. **Effect of factor X and phospholipid on the inactivation of factor X_a by ATIII.** *Panel A*, factor X_a (10 nM) was incubated with ATIII (1 μM) in the presence of varying amounts of factor X, Ca^{2+} (10 mM) and 100 μM phospholipid (●) or in the absence of phospholipid (○). *Panel B*, the same incubation but in the presence of varying amounts of phospholipid, Ca^{2+} (10 mM) and 1 μM factor X (●) or in the absence of factor X (○). The pseudo first-order rate constants of inactivation of factor X_a by ATIII were determined as described under "Experimental Procedures."

25 mol % PS, 75 mol % PC. The rate constants progressively decreased until apparent end levels of 0.1 and 0.3 min^{-1} in the presence of vesicles containing 25 mol % PS or 10 mol % PS, respectively (Fig. 3). From reported factor X-phospholipid-binding parameters (31, 32) it was calculated that at each phospholipid concentration used, virtually all available phospholipid-binding sites were saturated with factor X, *i.e.* 12.3 μmol of factor X bound/mmol of 25% PS, 75% PC and 6.7 μmol factor X bound/mmol of 10% PS, 90% PC. Thus, the fraction of factor X bound to phospholipid linearly increased with the phospholipid concentration. Although factor X and factor X_a interact competitively with the same phospholipid-binding sites, factor X, by virtue of its affinity for phospholipid and factor X_a, promotes the binding of factor X_a at the surface. Hence, an apparent inhibition of the inactivation reaction might be a consequence of a decreased fluid-phase factor X_a concentration concomitant with an increase in the concentration of phospholipid-bound factor X. Additional support for our notion that the factor X_a-factor X association rate at the surface might be the controlling element was obtained from an identical set of experiments but using a nonsaturating factor X concentration (0.2 μM). The observed rate constant of the inactivation reaction went through a minimum of 0.4 min^{-1} at 60 μM phospholipid (25% PS, 75% PC) and then increased with further increase in the concentration of the phospholipid until an almost noninhibited value (0.55 min^{-1}) at 400 μM of phospholipid.

Effect of Unfractionated Heparin and Pentasaccharide on Factor IX_a and Factor X_a Inactivation by ATIII during Factor X Activation—For the determination of the pseudo first-order reaction rate constants of factor IX_a and factor X_a inactivation during factor X activation by ATIII (200 nM) in the presence of UFH (0–120 ng/ml) or pentasaccharide (0–12 ng/ml), the same fit-procedure as already described was used. However, as the rabbit anti-factor IX IgG neutralized the UFH, but not pentasaccharide, we modified the conditions for the investigation of the effect of UFH. Instead of blocking the factor IX_a activity by the addition of anti-factor IX IgG, ATIII only was added, and factor IX_a inactivation was determined separately as described under "Experimental Procedures." In order to obtain the decay constant of factor X_a generated by factor IX_a, Equations 1–4 were fitted to the experimental data with the parameters V_{X_a} and k_{IX_a} set at values calculated from direct measurements according to Equation 6 and to $[IX_a]_t = [IX_a]_0 \exp(-k_{IX_a}t)$, respectively.

Our mathematical model for analysis of the data depends on heparin-dependent factor X_a and factor IX_a inactivation being a pseudo first-order reaction. This approach will only work if the protease concentrations remain far below saturation levels (1). Therefore, pseudo first-order rate constants had to be determined as a function of the protease concentration under the same experimental conditions. To this end, factor IX_a at different concentrations (40 and 80 nM) was incubated with ATIII (200 nM) and heparin (300 ng/ml) in 50 mM tris-HCl, pH 7.9, 175 mM NaCl, 5 mM $CaCl_2$, and 0.5 mg ovalbumin/ml. Linear first-order rate plots were obtained for at least 70% of the reaction. Rate constants ± S.E. were calculated from a linear least-squares fit using six time points in triplicate to be 0.203 ± 0.017 min^{-1} (40 nM factor IX_a) and 0.213 ± 0.011 min^{-1} (80 nM factor IX_a). We concluded that the apparent pseudo first-order rate constant of inhibition of factor IX_a under our conditions and within the range used was independent of the factor IX_a concentration. This finding also holds for the inactivation of factor X_a by ATIII (200 nM) in the presence of heparin (80 ng/ml). The rate constants were 0.756 ± 0.033 min^{-1} (10 nM factor X_a) and 0.759 ± 0.039 min^{-1} (20 nM factor X_a).

Fig. 4 shows the effect of UFH and pentasaccharide on the inactivation of factor X_a by ATIII, both for free solution and for factor X_a during factor X activation. The stimulating effect of UFH on the inactivation of purified factor X_a by ATIII (Fig. 4*A*) was the same either in the presence or in the absence of phospholipids and/or calcium, with a rate constant of 0.012 min^{-1} $(ng/ml)^{-1}$. However, during factor X activation the inhibition of factor X_a generated by factor IX_a was markedly reduced at heparin concentrations up to 40 ng/ml. At higher UFH concentrations the stimulating effect was the same as observed with purified factor X_a in free solution. Because this phenomenon was also observed in the presence of factor X alone, it might indicate that factor X and ATIII compete with each other for UFH. Indeed, when increasing amounts of factor X were added to a mixture containing factor X_a (10 nM), ATIII (200 nM), and 40 ng of UFH/ml, the rate constant of the inactivation reaction progressively decreased with increasing factor X eventually reaching the value of the heparin-independent ATIII-X_a reaction. The factor X concentration that neutralized half the amount of available UFH was 0.2 μM.

The stimulating effect of pentasaccharide on the inactivation of factor X_a by ATIII was 5-fold lower during factor X activation as compared to factor X_a inactivation in free solution (Fig. 4*B*). The rate constants were 0.014 min^{-1} $(ng/ml)^{-1}$ and 0.071 min^{-1} $(ng/ml)^{-1}$, respectively. Factor X alone did not have an effect on the pentasaccharide-dependent inactivation of factor X_a. Thus, the lower rate constant during

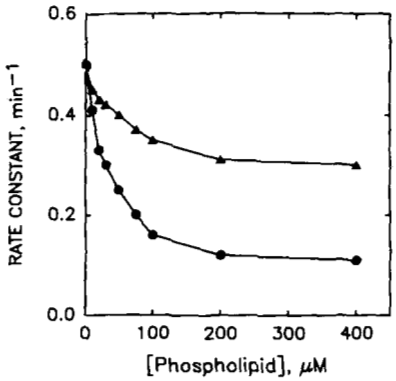

FIG. 3. **Effect of factor X and phospholipids of different compositions on the inactivation of factor X_a by antithrombin III.** Factor X_a (10 nM) was incubated with ATIII (1 μM) in the presence of factor X (10 μM), calcium (10 mM), and varying concentrations of phospholipid vesicles composed of 10 mol % PS, 90 mol % PC (▲), or 25 mol % PS, 75 mol % PC (●).

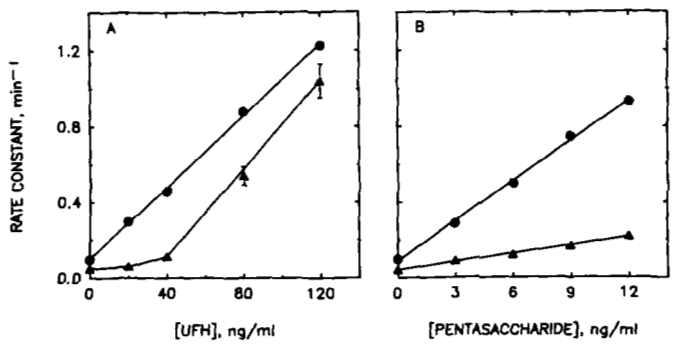

FIG. 4. **Pseudo first-order rate constants of inactivation of factor X_a by ATIII as a function of the UFH (*panel A*) and the pentasaccharide (*panel B*) concentration.** ●, factor X_a (10 nM) plus ATIII (200 nM) and ▲, factor X_a during factor X activation by [factor IX_a-phospholipid-Ca^{2+}] in the presence of ATIII (200 nM).

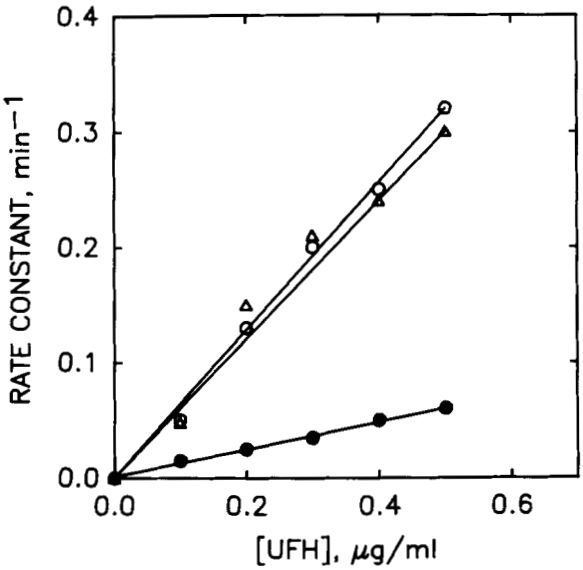

FIG. 5. **Pseudo first-order rate constants of inactivation of factor IX_a (80 nM) by ATIII (200 nM) as a function of the UFH concentration.** ●, factor IX_a alone; ○, factor IX_a during factor X activation; and △, factor IX_a in the presence of calcium (10 mM).

factor X activation was the result of the protective effect of phospholipid-bound factor X on the inactivation of factor X_a.

Pentasaccharide was unable to enhance the inactivation of factor IX_a, either in the absence or presence of calcium, phospholipid, and/or factor X. Fig. 5 shows that UFH had a very small effect on the inactivation of factor IX_a in free solution, $k = 0.11$ min^{-1} $(\mu g/ml)^{-1}$. However, during factor X activation a 6-fold higher rate constant of inactivation of factor IX_a was found, $k = 0.68$ min^{-1} $(\mu g/ml)^{-1}$. To establish the cause of this discrepancy, we examined the effect of UFH on factor IX_a inactivation in the presence of the individual components present in the factor X activation mixture. From Fig. 5 it is clear that the enhanced inactivation rate of factor IX_a by ATIII is solely due to the presence of calcium.

DISCUSSION

Several investigators have studied the inactivation of activated coagulation factors IX and X by ATIII (10–13). However, the majority of these studies dealt with the effect of ATIII on purified factor IX_a and factor X_a in free solution.

In the present paper we describe a method for the determination of decay constants under more physiological conditions by modeling the inactivation of factor IX_a and factor X_a *during* factor X activation. The validity of this model was confirmed by the close resemblance between the data computed by the fit-procedure and the experimentally obtained data points (Fig. 1). Furthermore, our experimental approach provided independent control of the velocity of factor X_a formation and the rate of inactivation of generated factor X_a. These values were in good agreement with the values as determined by the fit-procedure.

The second-order rate constant for factor X_a inactivation by ATIII thus obtained was 2.1×10^5 M^{-1} min^{-1}. Because of the much lower rate of inhibition of factor IX_a under these conditions when compared with factor X_a, we were unable to calculate the rate constant of inhibition of factor IX_a by ATIII. However, by making use of a factor IX_a assay and high ATIII concentrations, we found that the reaction of factor IX_a and ATIII proceeded with a second-order rate constant of 8.2×10^3 M^{-1} min^{-1}, about 25-fold slower when compared with the factor X_a/ATIII reaction.

As several authors have reported that human factor X_a (25) and bovine factor X_a (26) are protected from inactivation by ATIII in the presence of phospholipids, we compared the decay constant of factor X_a determined during factor X activation with the decay constant of free factor X_a. Indeed, we found that the second-order rate constant of factor X_a inactivation by ATIII was 5.0×10^5 M^{-1} min^{-1}, compared to a second-order rate constant of 2.1×10^5 M^{-1} min^{-1} during factor X activation. However, in contrast to Ellis *et al.* (24) and Walker and Esmon (26), but in accordance with Marciniak (27) and Jesty (15), we did not observe that factor X_a was protected from inhibition by phospholipids. Using previously reported binding data of the factor $X_{(a)}$-phospholipid interaction (31, 32), it can be calculated that in the presence of 100 μM of phospholipid (25 mol % phosphatidylserine, 75 mol % phosphatidylcholine) 90% of total factor X_a (10 nM) is bound to the phospholipid surface, and, yet, the pseudo first-order rate constant of inhibition of factor X_a is the same as that in the absence of phospholipid (Fig. 2). Therefore, the presence of phospholipid does not explain the lower decay constant of factor X_a during factor X activation. Surprisingly, factor X appeared to reduce the rate of the factor X_a/ATIII reaction. This phenomenon was only observed when both calcium and phospholipid were present. The similar effects of either increasing amounts of phospholipid at a fixed factor X concentration or increasing factor X at a fixed phospholipid concentration on the decay constant of factor X_a strongly suggest that phospholipid-bound factor X protected factor X_a from inhibition (Fig. 2).

In view of a factor X_a-catalyzed conversion of phospholipid-bound factor X to β-factor X (28–30) it seems reasonable to rationalize the observed inhibition of the inactivation reaction in terms of a competitive enzyme substrate interaction. Thus, the rate constant of the ATIII-factor X_a reaction will be attenuated by a factor of $1/(1 + [S]/K_m)$ and should approach zero at saturating substrate levels (33). However, a substantial increase in surface-bound factor X did not result in a further decrease of the apparent rate constant when factor X_a was quantitatively bound to the surface (Fig. 3). Moreover, the level to which the inactivation reaction could be inhibited appeared to be a function of the phosphatidylserine content of the phospholipid vesicle. Because the binding capacity of vesicles increases with increasing phosphatidylserine content (31) it seems that the local factor X concentration at the surface, rather than the bulk phospholipid-bound factor X concentration, is the effective substrate concentration for the competitive reaction.

At present, an alternative mechanism, in which the active site of factor X_a is not involved in the factor X_a-factor X interaction at the surface, can not be ruled out. That is, the decrease in the rate of inactivation of factor X_a could also reflect a reduced reactivity of factor X_a in the ternary complex of factor X-phospholipid-factor X_a toward ATIII.

The inactivation of factor IX_a was not affected by the accessory components (phospholipid, calcium) and factor X. Regardless of the presence of factor X, phospholipids, and/or calcium, the second-order rate constant for factor IX_a inactivation by ATIII was 8.2×10^3 M^{-1} min^{-1}. In view of the protection of factor X_a by phospholipid-bound factor X, it is quite striking that the interaction between factor IX_a and factor X at the surface does not compete with the ATIII-factor IX_a reaction. However, because the phospholipid-factor IX_a interaction would be one of the affecting elements, it is interesting to note that the binding constant of this interaction is substantially larger than the one reported for the factor X_a-phospholipid interaction (34).

We next examined the effect of two types of heparin, UFH, and synthetic pentasaccharide heparin, on the inhibition of factor IX_a and factor X_a during factor X activation. Pentasaccharide was included in our study because of reported effects of the non-ATIII-binding region of heparin molecules on factor X_a (25) and factor IX_a inhibition (13).

Comparing the inhibition of factor X_a during factor X activation in the presence of UFH and pentasaccharide, it is evident that a polysaccharide sequence in addition to the ATIII-binding sequence alters the kinetics of factor X_a inhibition in such a way that it diminishes the protection of factor X_a by phospholipid-bound factor X. It is feasible that the competitive interaction between factor X_a and phospholipid-bound factor X is counterbalanced more strongly by the interaction between factor X_a and UFH-ATIII because of the higher affinity of factor X_a for UFH-ATIII than for pentasaccharide-ATIII.

Pentasaccharide could not enhance the inhibition of factor IX_a by ATIII. Apparently, factor IX_a-binding regions on the heparin molecule are absolutely required for its catalytic function. The decay constant of factor IX_a increased linear with the concentration of UFH, $k = 0.65\ \text{min}^{-1}/\mu\text{g}$ of UFH/ml. To our surprise, the stimulatory effect of UFH on the inactivation of factor IX_a was about 6-fold higher during factor X activation as in free solution. It appeared that this stimulation was due to the presence of calcium (Fig. 5). The same phenomenon has been reported for human factor X_a (25). Calcium, because of its ability to induce structural changes in factor IX_a (35, 36), may affect the heparin-catalyzed reaction either by enhancing ternary complex formation or by increasing the intrinsic rate constant of the ATIII-factor IX_a reaction at the heparin surface.

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