The Use of Prothrombin(S525C) Labeled with Fluorescein to Directly Study the Inhibition of Prothrombinase by Antithrombin during Prothrombin Activation*

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Serine 525 of human prothrombin was mutated to cysteine and covalently labeled with fluorescein to make II(S525C)-fluorescein. Kinetics of cleavage of this derivative by prothrombinase are identical to those of wild-type prothrombin. Cleavage is coincident with a 50% increase in fluorescence intensity and the product is catalytically inactive. Thus, it allows convenient monitoring of prothrombin activation without generating active thrombin. The kinetics of inhibition of factor Xa (FXa) by antithrombin (AT) and AT-heparin were measured by monitoring activation of II(S525C)-fluorescein and the hydrolysis of the chromogenic substrate S2222 in the presence of AT. With S2222 as the substrate the rate constant for inhibition of FXa, Ca\(^{2+}\), and unilamellar vesicles of phosphatidylcholine and phosphatidylserine (75:25) (PCPS) vesicles by AT was 3.51 \times 10^2 \text{s}^{-1} \text{M}^{-1} when factor Va (FVa) was included the rate constant was 1.55 \times 10^3 \text{s}^{-1} \text{M}^{-1}. In the absence of FVa, II(S525C)-fluorescein had no effect on inhibition. When II(S525C)-fluorescein was the substrate, however, FVa at saturating concentrations profoundly protected FXa from inhibition by AT, increasing the half-life from 3 min with FXa, Ca\(^{2+}\), PCPS, and II(S525C)-fluorescein, to greater than 69 min when FVa was included. Thus, both FVa and prothrombin are necessary for this level of protection. In the absence of prothrombin, FVa decreased the second order rate constant for inhibition by the AT-heparin complex from 1.58 \times 10^3 \text{M}^{-1} \text{s}^{-1}, for FXa, Ca\(^{2+}\), and PCPS, to 7.72 \times 10^2 \text{M}^{-1} \text{s}^{-1}, II(S525C)-fluorescein and factor Va together reduced the rate constant to less than 1% of that for FXa, Ca\(^{2+}\), and PCPS. At a heparin concentration of 0.2 unit/ml, this corresponds to a half-life increase from 1 s to 136 s.

One of the key steps of coagulation involves activation of prothrombin to its active form thrombin by the multicomponent complex, prothrombinase. Prothrombinase comprises the serine protease factor Xa, the activated protein cofactor factor Va, calcium ions, and an appropriate cell membrane or phospholipid surface (1–5). Although factor Xa alone can slowly generate thrombin by an extremely inefficient reaction, the rate of thrombin generation is enhanced by several orders of magnitude by incorporation of the cofactor protein factor Va and the procoagulant surface (5).

The activities of the clotting serine proteases, such as thrombin, are regulated, in part, by plasma protease inhibitors of the serpin superfamily. Of these, antithrombin appears to be the most important. Antithrombin targets both the product of prothrombin activation, thrombin, and the enzyme responsible for the reaction, factor Xa. Inhibition of serine proteases by serpins occurs through the formation of a stable complex between the serpin and the active site of the serine protease. Inhibition by antithrombin is markedly enhanced by the glycosaminoglycan cofactor, heparin. Heparin accelerates rates of inhibition of both thrombin and factor Xa several thousand fold (6). Upon binding, heparin induces a conformational change in antithrombin (7–9), which is sufficient to accelerate the inhibition of factor Xa through an allosteric mechanism. The acceleration of thrombin inhibition, however, is dependent on the formation of a ternary complex between heparin, antithrombin, and thrombin (10–12).

Several studies demonstrated that incorporation of factor Xa into the prothrombinase complex changes the ability of the antithrombin-heparin complex to inhibit it. Calcium ion accelerates the heparin catalyzed inactivation of factor Xa by antithrombin through a template mechanism (13). Phospholipids and factor Va play protective roles and decrease the ability of the heparin-antithrombin complex to inhibit factor Xa both on synthetic surfaces (14–19) and in whole blood clots (20). Herault et al. (21), however, indicated that the antithrombin-heparin complex is an effective inhibitor for phospholipid/factor Va-bound factor Xa in solution and on blood cells. The substrate prothrombin also plays an important role in the protection observed when factor Xa is incorporated into the prothrombinase complex (14).

Prothrombinase activity is usually inferred through measurements of thrombin activity over time. Therefore, studying heparin-dependent antithrombin inhibition of prothrombinase is challenging because the inhibitor recognizes both the product of the reaction, thrombin, and the enzyme component of prothrombinase, factor Xa. To resolve this problem in this study, a mutant form of prothrombin (II(S525C)), in which serine 525 (the active site serine of thrombin) is replaced with cysteine, was expressed in a mammalian expression system, isolated, and covalently labeled at Cys\(^{525}\) with fluorescein. II(S525C)-fluorescein exhibits an increase in fluorescence intensity upon activation to thrombin but is catalytically inactive to both small and macromolecular substrates. Thus, it allows convenient monitoring of prothrombin activation in the presence of antithrombin without generating active thrombin. In this study inhibition of free factor Xa or factor Xa bound to vesicles and factor Va, by antithrombin or the antithrombin heparin complex was determined by monitoring over time the
hydrolis of the small factor Xa substrate S2222 or the activation of II(S525C)-fluorescein.

**EXPERIMENTAL PROCEDURES**

**Materials**

DNA restriction and modification enzymes were obtained from New England BioLabs (Mississauga, Ontario) or Life Technologies, Inc. (Burlington, Ontario) and *Pyrococcus furiosus* (Pfu) DNA polymerase was obtained from Stratagene (La Jolla, CA). Baby hamster kidney cells and the mammalian expression vector pNUT were graciously provided by Dr. Ross MacGillivray (University of British Columbia). Newborn calf serum, Dulbecco’s modified Eagle’s medium/F-12 nutrient mixture (1:1), Opti-MEM, penicillin/streptomycin, fetal bovine serum, glutathione (1 mM), and sodium citrate to a final concentration of 0.025 M, followed by addition of 50 μM ZnCl2, 10 μg/ml vitamin K1, and penicillin/streptomycin/Fungizone mixture. The medium was collected at 24-h intervals, supplemented with glutathione (1 mM), and stored at −20 °C.

**Cell Culture, Transfection, and Selection—**BHK cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 nutrient mixture (1:1) supplemented with 5% newborn calf serum during transfection and selection. Transfection of II(S525C)-cDNA was carried out using the calcium phosphate co-precipitation technique (28). After sixteen hours after transfection, the medium was supplemented with 0.44 mM methotrexate to select for pNUT. Fifteen days after transfection, individual colonies were screened for II(S525C) production by enzyme-linked immunosorbent assay with horseradish peroxidase-conjugated sheep anti-human prothrombin antibody. High expressing clones were seeded into F-12 medium supplemented with 0.44 mM methotrexate and stored at −20 °C.

**Bacterial Overexpression**

Bacterial overexpression was carried out in *E. coli* with 50 μM ZnCl2. The plasmid was digested with NotI and XhoI, followed by incubation with T4 DNA Polymerase, and ligated into the pNUT vector (27) at the SmaI site. This site is downstream of the zinc-inducible mouse metallothionein I promoter and upstream of the human growth hormone poladenylation signal. Proper orientation of the construct was determined using restriction digest analysis and DNA sequence analysis using oligonucleotides 3 and 4. The pNUT vector encodes a modified dihydrofolate reductase gene, which allows for selection under high methotrexate concentrations.

**SDS-PAGE Analysis of Cleavage of II(S525C)-fluorescein—**II(S525C)-fluorescein (800 nM) was activated with 50 μM PCPs, 5 mM FVα, 0.1 mM Fxα, and 5 mM CaCl2 in TBS. Fluorescence intensity was monitored at room temperature with an excitation wavelength of 495 nm and an emission wavelength of 535 nm, slit widths of 5 nm and 10 nm, respectively, and a 515-nm cut-off emission filter in place. Aliquots were removed from the reaction and added to an equal volume of 2 M guanidinium. Samples were cooled and then subjected to SDS-PAGE on a 5–15% minigel, which was subsequently photographed under UV light.

**Recombinant Protein Purification—**Stored medium was thawed at 4 °C and loaded on to XAD-2 (2.2 × 15 cm) and Q-Sepharose (1.4 × 8 cm) columns in tandem at either 4 °C or 21 °C. The Q-Sepharose column was then washed with 5 column volumes of 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4 (TBS), followed by elution of II(S525C) with 0.02 M Tris-HCl, 0.5 M NaCl. Protein containing fractions were identified using a Bio-Rad Assay and pooled. Fractions were collected until all protein was eluted from the column. Fluorescent labeling was performed directly on this pooled fraction by adding a 30 μM excess of 5-IAF from a 20 mM stock solution of 5-IAF in N,N-dimethyl formamide and incubating the sample for 2 h at room temperature in the dark. The resulting fluorescently labeled II(S525C)-fluorescein was separated from the excess label by dialysis against sodium citrate to a final concentration of 0.025 M, followed by addition of a 1.0 mM BaCl2 solution to a final concentration of 0.08 M. The solution was stirred at 4 °C for 1 h and centrifuged. The resulting pellet was washed with the supernatant from a parallel precipitation carried out in 0.02 M Tris-HCl, 0.5 M NaCl. The adsorbed protein was eluted by dissolving the barium citrate pellet in 0.2 M EDTA, pH 8.0 (pH of the original volume). The sample was then dialyzed against TBS, at 4 °C, and subjected to anion-exchange chromatography on a Pharmacia FPLC fast-protein liquid chromatography Mono-Q HR 5/5 column at 4 °C. This step was carried out to resolve fully γ-carboxylated species from partially γ-carboxylated species of II(S525C)-fluorescein (26, 29). The protein was eluted with a 0–30 mM CaCl2 gradient in TBS (50 ml per first column, flow rate 0.5 ml/min). The first peak containing the fully γ-carboxylated II(S525C)-fluorescein eluted at 15.0 mM CaCl2, whereas the second eluted between 18 and 25 mM CaCl2. Fractions from the first peak were pooled, and protein concentrations and labeling efficiency values were determined by absorbance readings at 280 and 495 nm, respectively.

**Oligonucleotides used in this study**

| No. | Primer name | Sequence and position |
|-----|-------------|-----------------------|
| 1   | S225C I     | 5'-GAAGGTACTGTTGGGGACCTTGTGTCATGA-3' (1705–1735) |
| 2   | S225C II    | 5'-TCATGACAGAGGGGTCGCAACGCTTCC-3' (1705–1735) |
| 3   | MTI Sequencing | 5'-ACATAAAAGGAGGCAAGCTG-3' (pNUT) |
| 4   | pA Sequencing | 5'-CCCCAGTGGTCTCCTGCGCCT-3' (pNUT) |

* Mutated nucleotides are in boldface.
For inhibition,  

\[
\frac{d[E\cdot I]}{dt} = k_d[I][E]  
\]  

(Eq. 2)  

For product formation,  

\[
[E] = \frac{d[E\cdot I]}{dt} \cdot \frac{1}{\alpha}  
\]  

(Eq. 4)  

In Equation 1, \( r \) is the rate of hydrolysis of S2222, \( k \) is \( k_{cat} \), \( [E]_p \) is the FXa concentration, \( [S] \) is the concentration of S2222 (250 \( \mu \)M), \( K_p \) is the Michaelis-Menten constant for hydrolysis of S2222, \( K_m \) is the Michaelis-Menten constant for the S2222 product, and \([P]\) is the concentration of the S2222 product. The \( K_m \) value for hydrolysis of S2222 was determined by measurement of initial reactions in the absence of product. Reactions containing S2222 at various concentrations, 0.1 nM FXa and 5 mM CaCl\(_2\), were monitored at 405 nm at room temperature. Initial rates were determined and plotted versus S2222 product, and a \( K_m \) value for the inhibition of FXa by the product of S2222 hydrolysis was determined according to the following equation, where \([P]\) is the concentration of S2222 product:  

\[
r = \frac{k[E]_p[S]}{K_m + [S] + \frac{K_p}{K_p} \times [P]}  
\]  

(Eq. 1)  

Equation 11 over time gives Equation 12,  

\[
\ln\left(\frac{[S]}{[S]_0}\right) = \frac{k[E]_p}{K_m + [S]}  
\]  

(Eq. 12)  

Equation 12 is rewritten as Equation 13,  

\[
\ln\left(\frac{[S]}{[S]_0}\right) = \beta \cdot t  
\]  

(Eq. 13)  

where \( \beta = k[E]_p/K_m + [S] \).  

Raw data were subjected to nonlinear regression to Equation 13 to find \( \beta \). Elimination of \( k[E]_p \) between Equations 10 and 13, yields Equation 14, from which the value of \( \alpha \) was calculated.  

\[
\alpha = \frac{\beta + 1}{\beta} \cdot \ln\left(\frac{[S]}{[S]_0}\right)  
\]  

(Eq. 14)  

The second order rate constant (\( k_2 \)) was then calculated using  

\[
k_2 = \frac{\alpha}{[I]}  
\]  

(Eq. 15)  

**RESULTS**  

Production and Characterization of II(S525C)-fluorescein—To obtain II(S525C), stable BHK cell lines expressing II(S525C) were grown in serum-free medium (Opti-MEM) in
triple flasks. Typically 4 liters of pooled conditioned media were used as the starting material for purification as outlined under “Methods.” II(S525C) behaved identically to wild-type recombinant prothrombin in all of the purification steps. II(S525C) was found to be present in conditioned media at a concentration of 5–10 mg/liter. Only 10% of it, however, was fully γ-carboxylated. II(S525C)-fluorescein was produced with a typical labeling efficiency of ~50%. Purified II(S525C)-fluorescein comigrated with plasma prothrombin when subjected to SDS-PAGE (data not shown). Upon activation of II(S525C)-fluorescein, an increase in fluorescence intensity of ~50% was observed, which was found to correlate well with cleavage (Fig. 1). The fluorescence intensity profile exhibited a transient maximum in intensity, which is indicative of the expected meizothrombin intermediate (26, 30). Additionally, upon cleavage, the band that retains the fluorescent properties corresponds to the B-chain of thrombin, which contains the active site serine. This confirms that the fluorescein moiety attaches specifically to Cys525 (Fig. 1). When compared with plasma prothrombin, II(S525C)-fluorescein activated at similar rates in both the presence and absence of FVa (Fig. 2). The thrombin generated from activation of II(S525C)-fluorescein was catalytically inactive toward small substrates, as determined by S2238 hydrolysis, in which II(S525C)-fluorescein was observed to have at least 130,000-fold less activity (data not shown). II(S525C)-fluorescein was also catalytically inactive toward macromolecular substrates such as Factor V, fibrinogen, and itself (data not shown).

**FIG. 1.** Increase in fluorescence intensity of II(S525C)-fluorescein upon activation correlates well with cleavage. 800 nM II(S525C)-fluorescein was activated with 50 μM PCPS, 5 nM FVa, 0.1 mM FXa, and 5 mM CaCl2 in TBS, 0.01% Tween 80. Fluorescence intensity was monitored as outlined under “Experimental Procedures.” In this figure the blank fluorescence has not been subtracted. Inset, aliquots were removed at the times indicated above, and concentrated. Reduced samples were run on a 5–15% gradient gel as outlined under “Experimental Procedures.” Bands were visualized by fluorescence.

**FIG. 2.** Activation of II(S525C)-fluorescein compared with plasma prothrombin in the presence and absence of FVa. A, 800 nM II(S525C)-fluorescein (open circles) or plasma prothrombin (closed circles) were activated with 50 μM PCPS, 5 mM FVa, 0.1 mM FXa, and 5 mM CaCl2 in TBS, 0.01% Tween 80. Aliquots were removed from the reactions and concentrated. Reduced samples were subjected to SDS-PAGE on 5–15% polyacrylamide gels. Densitometry was carried out to determine the amount of prothrombin remaining at each time point. B, II(S525C)-fluorescein (open circles) or plasma prothrombin (closed circles) were activated with 50 μM PCPS, 10 mM FXa, and 5 mM CaCl2 in TBS, 0.01% Tween 80. Aliquots were removed and treated as in A.
Fig. 3. S2222 subsampling assays to determine $k_2$. 200-μl reactions were set up containing 1.0 μM AT with 20 nM FXα alone (open circle), 20 nM FXα + 5 mM CaCl₂ (open circle), 20 nM FXα + 5 mM CaCl₂ + 50 μM PCPS (closed inverted triangle), 10 nM FXα + 5 mM CaCl₂ + 50 μM PCPS + 50 nM FVa (open inverted triangle), or 20 nM FXα + 5 mM CaCl₂ + 50 μM PCPS + 1 μM II(S525C)-fluorescein (closed square), in TBS, 0.01% Tween 80. Aliquots were removed and diluted in a 96-well plate. Absorbance at 405 nm was monitored, and initial rates were determined and plotted versus time. Corresponding slopes were used to calculate $k_2$ values.

Fig. 4. S2222 hydrolysis over time. Reactions containing 400 μM S2222 and 2 nM FXα + 5 mM CaCl₂, or 2 nM FXα + 5 mM CaCl₂ + 50 μM PCPS + 25 nM FVa, were set up in a 96-well plate in both the absence (solid line) and presence (dashed line) of 1.0 μM AT. Reactions were started by the addition of FXα and monitored at 405 nm until completion. End point data were analyzed to determine the second order rate constants.

Sampling are shown in Fig. 3. Two time courses of the reactions, along with their controls without AT, carried out with the substrate included (end point analysis) are shown in Fig. 4. In the subsampling experiments, plots of the natural logarithm of the residual activity versus time were linear and pseudo first order rate constants were measured from the slopes. In the end point analyses, reactions containing AT did not go to completion because the enzyme was consumed, which allowed determination of the pseudo first order rate constant for inhibition from the residual substrate concentration and the $v_{\text{max}}/K_m$ ratio for the control. Initial efforts to find the $v_{\text{max}}/K_m$ ratio by application of the integrated Michaelis-Menten equation were unsuccessful, because the time courses of product formation were first order, regardless of the initial substrate concentration, which is indicative of equal affinity binding of both the substrate and product to the enzyme. Thus, the $K_m$ was found by standard initial rate measurements and the $K_P$ for inhibition by product was found by measuring initial rates of substrate hydrolysis in the presence of the product at various concentrations (Fig. 5). $K_m$ was determined to be the same regardless of the presence or absence of Ca²⁺, PCPS, and FVa. The value inferred by non-linear regression of the data from Fig. 5 to the Michaelis-Menten equation was $234 \pm 40 \mu M$. The $K_P$ value for product inhibition obtained for the data inset in Fig. 5 was $338 \pm 14 \mu M$.

The $k_2$ values for the inhibition of FXα by antithrombin were determined for FXα alone, FXα + Ca²⁺, FXα + Ca²⁺ + PCPS, FXα + Cu²⁺ + PCPS + FVa, and FXα + Ca²⁺ + PCPS + II(S525C)-fluorescein. Table II summarizes the $k_2$ values determined using both methods. The values determined were in good agreement with each other. Thus, end point analysis was determined to be a valid method for measuring $k_2$ and was used in subsequent experiments with II(S525C)-fluorescein. Upon addition of Ca²⁺, FXα was more susceptible to inhibition by AT, which was expected (13). Addition of PCPS or PCPS + II(S525C)-fluorescein to the system had little to no effect when compared with FXα + Ca²⁺. Finally, addition of FVa decreased the second order rate constant by ~50% when compared with that for FXα + Ca²⁺ + PCPS. In the absence of FVa, II(S525C)-fluorescein had no effect on inhibition of FXα by AT.

Effects of FVa on AT Inhibition of Prothrombinase in the Absence and Presence of Prothrombin—II(S525C)-fluorescein is a useful substrate for monitoring AT-dependent inhibition of prothrombinase, because activation of the mutant can be easily monitored without the generation of active thrombin. For each experiment, a control experiment, containing no AT, was car-
Factor Xa Inhibition during Prothrombin Activation

![Graph](image)

**Fig. 5.** Determination of $K_m$ and $K_v$ values associated with S2222 hydrolysis. Reactions containing various amounts of S2222, 0.1 nM FXa, and 5 mM CaCl$_2$ in TBS, 0.01% Tween 80 were monitored at 405 nm. Initial rates were determined and plotted versus [S2222] to determine a $K_v$ value. Inset, reactions containing various amounts of S2222, 2 nM FXa, and 5 mM CaCl$_2$ in TBS, 0.01% Tween 80 were monitored at 405 nm in a 96-well plate, until completion. Additional S2222 (250 μM) was added, and the resulting reaction was monitored at 450 nm. Initial rates were determined and plotted versus [S2222 product] and a $K_v$ value was determined as outlined under “Experimental Procedures.”

Table II

| Conditions | $k_2$ values as determined by subsampling experiments and endpoint assays |
|------------|---------------------------------------------------------------|
| FXa        | $k_2 = 10^{-3} \text{ (µM}^{-1} \text{s}^{-1})$ |
| FXa + Ca$^{2+}$ | 2.30 ± 0.05 |
| FXa + Ca$^{2+}$ + PCPS | 3.51 ± 0.09 |
| FXa + Ca$^{2+}$ + PCPS + FVa | 3.23 ± 0.34 |
| FXa + Ca$^{2+}$ + PCPS + II(S525C)-fluorescein | 1.55 ± 0.02 |
| FXa + Ca$^{2+}$ | 3.44 ± 0.03 |

Effects of Heparin on AT Inhibition of Prothrombinase—
Heparin accelerates inhibition of FXa by AT by several orders of magnitude. Fig. 8 shows the calculated pseudo first order rate constants for a variety of heparin concentrations determined by monitoring II(S525C)-fluorescein activation by prothrombinase. The corresponding $k_2$ value was determined from the slope of the line to be $1.27 \times 10^6$ M$^{-1}$ s$^{-1}$. The inset in Fig. 8 illustrates the pseudo first order rate constants obtained for analysis of the heparin-dependent AT inhibition of FXa + FVa + PCPS + Ca$^{2+}$ determined by S2222 hydrolysis. The corresponding $k_2$ value was determined to be $7.72 \times 10^6$ M$^{-1}$ s$^{-1}$. The second order rate constant for FXa + Ca$^{2+}$ + PCPS was also determined in a similar manner and found to be $1.58 \times 10^5$ M$^{-1}$ s$^{-1}$. Table III summarizes the $k_2$ values determined in both the absence and presence of heparin. Heparin increased the $k_2$ for inhibition of FXa in the presence of Ca$^{2+}$ and PCPS by 5300-fold. This increase was also observed for FXa + Ca$^{2+}$ + PCPS + FVa as determined by S2222 hydrolysis. It was impossible to calculate a -fold difference for prothrombinase inhibition in the presence of II(S525C)-fluorescein, because prothrombinase appears to be completely protected at saturating levels of FXa in the absence of heparin under these circumstances but is inhibited with a second order rate constant of $1.27 \times 10^6$ M$^{-1}$ s$^{-1}$ upon heparin addition. Table III also summarizes the $k_2$ values in the presence of heparin relative to that for FXa + Ca$^{2+}$ + PCPS. The $k_2$ for prothrombinase in the presence of II(S525C)-fluorescein was found to be 0.8% of that for FXa + Ca$^{2+}$ + PCPS, implying profound protection of FXa, when it is incorporated into the prothrombinase complex and in the presence of prothrombin, in the presence of heparin. The $k_2$ for prothrombinase as determined by S2222 hydrolysis, was one-half that obtained with FXa + Ca$^{2+}$ + PCPS. This value was 60-fold greater than that obtained with prothrombinase plus II(S525C)-fluorescein. The profoundly smaller value observed when II(S525C)-fluorescein is the substrate again suggests that the presence of both FXa and prothrombin causes increased protection of FXa from AT, even in the presence of heparin.
Effects of the Concentration of Prothrombin on AT Inhibition of Prothrombinase—Reactions containing FXa, FVa, PCPS, Ca²⁺ and II(S525C)-fluorescein at various concentrations, in the presence and absence of 2 μM AT, were monitored at excitation and emission wavelengths of 490 and 538 nm, respectively, with a 515-nm emission filter in place. Reactions were started by addition of FXa.

Results in Table III indicate that the k₂ value in the absence of prothrombin should be 7.72 × 10⁶ M⁻¹ s⁻¹, which is 50-fold higher than the average k₂ value in Fig. 9. Thus, prothrombin does appear to play a protective role, but, perhaps similar to FVa, the protective effects of prothrombin saturate at very low concentrations. Unfortunately, it is impossible to accurately monitor activation of II(S525C)-fluorescein at concentrations below 50 nM, because the change in fluorescence is too small.

**DISCUSSION**

We have expressed a variant of prothrombin in which the serine of the thrombin catalytic triad has been replaced with a cysteine that was subsequently labeled with fluorescein. Characterization of the change in fluorescence upon cleavage, the rate of activation in the presence and absence of FVa and the absence of activity upon activation of this variant showed it to be a good model substrate to investigate its activation by the enzyme FXa in the presence of the physiological inhibitor antithrombin. This variant allows easy monitoring of the conversion of prothrombin to an inactive thrombin and would be a useful tool for monitoring prothrombin activation in the absence of all thrombin feedback reactions, such as the activation of FV, and cleavage of itself.

It has been well established that FXa is less susceptible to inhibition by antithrombin and the antithrombin-heparin complex when it is incorporated into the prothrombinase complex (14–20). The extent of this protection and the mechanism behind it, however, have not been clearly established. Inhibition of FXa by antithrombin and the antithrombin-heparin complex under various conditions was quantitatively determined in this study. In the absence of FVa, the combination of phospholipid vesicles and prothrombin had no effect on the second order rate constant of inhibition of FXa by antithrombin; FVa in the absence of prothrombin showed only a modest effect, decreasing the rate constant approximately 2-fold. Upon addition of both FVa and prothrombin, however, the second order rate constant decreased to an immeasurable level, suggesting that, when both FVa and prothrombin are present, prothrombinase is extremely protected from antithrombin, with its half-life increasing from 3 min in the absence of FVa to greater than 69 min with prothrombin and FVa. Upon addition of heparin, FVa in the absence of prothrombin was again observed to decrease the second order rate constant by 50%. In the presence of both FVa and prothrombin, the second order rate constant decreased to 0.8% of that for FXa + Ca²⁺ + PCPS, implying profound protection of prothrombinase even in the presence of heparin.
presence of heparin. In the presence of heparin at 0.2 unit/ml (40 nM), the half-life increased from 1 s without prothrombin to 136 s with it.

Prothrombin clearly effects the protection of prothrombinase. Previous studies have suggested that the thrombin produced at the catalytic surface consumes antithrombin and therefore lowers its concentration in the vicinity of the prothrombinase complex (15). In the present study however, active thrombin was not produced and no change in the fluorescence of IIa(S525C)-fluorescein was observed upon antithrombin addition, implying that the variant used in this study does not associate with and therefore does not consume antithrombin.

However, the remarkable extent to which FXa is protected from inhibition by both antithrombin and the antithrombin-heparin complex also cannot be rationalized based on straightforward competition between prothrombin and antithrombin according to a simple Michaelis-Menten model. Thus, some other aspect of the conversion of prothrombin to thrombin by prothrombinase is responsible for the protection. One possibility is that the interactions of FXa with both prothrombin and antithrombin require an exosite on FXa distinct from the S1 subsite of the enzyme. That such an exosite exists on FXa is supported by the data of Krishnaswamy and Betz (31). If this rationalizes the protection, the exosite is not recognized by

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**TABLE III**

| Conditions                                      | −Heparin | +Heparin | Increase | Relative $k_2$ |
|------------------------------------------------|----------|----------|----------|---------------|
| FXa + Ca$^{2+}$ + PCPS                         | $2.97 \times 10^2$ | $1.58 \times 10^7$ | 5300     | 1             |
| FXa + Ca$^{2+}$ + PCPS + FVa                    | $1.49 \times 10^2$ | $7.72 \times 10^6$ | 5200     | 0.5           |
| FXa + Ca$^{2+}$ + PCPS + FVa + II(S525C)-fluorescein | $<0.083 \times 10^2$ | $1.27 \times 10^5$ | N/A      | 0.008         |

$a$ $k_2$ values are relative to $k_2$ for FXa + Ca$^{2+}$ + PCPS in the presence of heparin.

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FIG. 8. Effects of heparin on the pseudo first order rate constant. Reactions containing heparin at various concentrations, 2 μM AT, 60 nM FXa, 50 μM PCPS, 5 nM FVa, 1 μM II(S525C)-fluorescein, and 5 mM CaCl$_2$ in TBS, 0.01% Tween 80, were monitored until completion at excitation and emission wavelengths of 490 nm and 538 nm, respectively, with a 515-nm emission filter in place. Reactions were started by addition of prothrombinase. End point analysis was carried out as outlined under “Experimental Procedures” to determine the pseudo first order rate constants, which were plotted versus [heparin]. The corresponding slope was used to calculate $k_2$. Inset, reactions containing heparin at various concentrations, 2 μM AT, 15 nM FXa, 50 μM PCPS, 50 nM FVa, 400 μM S2222, and 5 mM CaCl$_2$ in TBS, 0.01% Tween 80, were monitored until completion and treated as above.

FIG. 9. Effects of prothrombin concentration on $k_2$. Reactions containing 60 μM FXa, 5 nM FVa, 50 μM PCPS, and II(S525C)-fluorescein at various concentrations, in the presence and absence of 2 μM AT and 60 nM heparin were monitored until completion at excitation and emission wavelengths of 490 nm and 538 nm, respectively, with a 515-nm emission filter in place. End point analysis was carried out as outlined under “Experimental Procedures,” and $k_2$ values were determined.
prothrombin in the absence of FVa, because prothrombin alone did not protect FXa. Alternatively, the protection is inherent in the dynamics of the catalytic cycle. Conceivably, for most of the cycle, FXa is in a conformation or state in which it does not recognize antithrombin. If this is so, then FXa would be accessible for only about 1% of the cycle, because the rate constant for inhibition decreases by a factor of $\approx 100$ for inhibition of FXa by the antithrombin-heparin complex. Regardless of the mechanistic reason for the protection, the fact that it exists suggests that prothrombin activation cannot be accounted for by simple substrate competition and the Michaelis-Menten model.

The serine proteases of other multicomponent enzymatic complexes of the coagulation cascade (factor VIIa and factor IXa) are also inhibited by antithrombin. Perhaps they are similarly protected from inhibition in the presence of the substrate (factor X), and thus the phenomenon reported here for prothrombinase may apply generally to all of the reactions of the cascade. If this is the case, the reactions of the cascade localized at the site of injury would be relatively protected from antithrombin, whereas enzymes that might escape would be susceptible to inhibition, thereby both allowing efficient thrombin formation locally, while preventing or attenuating it systemically.

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