Kinetics of Blood Coagulation Factor Xα Autoproteolytic Conversion to Factor Xαβ

EFFECT ON INHIBITION BY ANTITHROMBIN, PROTHROMBINASE ASSEMBLY, AND ENZYME ACTIVITY*

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Autoproteolysis of blood coagulation factor Xα (FXα) results in the excision of a 4-kDa fragment (β-peptide) from the intact subform, factor Xαβ (FXαβ), to yield factor Xαβ (Fβ). In the preceding paper, we showed that generation of Fβ leads to expression of a plasminogen binding site. Fβ may consequently participate in fibrinolysis; therefore, the timing of subform conversion compared with thrombin production is important. In the current study we evaluated the kinetics of Fβ generation, which showed that autoproteolysis of FXαβ followed a second order mechanism where FXαβ and Fβ behaved as identical enzymes. Rate constants of 9 and 172 min⁻¹ s⁻¹ were derived, respectively, in the absence and presence of FXαβ binding to procoagulant phospholipid. Under identical conditions the latter is estimated to be 6 orders of magnitude slower than thrombin generation by prothrombinase. Since heparin binding and prothrombin recognition have been previously attributed to a region of FXα proximal to the β-peptide, functional comparisons were conducted using homogeneous and stabilized preparations of FXαβ and FXαβ. Comparisons included 1) the recognition of small substrates; 2) the rate of interaction with antithrombin/heparin; 3) the assembly of prothrombinase; and 4) the activation of prothrombin by prothrombinase. Although the β-peptide neighbors a probable functional region in FXαβ, conversion to Fβ was not observed to influence these functions. The data support a model where FXαβ is predominantly responsible for thrombin generation and where slow conversion to Fβ coordinates coagulation and the initiation of fibrinolysis at sites of prothrombinase assembly.

The established function of coagulation factor Xα (FXα) is to generate thrombin. In association with the cofactor Va (FVa), procoagulant phospholipid (PCPS), and Ca²⁺ to form the prothrombinase complex, FXα activity is accelerated to a level that is required to overcome anticoagulant barriers (1, 2). One of the anticoagulants that regulates FXα is antithrombin (AT), which is a member of the serpin family of proteins (3). AT functions by forming a 1:1 irreversible complex with a susceptible serine protease and utilizes heparin as a cofactor to increase its inhibitory activity by several hundred-fold in the case of FXα (4). Recently, a new function for FXα has been described (5). The evidence suggests that FXα is capable of interacting with the fibrinolysis zymogen, plasmin. In doing so, FXα enhances the tissue plasminogen activator (tPA)-mediated generation of plasmin by approximately 2 orders of magnitude.

In the preceding paper we demonstrated that autoproteolytic excision of a small COOH-terminal peptide (β-peptide) from the intact FXα subform, FXαβ, to yield Fβ, was necessary for the expression of a plasminogen binding site. Combined with earlier observations (5–7), this finding fits into a regulatory model that coordinates the participation of FXαβ in coagulation and subsequently in fibrinolysis. Whether FXαβ or Fβ is the prevalent enzyme during thrombin production has not been previously reported. Therefore, an assumption made in the model is that the conversion of FXαβ to Fβ is slow relative to the generation of thrombin, which is necessary to prevent the initiation of fibrinolysis prior to sufficient clot formation.

The process of FXαβ conversion to Fβ on procoagulant and anticoagulant functions has not been investigated in adequate detail. The only direct studies comparing FXα subform activity used clotting assays containing PCPS, and the results were contradictory. Some of the reports supported the conclusion that the specific activity of FXαβ is greater than that of FXβ (8, 9), while others showed that the subforms behave the same (10, 11). These inconsistencies could be explained by the known acceleration of FXαβ autoproteolysis by PCPS (10, 12–14), which may make FXβ the effective enzyme in some clotting assays regardless of the subform that is initially present.

In an effort to localize important functional areas in FXα, a COOH-terminal region that closely neighbors the β-peptide has been previously identified. The functions that were implicated are the binding of the anticoagulant cofactor, heparin (15), and recognition of the prothrombinase substrate, prothrombin (16). Due to proximity, it is conceivable that excision of the β-peptide may influence these functions. Any such influence would be compounded when fibrinolysis is triggered, since, as the preceding paper demonstrated, plasmin generates a species indistinguishable from FXβ at least 3 orders of magnitude faster than autoproteolysis.

In the current study, the kinetic mechanism of FXβ production was derived to quantitatively establish whether the timing of FXαβ autoproteolysis supports the proposed fibrinolytic pathway. Furthermore, the potential influence of subform conversion on the acceleration of AT by heparin (15) and the discrepancy in the literature regarding clotting activity (8–11) were addressed by comparing homogeneous FXαβ and FXβ in unambiguous purified systems. The data presented here...
clearly show that Fxa must be the prevalent enzyme during the initial stages of thrombin generation, and excision of the $\beta$-peptide has no effect on inhibition by AT/heparin or prothrombinase assembly and activity.

**EXPERIMENTAL PROCEDURES**

Chemicals and Reagents—HEPES, para-aminobenzamidine (pAB) (Sigma), Glu-Gly-Arg-chloromethylketone (EGRck, Calbiochem), dapsylarginine-N-(3-ethyl-1-S-pentanediyl)amide (DAPA, Haematologic Technologies, Inc.), heparin (5-kDa average molecular weight, Calbiochem), and benzoyl-Arg-Glu (piperidyl)-Arg-p-nitroanilide (S-2337, Helena Laboratories). Protein Preparation—Bovine factor X (FX), prothrombin (17, 18), and AT (19) were purified as described previously. Bovine FVa was commercially prepared (Haematologic Technologies) according to established protocols (20, 21).

Stable FXa was generated by incubating factor X with the purified activator from Russell’s viper venom (RVV, Haematologic Technologies) (22) in 20 mM HEPES, 150 mM NaCl, 2 mM CaCl$_2$, pH 7.2 (HBS) at $37^\circ$C in the presence of a 100-fold molar excess of the covalent inhibitor, EGRck (EGR-FXa) or 5 mM of the noncovalent inhibitor, pAB (pAB-FXa). Excess EGRck was removed by Sephadex G-25 gel filtration chromatography (Pharmacia Biotech Inc.) in 25 mM citrate, 100 mM NaCl, pH 6.0. The EGR-FXa was then loaded onto a DEAE-Cellulose (Whatman DE-52) column equilibrated in the same buffer to remove the RVV and was eluted by including 1.0 M NaCl. The RVV was separated from the pAB-FXa in the same way except that all buffers contained 5 mM pAB, and the activation mixture was diluted 15-fold in start buffer and then loaded directly onto DEAE-Cellulose. The protein peak that eluted from DEAE-cellulose was concentrated by precipitation with 75% saturated ammonium sulfate and resuspended in a minimum volume of HBS (containing 5 mM pAB for the pAB-FXa preparation). Any residual factor X and activation fragments were removed by Sephadex G-75 (Pharmacia) gel filtration chromatography in the same buffer. The protein elution peak containing EGR-FXa was concentrated by 75% ammonium sulfate precipitation and resuspended in HBS, 50% glycerol for storage at $-70^\circ$C directly after elution.

To produce FXa, factor X was activated by treatment with RVV and isolated by benzamidine-Sepharose affinity chromatography (23). The quantitative conversion to FXa was achieved by dialysis at 0.5 mg/ml for 8 h in HBS, 2 mM Ca$^{2+}$ at $22^\circ$C. FXa was then treated with a 100-fold molar excess of EGRck to produce EGR-FXa and subjected to G-25 chromatography as above.

The complete inhibition of both FXa subforms (1.5 $\mu$M) by chloromethylketone was confirmed by the lack of detectable amidolytic activity (S-2337, Molecular Devices) with full scale set to 0.03 optical density units over 15 min. EGR-FXa was radioiodinated using Iodogen (Pierce) and chromatographically desalted (Exclusion 5S, Pierce) to remove unincorporated I$^{131}$.$^{131}$. The homogeneity of all proteins was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (24) and where applicable by autoradiography using X-Omat AR film (Kodak) and Quanta III intensifying screens (DuPont). Immediately prior to experimentation, all proteins were dialyzed (Spectrope 2 tubing, Spectrum Medical Industries) into HBS, or when pAB was to be used as a reporter group, the pAB-FXa and FXa were dialyzed into HBS containing 80 $\mu$M pAB at $4^\circ$C.

The molar concentration of proteins was determined spectrophotometrically using E$_{280}$ = 2.6 $\mu$M$^{-1}$cm$^{-1}$ and molecular weights of 1.24 and 55,000 for factor X (25); 1.24 and 45,000 for FXa (11, 26); 1.24 and 42,000 for FXa (11, 26); 1.44 and 72,000 for prothrombin (27, 28); 1.74 (20) and 90,000 (29) for FVa; and 0.65 and 58,000 for AT (30). The optical density at 280 nm was corrected for Rayleigh light scatter by subtracting 1.7 times the absorbance at 320 nm (31). For chemically modified proteins or proteins containing pAB, the concentrations were measured colorimetrically using a cupric bicinchoninic acid detection system (BCA, Pierce) and confirmed by quantitative amino acid composition by Dr. A. Korsak, University of Texas at Galveston, Protein Chemistry Laboratory.

Kinetics of FXa Subform Conversion—The autoproteolytic subform conversion of FXa was followed by densitometry of Coomassie Brilliant Blue-stained SDS-PAGE (12% acrylamide) at $22^\circ$C in HBS, 2 mM Ca$^{2+}$. Four initial concentrations of FXa were used either in the presence or absence of synthetic unilamellar phospholipid vesicles composed of 75% phosphatidylcholine and 25% phosphatidylserine (PCPS), and the data were globally fit to integrate rate laws that describe three kinetic models.

**Models 1-3**

Models 1 and 2 represent simple unimolecular and bimolecular reactions, respectively, where $k$ is the rate constant and $\alpha$ and $\beta$ are abbreviations for FXa and FXa. Integration of the corresponding rate laws yield the equations (32),

$$[\beta] = [\text{FXa}] - \frac{1}{e^t} \quad \text{(Eq. 1)}$$

and

$$[\beta] = [\text{FXa}] - \frac{1}{k_1 + k_2 + k_3} [\text{FXa}] \quad \text{(Eq. 2)}$$

where $[\beta]$ is the concentration of FXa at any time (t) and [FXa] is the total concentration of FXa plus FXa at t (i.e. for $t = 0$, [FXa] = $[\alpha_0]$). The third model includes FXa in addition to FXa as an autoproteolytic enzyme in the reaction mixture and gives the following integrated rate law, assuming that the sequential rate constants are equal:

$$[\beta] = [\text{FXa}] - [\text{FXa}] e^{-kt} \quad \text{(Eq. 3)}$$

$pAB$ Binding to FXa Subforms—The interaction of pAB with FXa or FXa was monitored by inhibition of amidolytic activity. In these experiments, four concentrations of chromogenic substrate S-2337 were combined with a range of pAB concentration (0.1-90 $\mu$M) in a 96-well plate. The reaction was initiated by adding the appropriate FXa subform (4 nM final) in HBS containing 0.1% (w/v) PEG 8000. Color development (405 nm) was monitored at 5-s intervals using a kinetic microplate reader ($V_{max}$, Molecular Devices) at room temperature. Initial reaction velocities were obtained by regression of the linear phase of the progress curves (usually the first 40 s). Inhibition constants ($K_i$) were derived by globally fitting the data to the conventional Michaelis equation describing competitive inhibition (32, 34).

Interaction of FXa Subforms with AT/Heparin—The interaction of AT with either FXa subform was followed by the quenching of pAB fluorescence upon displacement from the serine protease active site, as previously reported (35). In our experiments, the concentrations of pAB-FXa or pAB-FXa (0.2 $\mu$M), AT (0.4 $\mu$M) and pAB (80 $\mu$M) were kept constant in the presence of various concentrations of heparin (1-44 $\mu$M) in HBS. The reaction was initiated by the rapid manual addition of a small volume of AT. pAB fluorescence was continuously monitored in ratio mode using an SLM 8000c spectrophotofluorometer (SLM Instruments), at $25^\circ$C with excitation and emission at 330 nm (bandpass, 4 nm) and 365 nm (bandpass, 8 nm), respectively. The fluorometer was modified to contain a temperature-regulated rhodamine reference turret. To reduce scattered light artifacts, buffer was filtered through a 0.45-um membrane and proteins were centrifuged for 2 min at 12,000 $\times$ g. The total reaction volume was 0.6 ml in a 0.5-cm cuvette. Initial reaction velocities at each concentration of heparin were obtained by regression of the linear phase of the fluorescence progress curves (first 60 s) and were corrected for the inhibitory effect of pAB by using the derived K, values as described (35). In these experiments, we were restricted to amounts of protein and cofactor that gave sufficiently slow initial rates to be measured after manual mixing.

Prothrombinase Assembly—The incorporation of EGR-FXa subforms into prothrombinase was followed by inhibition of thrombin production. In these experiments, the concentrations of active FVa (3 nm), FVa (3 nm), prothrombin (1.4 $\mu$M), and PCPS (0.6 $\mu$M) (2) were kept constant. Thrombin generation was continuously monitored by following the fluorescence enhancement of DAPA (3 $\mu$M) at $25^\circ$C in HBS with a 2.0-ml total reaction volume and initiated by adding PCPS. Excitation and emission wavelengths were at 330 nm (bandpass 8 nm) and 565 nm (bandpass 16 nm), respectively. The maximum rate of prothrombinase activity was determined by regression of the initial linear region of each thrombin generation curve (first 60 s) at varying concentrations of EGR-FXa or EGR-FXa. Scattered light artifacts were minimized with a 500-nm-long pass filter (Corion LL-500-S-V447) in the emission
channel. To further reduce scattered light artifacts, proteins and buffer were treated as described above.

Prothrombinase Activity—To determine the effect of FXa subform conversion on prothrombinase activity, the rate of prothrombin activation by prothrombinase composed of either pAB-FXa or pAB-FXaβ (1 nM), factor Vα (1 nM), and PCPS (0.6 μM) was measured using DAPA as described above. In these experiments, the prothrombin concentration was varied.

RESULTS

FXa Subform Preparations—The technical difficulty in comparing the biochemistry of FXa subforms is the continuous autoproteolytic conversion of FXaα to FXaβ. This is especially true in the presence of procoagulant phospholipid, which accelerates the conversion. To avoid this complication, we prepared FXaα in the presence of EGRck, an irreversible active site inhibitor. The SDS-PAGE pattern of the resulting EGR-FXaα is shown in Fig. 1, lane 1. Treatment of the FXa in this way is useful to investigate certain macromolecular interactions but obviously precludes studies that depend directly on the active site. Therefore, to compare prothrombinase activity or the rate that AT associates with the active site of the FXa subforms, FXaα was prepared in the presence of pAB, which is a reversible serine protease inhibitor. Fig. 1, lane 3, shows the pAB-FXaα produced in this way. Both forms of stabilized FXaα exhibited less than 5% contaminating FXaβ.

The production of homogeneous FXaβ from FXaα was complicated by additional autoproteolytic cleavages that are known to occur (12, 37). Since these inhibit the procoagulant activity of FXa, the resulting products were not evaluated in the current studies. To produce FXaβ we found that the solution phase conversion of FXaα minimized further autoproteolysis, which at 0.5 mg/ml, 22 °C, 2 mM Ca2+, and 8 h of incubation, approximately 95% is FXaα. The preparation of FXaβ was accomplished while simultaneously dialyzing. This removed the β-peptide, as confirmed by NH₂2-terminal sequence analysis of the final product. As a positive control, the β-peptide was detectable if the subform conversion was done without dialysis. We found that preparation of human FXaβ is not practical by this method because further autoproteolytic processing was found to be approximately 5-fold faster compared with bovine FXaβ (data not shown).

Kinetics of Subform Conversion—To establish the least complicated mechanism that adequately describes autoproteolytic FXaβ production, kinetic data were fit to integrated rate laws that describe three reaction mechanisms of increasing complexity. Fig. 2 compares the concordance of the data to these models in the absence (upper panels) and presence (lower panels) of PCPS. As can be seen, neither a first order (panels A and B, Model 1) nor a simple second order (panels C and D, Model 2) reaction satisfactorily represented the data. However, the kinetic data were fit well by the equation describing Model 3. The rate constants that were derived from the fit data were 9 and 172 M⁻¹ s⁻¹ in the absence and presence of excess PCPS, respectively. The rate of human FXaα autoproteolysis in the presence of PCPS (shown in the preceding paper, Fig. 2) was observed to be approximately the same as bovine FXaα.

To provide experimental evidence in support of the two sequential rate constants being equivalent, which is implicit in Model 3, we followed the subform conversion of EGR-FXaα as a substrate at a single concentration. No difference in the kinetic profiles was observed when either catalytic amounts of FXaα or FXaβ were used as the enzyme (data not shown).

Small Substrate Recognition—The interaction of AT with the active site of FXa (35) or thrombin (38) using pAB as a fluorescent probe has been investigated extensively. These authors demonstrated that pAB is competitively displaced by AT, with subsequent fluorescence quenching being directly proportional to the amount of AT-protease complex that is formed. In the current work, we used the same signal to evaluate the rate of association of AT with pAB-FXaα or pAB-FXaβ at various concentrations of heparin. As an initial step it was necessary to establish whether the pAB bound equally to the two subforms. Presented in Fig. 3 are kinetic plots showing the effect of pAB on chromogenic substrate cleavage (S2337) by either FXaα or FXaβ. K₅ values were derived by fitting the data to the Michaelis equation describing competitive inhibition (32) and yielded nearly identical values of 43 μM for FXaα and 38 μM for FXaβ (±15%). Therefore, differences in pAB binding to the FXa subforms would not be a variable in the experiments designed to measure the relative rate of pAB displacement by AT from FXaα or FXaβ. This confirmed that a direct comparison of AT-Xa subform complex formation measured by pAB fluorescence is valid. The somewhat lesser reaction velocities observed for FXaβ can be accounted for by the small amount of secondary autoproteolytic products that are contained within the FXaβ preparations, which are inactive (12, 37). These observations are in agreement with less rigorous studies, which
showed that the amidolytic activity of FXa\textsubscript{α} and FXa\textsubscript{β} was the same (8).

The amidolytic experiments were conducted in the absence of procoagulant phospholipid and at relatively low concentrations of FXa\textsubscript{α}. For these reasons, even at the lowest experimental concentration of inhibitor, pAB (1 \textmu M), subform conversion of \textsuperscript{125}I-pAB-FXa\textsubscript{α} was not detected during the experiment (data not shown).

Inhibition by AT—To determine the effect of subform conversion on the heparin-dependent acceleration of AT-FXa binding, the amount of pAB-FXa\textsubscript{α} or pAB-FXa\textsubscript{β}, AT, and pAB was held constant, while heparin was titrated at rate-limiting concentrations. The initial velocities plotted in Fig. 4 showed that the rates of FXa\textsubscript{α}AT complex formation are the same in the presence or absence of heparin for both FXa subforms. Therefore, the cofactor effect of heparin on inhibition of FXa\textsubscript{α} or FXa\textsubscript{β} by AT is also equivalent. The apparent \(k_{cat}/K_m\) values derived from these data by the method of Craig et al. (35) were 2.8 \times 10^4 and 2.4 \times 10^4 M^{-1} \text{ s}^{-1}, respectively. It should be noted that the concentration of AT used by these authors to ensure that it was not limiting was 4-fold higher than in our experiments. Although the basic conclusion that FXa\textsubscript{α} and FXa\textsubscript{β} associate equally with AT in the presence of heparin is valid, our values for \(k_{cat}/K_m\) may be moderately underestimated.

Assembly of Prothrombinase—The incorporation of FXa subforms into prothrombinase complexes was evaluated using EGR-FXa\textsubscript{α} or EGR-FXa\textsubscript{β} to inhibit thrombin generation by prothrombinase. Several other laboratories have demonstrated that the inhibition is competitive (2). In our experiments, FXa\textsubscript{β} was selected as the active enzyme rather than FXa\textsubscript{α} to eliminate any variability we may have observed due to possible subform conversion of the functional enzyme. Fig. 5 compares the initial rate of thrombin generation at various concentrations of EGR-FXa\textsubscript{α} or EGR-FXa\textsubscript{β}. The data revealed that the association of the two active site-blocked FXa subforms with FVa/PCPS, causing displacement and inhibition of the active FXa\textsubscript{β}, is identical. The concentration required to achieve 50% inhibition was approximately 4 \textmu M for both subforms.

The similarity that we observed between the two subforms in the prothrombinase inhibition experiments may have been caused by the phospholipid-accelerated conversion of EGR-FXa\textsubscript{α} to EGR-FXa\textsubscript{β} by the functional enzyme that was present. To investigate this possibility, a trace amount of \textsuperscript{125}I-EGR-FXa\textsubscript{α} was included in the reaction mixture containing the highest amount of EGR-FXa\textsubscript{α} (44 \textmu M) and sampled over time for subsequent analysis by SDS-PAGE. At each time point, the reaction was stopped by denaturation in Laemml sample buffer containing SDS. The autoradiograph presented in Fig. 6 demonstrated that conversion does not take place over the course of the experiment (60 s) and was not significant until approximately 1 h of incubation. In separate experiments we ascertained that iodination was not inhibitory to subform conversion (data not shown).

Prothrombinase Activity—Having established that the equilibria governing prothrombinase assembly are independent of the FXa subform, we next determined if the ability to activate prothrombin was different. In these experiments, thrombin production by prothrombinase formed with either pAB-FXa\textsubscript{α} or pAB-FXa\textsubscript{β} was monitored using DAPA at different concentrations of the substrate prothrombin. The FXa subforms were sufficiently diluted to make the inhibitory effect of residual pAB (10 \textmu M) negligible. Fig. 7 shows the kinetic profiles used to derive apparent \(K_m\) values of 1.2 \textmu M for FXa\textsubscript{α} and 1.4 \textmu M (± 15%) for FXa\textsubscript{β} by nonlinear least squares fit to the Michaelis-Menten equation. These values are higher than those previously reported (39, 40) and may be complicated by the rela-
FXa conversion and prothrombinase activity in experiments usingFXa were directly supported by simultaneously following subform
teleolytic conversion of FXa respectively low concentration of PCPS used in the assay.
Prothrombin (0.2–2.0 μM) activation, was initiated by the addition of
PCPS (600 nM) in the presence of either FXα (●) or FXαβ (○) (1 nM)
and continuously monitored using DAPA (3.0 μM) as a
thrombin-specific fluorescent probe. Shown are averages of at least
duplicate initial velocities with standard deviation. The data were iter-
atively fit to the Michaelis-Menten equation.

tively low concentration of PCPS used in the assay. Nevertheless, the data are clearly consistent with the conclu-
sion that conversion of FXα to FXαβ does not significantly
influence prothrombinase activity.

To verify that the active FXα was at an adequately low
concentration to make subform conversion negligible over the
time frame of the experiment (60 s), an identical parallel
experiment was performed using radiiodinated FXα at the
highest and lowest concentrations of prothrombin. After elec-
trophoresis and autoradiography of samples taken over 30 min
we observed no generation of FXαβ (data not shown). This was
found to be independent of prothrombin and FVa.

DISCUSSION

In the current study, a mechanism describing the autopro-
 teaseolytic conversion of FXα to FXαβ was derived. The simplest
kinetic model to satisfactorily fit the data was a sequential
second order process and included FXα and FXαβ as equiva-
lent enzymes (see “Experimental Procedures,” Model 3). Exper-
imental evidence that the reaction involved two molecules of
FXα was first obtained by Jesty et al. (10) using diisopropyl
fluorophosphate-inhibited FXα as a substrate for active FXα.
We found that an identical model described the kinetic data
either in the absence or presence of PCPS, with the latter being
2-fold faster. Compared with the generation of thrombin by
prothrombinase, which occurred at approximately 6 nm throm-
bin/s (Fig. 5), the same concentration of FXα in the presence of
PCPS is expected (from Equation 3, at t = 60 s) to convert to
FXαβ more slowly by 6 orders of magnitude. These conclusions
were directly supported by simultaneously following subform
conversion and prothrombinase activity in experiments using
catalytic amounts of 125I-FXα as the enzyme. The data re-
vealed no generation of FXαβ, although significant thrombin
was produced. The presence of FVa was not observed to affect
the rate of FXα autoproteolysis (data not shown). To our
knowledge these are the first studies to demonstrate that FXα
is the prevalent enzymatic component of prothrombinase dur-
ing the initial stages of thrombin production.

In the preceding paper data are presented that show FXαβ but not FXα is capable of functioning as a plasminogen recep-
tor. Since the formation of plasminogen-FXα complexes is
known to accelerate plasin production by tPA (5), autopro-
teolysis was suggested to provide a mechanism to initiate plas-
minogen activation directly at sites of prothrombinase as-
semble. The finding here that autoproteolytic generation of FXαβ is slow relative to thrombin production fits into this mechanism
by coordinating the sequential expression of procoagulant and
profibrinolytic activity.

Given the slow relative rate of FXα autoproteolysis com-
pared with thrombin production by prothrombinase, one would
predict that other possible regulatory functions for excision of
the β-peptide (bovine, 2 residues 433–449 (14); human, residues
436–447 (41)) would evolve to favor anticoagulant processes.
This supposition was supported in the literature by the position of
the heparin binding site on FXα that has been inferred from
similarities to thrombin (15). Through the introduction of point
mutations (42) and by chemical protection (43), three basic
amino acids near the COOH terminus of thrombin were previ-
ously identified. The latter two residues line up with basic
residues in FXα (bovine, Lys706 and Arg720, human, Lys726 and
Lys728) when comparing crystal structures (15). Furthermore,
FXα (bovine and human) contains several other flanking basic
amino acids that may participate. Due to the proximity of these
to the domain within FXα corresponding to the β-peptide
(β-domain), we hypothesized that subform conversion may in-
fluence heparin binding to FXα and consequently affect inhibi-
tion by AT. We therefore investigated the inactivation of FXα by
AT in the presence of the cofactor heparin. Our experiments
showed that FXα and FXαβ form complexes with AT at the
same rate. Moreover this similarity was not dependent on the
concentration of heparin.

Through the use of synthetic peptides corresponding to the
FXα primary structure, a COOH-terminal heavy chain region
that is conserved between species (bovine, residues 411–420;
human, residues 415–424) has been shown to inhibit thrombin
generation by prothrombinase (16). The nearness of this to the
β-domain suggested that FXαβ conversion may have an effect
on prothrombinase function. Due to the slow relative rate of
FXαβ production, we predicted that subform conversion may
attenuate prothrombinase activity. To test this directly, we
chemically inhibited the autoproteolysis of FXα during ex-
periments. The data showed that neither FXαFVaCa2+-PCPS
complex assembly nor recognition of prothrombin as a sub-
strate was dependent on whether FXα or FXαβ was present.
This is an important consideration for the pharmacological
development of FXα modified at the active site, which has been
used as an effective anticoagulant (44, 45).

Cumulatively, our observations are consistent with the β-domain
of FXα not participating in small substrate recognition,
hibition by AT/heparin, prothrombinase assembly, or
thrombin activation. These conclusions do not preclude the
possibility that release of the β-peptide is associated with other
procoagulant regulatory functions. As an example, a COOH-
terminal region (bovine, 2 residues 413–427; human, residues
2 Numbering based on the amino acid sequence predicted from FX
cDNA (33).
417–431) has been connected with the recognition of the FXa subform conversion with activation. Acknowledgments—We acknowledge Neil Cave and Yvonne Coulombe for technical assistance, Tina Raynor for reading the manuscript, and Drs. William Sheffield and Catherine Dalzell for helpful discussions. Sriram Krishnaswamy is owed special thanks for critical suggestions regarding data analyses.

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