The effects of the stoichiometric inhibitors tissue factor pathway inhibitor (TFPI), antithrombin-III (AT-III) and heparin cofactor-II (HC-II) on thrombin generation were evaluated in a reaction system composed of coagulation factors VIIa, X, IX, VIII, and V and prothrombin initiated by tissue factor (TF) and phospholipids. Initiation of the reaction in the absence of inhibitors resulted in explosive thrombin generation for factor VIIa-TF concentrations varying from 100 to 0.25 pM with the lag time or initiation phase of thrombin generation increasing from 0 to 180 s with decreasing factor VIIa-TF concentrations. During the propagation phase, prothrombin is quantitatively activated to 1.4 μM α-thrombin. At normal plasma concentration (2.5 mM) full-length recombinant TFPI prolonged the initiation phase of thrombin generation 2-fold, and the rate of thrombin generation in the propagation phase of the reaction was 25–50% that of the uninhibited reaction when the reaction was initiated with 1.25–20 pM factor VIIa-TF. Inhibition of the reaction by TFPI is associated with a delay in factor V activation. In the presence of TFPI no explosive thrombin generation was observed when factor VIII was omitted from reactions initiated by factor VIIa-TF concentrations ≤20 pM. This indicates that in the presence of TFPI the factor IXα-facor VIIIα pathway becomes essential at low factor VIIα-TF concentrations. In the reconstituted system, AT-III (3.4 μM) did not prolong the initiation phase of thrombin generation when the reaction was initiated with 1.25 pM factor VIIα-TF, nor did AT-III delay factor V activation. The rate of thrombin formation in the presence of AT-III was reduced to 30% that of the uninhibited reaction, and the α-thrombin formed was rapidly inhibited subsequent to its generation. The addition of HC-II alone at its physiological concentration (1.38 μM) to the procoagulant mixture did not alter the rate or extent of thrombin generation. Subsequently, the thrombin formed was slowly inhibited by HC-II. The slow inactivation of thrombin by HC-II does not contribute to thrombin inhibition in the presence of AT-III. In contrast, the combination of physiological levels of AT-III and TFPI inhibited explosive thrombin generation initiated by 1.25 pM factor VIIα-TF completely. The absence of prothrombin consumption indicated that the combination of TFPI and AT-III is able to prevent the formation of prothrombinase activity at low factor VIIα-TF concentrations. The data indicate that TFPI potentiates the action of AT-III by decreasing the rate of formation and thus the amount of catalyst formed in the reaction, enabling AT-III to effectively scavenge the limited traces of factor IXα and factor Xα formed in the presence of TFPI. The initiation of thrombin generation by increasing factor VIIα-TF concentrations in the presence of physiological concentrations of TFPI and AT-III showed dramatic changes in the maximal rates of thrombin generation over small changes in initiator concentration. These data demonstrate that significant thrombin generation becomes a “threshold-limited” event with regard to the initiating factor VIIα-TF concentration in the presence of TFPI and AT-III.

The extrinsic pathway of blood coagulation involves the activation of multiple coagulation factors leading to thrombin generation. The procoagulant reaction starts with the binding of activated factor VII (factor VIIa) to its cofactor, tissue factor (TF). TF is an integral membrane protein that is exposed as a result of vessel wall injury or cytokine activation of endothelial cells or peripheral blood monocytes. The membrane-bound factor VIIα-TF enzyme complex activates the zymogens factor X and factor IX by limited proteolysis (1). Factor IXα combines with factor VIIIα on the membrane surface to form a second complex that activates factor X. Once activated, factor Xα associates with factor Va on a membrane surface to form prothrombinase, which converts prothrombin into thrombin. (For reviews on blood coagulation and membrane-dependent reactions in blood coagulation see, respectively, Refs. 2 and 3). The thrombin initially formed accelerates further thrombin generation by feedback activation of the procofactors factor V and factor VIII. Thrombin may also activate factor XI (4, 5), which, in turn, activates more factor IX. Deficiencies in factors VII, X, IX, V, or VIII or prothrombin are associated with abnormal bleeding. Factor XI-deficient individuals rarely suffer from spontaneous bleeding; however, homozygotes may require replacement therapy during significant surgical challenge. Thrombin also activates platelets, which secrete their granule contents and aggregate upon activation. In addition, thrombin cleaves fibrinogen to generate the fibrin network and activates the protransglutaminase factor XIII. The fibrin-platelet aggregate, stabilized by factor XIIIa-catalyzed cross-links, forms the hemostatic plug, which maintains the integrity of the circula-

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The abbreviations used are: TF, tissue factor; TFPI, tissue factor pathway inhibitor; AT-III, antithrombin-III; HC-II, heparin cofactor-II; FPR-ck, d-phenylalanyl-L-arginine chloromethyl ketone; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; F1, fragment 1; F2, fragment 2; IIa, α-thrombin.
Regulation of Tissue Factor-initiated Thrombin Generation

In normal hemostasis, the procoagulant system is in balance with anticoagulant systems involved in the termination of the hemostatic reaction and the fibrinolytic system, which dissolves clots once they are formed. The anticoagulant systems consist of several stoichiometric protease inhibitors, the tissue factor pathway inhibitor (TFPI), antithrombin-III (AT-III), and heparin cofactor-II (HC-II), and the dynamic protein C pathway, which involves thrombin, activated protein C, protein S, and thrombomodulin.

TFPI is a reversible, active site-directed inhibitor of factor Xa, which regulates coagulation by inhibiting factor VIIa in a factor Xa-dependent manner (for a review on TFPI see Ref. 6). The TFPI-factor Xa complex binds to the factor VIIa-TF complex, resulting in the formation of a TF-factor VIIa-TFPI-factor Xa quaternary complex (7). Although no human deficiencies have been reported, the in vitro relevance of TFPI is supported by experiments that showed the sensitization of rabbits to TF-triggered disseminated intravascular coagulation after immunodepletion of TFPI (8).

AT-III is a serine protease inhibitor whose importance in hemostasis is confirmed by the association of thrombosis with heterozygous AT-III deficiency (for reviews on AT-III see Refs. 9 and 10). AT-III inhibits thrombin, factor Xa, factor IXa, factor VIIa, factor XIIa, and factor XIa in vitro by forming covalent complexes in which the active site of the protease is trapped. The action of AT-III is potentiated by heparinoids. The rate of inhibition by AT-III and the potentiation of the inhibition reaction by heparinoids varies for each target protease (10). Factor Xa is protected from inactivation by AT-III when in a membrane-associated complex with factor Va (11, 12). In contrast, factor VIIa inactivation by AT-III is only significant when the protease is bound to TF (13). The inactivation of TF-bound factor VIIa by AT-III probably involves the opening of the active site of factor VIIa upon TF binding, allowing factor VIIa inactivation by the active site-directed AT-III. The low enzymatic activity of free factor VIIa provides a mechanism by which traces of factor VIIa may circulate in blood (14).

HC-II is a serine protease inhibitor circulating in blood plasma at a concentration of ∼1.2 μM (15). Thrombin is the only procoagulant reported to be inhibited by HC-II. The inhibition of thrombin by HC-II is potentiated by heparin and by dermatan sulfate (for a review on HC-II see Ref. 16). A heterozygous deficiency of HC-II (activity levels 60%) is found in approximately 1% of the healthy population and does not appear to be a risk factor for thrombosis. A homozygous deficient individual has not yet been identified (16).

We have described reconstituted empirical (17) and mathematical (18) models for the tissue factor pathway to thrombin using purified coagulation factors and computer simulations based upon the known kinetic constants for the reactions thought to be essential in the procoagulant scheme. The reaction could be divided into two phases, an “initiation phase,” in which factor V and factor VIII were quantitatively cleaved and trivial amounts of factor Va and factor IXa were produced, and a “propagation phase” in which prothrombin was quantitatively activated (17). As the concentration of initiator (factor VIIa-TF) was reduced, the initiation phase was prolonged while the rate of thrombin generation in the propagation phase varied by only 5-fold over a 1000-fold range of factor VIIa-TF concentration. The initiation phase was shortened when the reaction was initiated in the presence of factor Va while the propagation phase was dependent upon factor VIII and factor IX at factor VIIa-TF concentrations below 100 μM. The data obtained with the reconstituted empirical model using purified coagulation factors were reasonably approximated by the mathematical model. The data presented here extend the empirical tissue factor pathway to thrombin studies to include TFPI, AT-III, and HC-II.

MATeRIALS AND METHODS

Reagents—Phosphatidylserine from bovine brain, phosphatidylycholine from egg yolk and Heps were purchased from Sigma. 3-Pheynylalaninyl-t-arginine chloromethyl ketone (FPR-ck) and the biotinylated product were provided as gifts by Dr. R. Jenny (Haematalogic Technologies Inc., Essex Junction, VT). Spectrozyme TH and Spectrozyme Xa were purchased from American Diagnostica, Inc. Q-Sepharose FF was obtained from Pharmacia Biotech Inc. All other reagents were of analytical grade. Mouse monoclonal antibodies aHFV-17, aHPC-2, and aHFX-10 were provided by Dr. William Church (Thrombosis Center Antibody Core Facility, Department of Biochemistry, University of Vermont).

In the experiments described, we mix together a potent mixture of enzyme and cofactor precursors together. The formation of spontaneous activation requires extraordinary levels of purity. Contaminating enzymes at femtomolar to picomolar levels cannot be tolerated. Human coagulation factors X and IX and prothrombin were isolated from fresh frozen plasma using the methods of Bajaj et al. (19). All steps were performed at 4 °C. Factor X and factor IX were passed over a polycyonal anti-human prothrombin-1 antibody immunosorb column in 20 mM Tris, 150 mM NaCl, 1 mM benzamidine pH 7.4 (TBS/benz) and concentrated on a Q-Sepharose FF column. Traces of factor VII were removed by washing the Q-Sepharose columns with 10 mM CaCl₂, 50 mM NaCl, 20 mM Tris, 1 mM benzamidine, pH 7.4. After the columns were washed with buffer without CaCl₂, factor X and factor IX were eluted with TBS/Benz containing 1 mM NaCl. Prothrombin was passed over anti-factor X (αHFX-10) and anti-protein C (αHPC-2) immunosorb columns and was concentrated and depleted of trace amounts of factor VII using Q-Sepharose as described for factor X and factor IX. Prothrombin was eluted from the Q-Sepharose column with 20 mM CaCl₂, 150 mM NaCl, 20 mM Tris, 1 mM benzamidine. To inhibit traces of active enzymes in the zymogen preparations, the preparations of factor IX and prothrombin were treated with 50 mM glycerol and 10 mM Tris at 37 °C for 1 h. 50 mM Tris containing 1 mM CaCl₂ was added to the Factor IX preparation to maintain a factor IX procoagulant reported to be inhibited by HC-II. The inhibition of thrombin by HC-II is potentiated by heparin and by dermatan sulfate (for a review on HC-II see Ref. 16). A heterozygous deficiency of HC-II (activity levels 60%) is found in approximately 1% of the healthy population and does not appear to be a risk factor for thrombosis. A homozygous deficient individual has not yet been identified (16).

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FIG. 1. Effect of TFPI on thrombin generation initiated with varying factor VIIa-TF concentrations. Thrombin generation is initiated by 0.25 ( ), 1.25 ( ), 5 ( ), 20 ( ), and 100 pM (○) factor VIIa-TF. Thrombin generation curves in the absence of inhibitor are shown in A, and the thrombin generation curves in the presence of 2.5 nM TFPI are shown in B. TFPI prolongs the lag phase and reduces the rate of thrombin generation during the propagation phase, resulting in curves shifted to the right in the presence of TFPI (compare A and B).

FIG. 2. Effect of TFPI on the lag time and the maximal rate of thrombin generation as a function of the factor VIIa-TF concentrations. Lag times were estimated by the intersection of the line through the linear part of the thrombin generation during the propagation phase and the x axes. The data is obtained from the thrombin curves shown in Fig. 1. A, the lag times in minutes in the control reactions (●) and in the presence of 2.5 nM TFPI ( ). B, the maximal prothrombinase concentrations in the control reactions (●) and in the presence of 2.5 nM TFPI ( ).

In order to follow thrombin generation via active site blotting, samples were subjected to SDS-PAGE on 4–12% polyacrylamide gels under conditions described by Laemmli (23). Following SDS-PAGE, the proteins were transferred to nitrocellulose membranes for immunoblot or active site blot analysis using techniques described by Towbin et al. (24). Membranes were blocked for nonspecific binding with 5% nonfat dry milk in TBS containing 0.05% Tween and probed for prothrombin activation products using techniques described by Towbin et al. (24). Membranes were blocked for nonspecific binding with 5% nonfat dry milk in TBS containing 0.05% Tween and probed for prothrombin activation products using a polyclonal burro anti-human prethrombin-1 antibody (4 μg/ml) (17) or probed for factor V and the factor Va heavy chain using a murine anti-human factor V monoclonal antibody (aHPV-17, 10 μg/ml). The reactive bands were visualized with peroxidase-conjugated goat antihorse IgG or peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution, Southern Biotechnology Associates, Inc.) using the chemiluminescent substrate Luminol (Renaissance chemiluminescent reagent, DuPont). Kodak X-Omat film was exposed to light emitted from the hydrolysis of the added Luminol substrate. Active site blots were blocked with 0.5% Tween in TBS and stained for incorporation of biotinylated-FPR-ck using the Vectastain ABC kit (Vector Laboratories, Inc.), which employs the multiple biotin binding sites on avidin to link peroxidase-conjugated biotin to the active site-incorporated biotinylated FPR-ck. Bound peroxidase was visualized using the chemiluminescent technique as described above.

RESULTS

Effect of TFPI on Thrombin Generation by Varying Initiator Concentrations—The effect of full-length TFPI on thrombin generation initiated by the addition of factor VIIa-TF-PCPS to a mixture of prothrombin and factors X, IX, V, and VIII was...
studied over a wide range of initiator (factor VIIa-TF) concentrations. As previously reported, in the absence of TFPI, the reaction profile can be divided into two phases, an initiation or lag phase and a propagation phase in which prothrombin is quantitatively cleaved to thrombin (Fig. 1A). In the absence of TFPI the rate of thrombin generation in the propagation phase increased only 5-fold when the initiator concentration was varied from 0.25 to 100 pM (Fig. 1A). However, increasing the factor VIIa-TF concentration shortened the initiation phase of the reaction from 180 s at 0.25 pM initiator to almost no lag with 100 pM factor VIIa-TF. The addition of 2.5 nM TFPI (the approximate physiological concentration) resulted in extension of the initiation phase (Fig. 1B) and a reduction in the rate of thrombin generation during the propagation phase. A plot of the duration of the lag time, estimated by the intersection of the line through the linear part of the propagation phase and the x axis, versus the factor VIIa-TF concentration revealed that TFPI doubled the lag time, independent of the factor VIIa-TF concentration (Fig. 2A). However, explosive thrombin generation via ~100 pM prothrombinase still occurred during the propagation phase, even at 0.25 pM factor VIIa-TF (Fig. 2B, squares).

To investigate whether the effect of TFPI was a result of direct inhibition of factor Xa or the inhibition of factor VIIa-TF activity, an experiment was performed in which prothrombinase activity was initiated by the addition of various factor Xa concentrations to a mixture of 200 pM PCPS and factor IX, factor V, and prothrombin (Fig. 3). The maximum concentration of prothrombinase formed was calculated from the linear part of the thrombin generation curve. TFPI (Fig. 3, open circles) reduced prothrombinase activity by 50–65% when the reaction was initiated with either 200 or 400 pM factor Xa. At 100 pM factor Xa (Fig. 3, open circles), TFPI (2.5 nM) accounts for 90% inhibition of the rate of thrombin generation. The inhibition of prothrombinase activity became negligible when the factor Xa concentration approached the TFPI concentration (2 nM factor Xa and 2.5 nM TFPI).

**Effect of TFPI on Thrombin Generation in the Absence of Factor VIII**—The influence of the factor IXa-factor VIIIa pathway on factor Xa generation in reactions initiated by factor VIIa-TF in the presence of TFPI was studied by omitting factor VIII, thus mimicking the condition of hemophilia A. The experiment performed was otherwise identical to that shown in Fig. 1. Similar results were obtained when factor IX was omitted from the mixture (results not shown). Thrombin generation curves in the absence of factor VIII are shown in Fig. 4.

The propagation rate is decreased in the absence of factor VIII; however, traces of factor VIIa-TF (0.25 pM) ultimately result in accumulation of significant prothrombinase (Fig. 4A, inverted triangles). Significant TFPI inhibition of thrombin formation is observed in the absence of factor VIII (Fig. 4B). In the presence of 2.5 nM TFPI, concentrations of factor VIIa-TF below 5 pM (triangles) did not result in significant thrombin generation. This implies that the explosive thrombin generation that occurs at ≤5 pM factor VIIa-TF in the presence of factor VIII in Fig. 1B is completely dependent upon the factor VIIa-Xa pathway. At 20 pM factor VIIa-TF in the absence of factor VIII (Fig. 4B, squares) the maximum rate of thrombin generation was inhibited 93% by TFPI. The virtually linear rate of thrombin generation of 50 nM min⁻¹ observed at 20 pM factor VIIa-TF suggests that a limited amount of factor Xa is formed before the factor VIIa-TF activity is eliminated. This rate of thrombin generation corresponds to the presence of 10 pM prothrombinase. From the data shown in Fig. 3 it follows that ~90% of the factor Xa formed is inhibited by TFPI under these conditions, indicating the formation of approximately 100 pM factor Xa in total. The rate of thrombin generation by 20 pM factor VIIa-TF in the absence of TFPI indicates the presence of 150 pM factor Xa after 1 min. Based upon the rate of 150 pM min⁻¹ of factor Xa generation in the absence of TFPI, a concentration of 1500 pM factor Xa is expected at 10 min if TFPI had not inhibited the factor VIIa-TF activity. Instead, only 100 pM factor Xa is produced, consistent with a 93% inhibition of factor Xa generation at 10 min. The rate of thrombin generation, however, does not significantly change after the first 2 min of the reaction, leading to the conclusion that following the initial formation of 100 pM factor Xa during the first 2 min of the reaction, TFPI completely inhibited subsequent factor Xa generation. These data provide evidence that in the reconstituted model TFPI functions by inhibiting both factor Xa and factor VIIa-TF activity. Explosive thrombin generation does occur in the presence of TFPI and the absence of factor VIII at factor VIIa-TF concentrations ≥100 pM (Fig. 4B, circles).

A comparison of the effect of TFPI on thrombin generation in the absence and presence of factor VIII reveals interesting observations regarding the influence of TFPI in hemophilia A. A plot of the maximum effective prothrombinase concentration formed during the reactions versus the initiating factor VIIa-TF concentration (Fig. 5, data obtained from Figs. 1 and 4) shows that in the hemophilia A situation (Fig. 5B) TFPI (open squares) inhibits thrombin generation at low factor VIIa-TF to less than 1% of the rate observed in its absence (filled circles). In comparison, when factor VIII is present (Fig. 5A) the effect of TFPI (open squares) on the formed prothrombinase activity is limited. These results emphasize the importance of the factor VIIIa-factor IXa pathway in overcoming TFPI-mediated inhibition of factor VIIa-TF-initiated thrombin generation and also demonstrates the potency of TFPI in inhibiting thrombin generation at low initiator concentrations.

**Concentration Dependence of TFPI Inhibition of Thrombin Generation**—Thrombin generation initiated by factor VIIa-TF (1.25 pM) in the presence (Fig. 6A) and absence (Fig. 6B) of factor VIII was only inhibited at concentrations of TFPI ≥0.5
bin generation was inhibited 60% by 1 nM TFPI, and a slight increase in the lag time was observed (Fig. 6B). When factor VIII was present, thrombin generation proceeded primarily by cleavage of prothrombin and meizothrombin-des-F1. These data, together with the lack of prethrombin-2, indicate that thrombin generation proceeded primarily by cleavage of prothrombin at Arg<sup>271</sup>, forming meizothrombin, followed by cleavage at Arg<sup>271</sup>, resulting in the formation of α-thrombin. Accordingly, active site blotting using biotinylated-FPR-ck (Fig. 10A) shows formation of the meizothrombin-derived meizothrombin-des-F1. Films exposed for a longer interval demonstrate the initial generation of meizothrombin (not shown). Prothrombin is also subject to cleavage by the various thrombins at Arg<sup>271</sup> (25). This results in the formation of prethrombin-1 that lacks the GLA domain containing fragment 1. The inability to interact with a membrane via the GLA domain results in reduced rates of activation of prethrombin-1 and meizothrombin-des-F1 by prothrombinase.

The addition of 2.5 nM TFPI (Fig. 7, open circles) to the reaction increased the initiation phase from 60 to 150 s and reduced the rate of thrombin generation in the propagation phase by 70%. The point of total prothrombin consumption was delayed to 10 min as shown by immunoblotting (Fig. 8B), which is consistent with the thrombin activity data. TFPI did not seem to change the thrombin generation pathway, since meizothrombin products prevail on the reduced immunoblot and on the nonreduced active site blot in the presence of TFPI (Figs. 9B and 10B). Maximum thrombin activity is reached (Fig. 7) when a considerable amount of the M<sub>r</sub> 50,000 component is present in the reaction (Fig. 8B, 10 min). These data suggest that the majority of the M<sub>r</sub> 50,000 intermediate is meizothrombin-des-F1, which expresses activity toward the synthetic substrate in the thrombin activity assay.

The addition of 3.4 μM AT-III (Fig. 7, open squares) to the reaction in the absence of TFPI did not significantly influence the lag time of thrombin generation. However, AT-III reduced the rate of appearance of active thrombin by 70%.
activity reached a peak of 600 nM after 3 min, followed by a decline of thrombin activity with an observed $t_{1/2}$ of approximately 60 s. At 10 min, essentially all thrombin activity was inhibited. Immunoblotting for prothrombin products showed that AT-III reduced the rate of prothrombin consumption by $\sim 50\%$, resulting in a slight delay in thrombin consumption compared with the control reaction (Fig. 8, compare A and C). After the appearance of thrombin activity a $M_f$ 97,000 immunoactive band appeared, indicating formation of an $\alpha$-thrombin-AT-III complex. Upon longer exposure of the immunoblot, a reactive band was also detected at $M_f$ 120,000, indicating the formation of a meizothrombin-des-F1-AT-III complex. The increase in intensity of the $\alpha$-thrombin-AT-III band parallels the decrease in thrombin activity. Similarly, the transient incorporation of biotinylated FPR-ck into the different forms of thrombin (Fig. 10C) also parallels the peak of thrombin activity (Fig. 7, open squares). In general, the formation of thrombin-AT-III complexes paralleled the decline in thrombin activity; however, a significant amount of noncomplexed, free thrombin B-chain is observed in the immunoblot after 20 min (Fig. 9C). No thrombin activity is detectable at this stage of the reaction, indicating that this thrombin, while inhibited by AT-III during the reaction, is present as an SDS-reversible thrombin-AT-III complex.

Thrombin generation was nearly abolished by the combination of 2.5 nM TFPI and 3.4 $\mu$M AT-III when the reaction was initiated by 1.25 pM factor VIIa-TF (Fig. 7, open diamonds). However, some active thrombin was still generated at a rate of 1.4 nM min$^{-1}$. A maximum of 14 nM thrombin was reached at 10 min, after which a stable level of thrombin was maintained for up to 15 min. This was followed by a slow decline in thrombin activity so that after 20 min 11 nM thrombin was still present. Significant prothrombin consumption was not observed, and densitometry of the prothrombin band showed no significant decrease over the 20-min period evaluated (Fig. 8B). Meizothrombin-des-F1 and prethrombin-1 were formed in time, and on longer exposures of the immunoblot a reactive band was also detected at $M_f$ 97,000 indicating formation of meizothrombin-AT-III complexes. Consistent with this is the presence of prothrombin fragment 2-A observed starting at 7 min on the reduced immunoblot (Fig. 9D). The relatively stable activity of the thrombin formed under these conditions seems to be the result of the activation of prothrombin and the inactivation of thrombin occurring at similar rates, producing a thrombin steady state.

TFPI and AT-III when added individually decrease the maximum rate of thrombin generation by 70%. The thrombin generation rate in the presence of both TFPI and AT-III was expected to be 10% of the control, or 100 nM/min, if the effects of TFPI and AT-III were multiplicative. However, the observed thrombin generation rate was 1.4 nM/min in the presence of both TFPI and AT-III. This result indicates that TFPI and AT-III together are 70-fold more potent at inhibiting the reaction than the inhibitors acting separately. Thus, the two inhibitors, acting in concert, seem to provide a synergistic inhibitory effect.

In the absence of inhibitors, factor V activation is a major event occurring during the initiation phase of the reaction (17). The effects of TFPI and AT-III on factor V activation were studied by immunoblotting with monoclonal $\alpha$HFV-17, which recognizes intact factor V and the factor Va heavy chain (residues 307–506). In the control experiment and in the presence of AT-III, factor V was partially activated at 30 s (Fig. 11A and C). At 60 s, complete cleavage of factor V had occurred, indicated by the disappearance of single chain factor V ($M_f$ 330,000) and the appearance of the heavy chain of factor Va ($M_f$ 105,000) (Fig. 11, A and C). When TFPI was added, factor V cleavage was delayed until 60 s but complete at 120 s (Fig. 11B). The activation of factor V in reactions with the combination of AT-III and TFPI was only slightly slower than that observed.
The influence of varying factor VIIa concentration (based on the TF concentration) on thrombin generation is insignificant in the presence of the faster inhibitor AT-III (11, 12). Thus, subsequently to its activation, factor Va reduces the potential for factor Xa from inactivation by AT-III (11, 12). Thus, subsequent to its activation, factor Va reduces the potential for factor Xa to inhibit factor Xa. The rate of factor Xa formation at the later stages of the reaction is probably limited by the concentration of active factor VIIIa in the reaction. Factor VIIIa activity is only transiently present because of the dissociation of its polypeptide chains (26–28) and/or proteolytic inactivation (29–33). The observation that the ability of AT-III to inhibit factor Xa is restricted to the early beginning of the reaction. This observation is consistent with the conclusion that factor Va protects factor Xa from inactivation by AT-III (11, 12). Thus, subsequent to its activation, factor Va reduces the potential for AT-III to inhibit factor Xa. The rate of factor Xa formation at the later stages of the reaction is probably limited by the concentration of active factor VIIIa in the reaction. Factor VIIIa activity is only transiently present because of the dissociation of its polypeptide chains (26–28) and/or proteolytic inactivation (29–33). The observation that the ability of AT-III to inhibit factor Xa generation is limited to the initial stage of the reaction is of major importance with respect to the proposed synergistic action of AT-III and TFPI. TFPI significantly delays factor V activation, thereby increasing the ability of AT-III to inhibit factor Xa.
thrombin factor IXa activation. In the presence of TFPI as the only inhibitor, the effect on the reaction, which is associated with delayed factor V generation, shows that TFPI exerts a relatively rapid inhibitory phase. The delay in the propagation phase of thrombin reducing the rate of thrombin generation during the propagation phase makes it difficult to determine the "lag" time, or initiation phase, of thrombin generation and factor Xa, resulting in a bolus of prothrombinase activity independent of the initiator concentration. In the absence of the factor IXa-factor VIIIa complex, TFPI reduces the maximally formed prothrombinase activity to 1% of that observed in the absence of the inhibitor at low (≤5 pM) factor VIIIa-TF concentrations. Thus, the propagation phase of thrombin generation in the presence of TFPI is totally dependent upon factor IXa-factor VIIIa activity at low concentrations of factor VIIIa-TF. These results provide quantitative support for the hypothesis that the failure of the hemostatic response upon injury in patients with hemophilia A or B is, in part, caused by the inactivation of low concentrations of factor VIIIa-TF by TFPI (34). In flow studies using purified proteins Repke et al. (35) showed that in the presence of TFPI, ongoing factor Xa generation by factor VIIIa-TF was only obtained in the presence of the factor IXa-factor VIIIa pathway.

Several groups have reported the rate constants for the inhibition of factor Xa by full-length TFPI (36–38), and a recent study by Jesty et al. (39) reports the rate constants for the inhibition of the factor VIIa-TF complex by the preformed factor Xa-TFPI complex. The complexity of the procoagulant reactions (no steady state conditions established and enzyme occupation by multiple substrates) combined with the complexity of the inhibition reactions by TFPI makes it difficult to determine whether the observed inhibition of the reaction by TFPI can be explained by the reported kinetic constants. Significant (~90%) inhibition of factor VIIa-TF activity by TFPI-factor Xa was observed by Jesty et al. (39) after 60 s. This rapid effect of TFPI on factor VIIa-TF is in agreement with the rapid effect of TFPI on the initiation of the reaction in the reconstituted model.

AT-III inhibits serine proteases by trapping the enzyme at an intermediate stage of proteolysis of the Arg403–Ser404 peptide bond (40–42). Speculation based upon the relatively high (μM) physiological concentrations of AT-III compared with the trace amounts (2.5 nM) of TFPI in the circulation have suggested a potential role for AT-III as an inhibitor of factor VIIa-TF. However, no empirical comparisons on the relative potencies of TFPI and AT-III as inhibitors of factor VIIa-TF-dependent thrombin generation have been reported. The inhibitor AT-III displays a completely different profile of inhibition compared with TFPI. At 1.25 μM factor VIIa-TF, no increase in lag time is observed, while the rate of prothrombin consumption during the propagation phase was reduced by 50% in the presence of physiological concentrations of AT-III. These data demonstrate that full-length TFPI is the major inhibitor of factor VIIa-TF-initiated thrombin formation, while AT-III has no significant influence upon the initiation phase of the reaction, which is almost totally a function of the factor VIIa-TF concentration. The inhibition of factor VIIa bound to TF by AT-III (13) is too slow to significantly inhibit the reaction during its early phase.

In experiments initiated with 5 pM factor VIIa-TF, AT-III inhibited factor Xa generation by 50% during the initiation phase. This is consistent with a slight decrease in prothrombin consumption observed in the presence of AT-III. However, the reduced amounts of factor Xa and factor IXa generated by factor VIIa-TF when TFPI is present seem to become efficiently
scavenged by AT-III, thus preventing explosive thrombin generation (Fig. 7, open diamonds).

Factor Va has been reported to protect factor Xa from inactivation by AT-III (11, 12). Factor V is cleaved by 2 min in the reaction and the picomolar amounts of factor Xa generated contemporaneously are saturated by this excess of factor Va and thus relatively protected against inactivation by AT-III. However, factor Xa inactivation can occur prior to factor V activation and/or prothrombinase complex formation. In this respect, the observed delay of factor V activation in the presence of TFPI may play an important role in the potentiation of the action of AT-III by TFPI (Fig. 7).

In the absence of TFPI, protection of factor IXa by factor VIIIa against inactivation by AT-III is probably of less importance because of the relatively low concentration and the transient presence of active factor VIIIa due to its dissociation (26–28) and/or proteolytic inactivation (29–33). In contrast to prothrombinase, the amount of factor VIIIa cofactor is probably the limiting factor for the formation of the factor IXa-factor VIIIa complex. In the presence of TFPI, however, the observed inhibitory effect of AT-III is probably also due to scavenging of the trace amounts of factor IXa generated. In this model the threshold for explosive thrombin generation in the presence of 2.5 nM TFPI and 3.4 μM AT-III is 20 pM factor VIIa

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FIG. 10. Active site blot of the experiment described in Fig. 7 using biotinylated-FPR-ck showing the thrombin species with an active site at different times in the reaction. Reaction time is indicated in minutes above the gel lanes. A, control without inhibitors; B, 2.5 nM TFPI; C, 3.4 μM AT-III; D, 3.4 μM AT-III, 2.5 nM TFPI. mIIa, meizothrombin (M, 72,000); mIIa des-F1, meizothrombin-des-fragment 1 (M, 50,000); IIa, α-thrombin (M, 38,500).

FIG. 11. Effect of AT-III and TFPI on the activation of factor V during the reaction as followed by the disappearance of intact factor V (FV, M, 330,000) and appearance of the factor Va heavy chain (HC, M, 105,000) by immunoblotting with monoclonal antibody aHFV-17 as described under “Materials and Methods.” Reaction time is indicated in minutes above the gel lanes. Samples of the experiment shown in Fig. 7 were withdrawn and quenched in SDS. A, control without inhibitors; B, 2.5 nM TFPI; C, 3.4 μM AT-III; D, 3.4 μM AT-III, 2.5 nM TFPI.
tions, such claims of synergism must be made with caution without full knowledge of the mechanisms involved (43). At present the complexity of the reaction does not allow a proof of true synergism between TFPI and AT-III.

The major inhibitory effect of TFPI on factor VIIa\textsubscript{TF}-initiated thrombin generation in the reconstituted model predicts that a TFPI deficiency would be a major risk factor for thrombosis. Titration of the effect of TFPI on thrombin generation revealed that TFPI exerts a significant inhibitory effect at 1 nM (Fig. 6). This TFPI concentration is approximately 50% of the normal plasma concentration, suggesting that an individual with a 50% TFPI level would derive a significant inhibitory benefit. Our data suggest, however, that a homozygous TFPI deficiency would result in massive thrombin formation, which may not be compatible with life. However this hypothesis does not take into account that the dependence on TFPI of inhibition of factor VIIa\textsubscript{TF}-initiated thrombin generation might be less in the presence of the dynamic protein C pathway.

Experiments with physiological concentrations of HC-II (1.38 \mu M) predict an insignificant role for this inhibitor as compared with AT-III. Thrombin generation proceeds in a similar fashion whether or not HC-II is present. Although a marginal effect of HC-II was observed on the activity of thrombin after the quantitative activation of prothrombin, no additional thrombin-inhibitory potential was observed when HC-II was combined with physiological concentrations of AT-III. This result supports the hypothesis that HC-II is not important as a coagulation inhibitor and is in agreement with the lack of a thrombotic tendency in individuals with reduced HC-II levels (16).

The combined effects of TFPI and AT-III prevent explosive thrombin generation by traces of factor VIIa-TF in the fully reconstituted system. The dramatic change in the rate of thrombin generation over a small change in the initiating factor VIIa-TF concentration (Figs. 13 and 14) clearly demonstrates that significant thrombin generation becomes a threshold-limited event with regard to the initiating factor VIIa-TF concentration in the presence of TFPI and AT-III. The presence of traces of fibrinopeptide A, prothrombin F1-2, and thrombin AT-III complexes in the plasma of normal individuals indicates that very low, but constant triggering of the coagulation cascade occurs in the unperturbed circulation. The present data suggest that the low level of basal activation of the coagulation system is largely controlled by the combined action of inhibitors like TFPI and AT-III, which prevent this apparent basal activity from turning into massive thrombin formation.

**FIG. 12.** Effect of HC-II on thrombin generation compared and combined with AT-III. Thrombin generation was initiated with 1.25 pM factor VIIa-TF. ○, control; ■, 1.38 \mu M HC-II; □, 3.4 \mu M AT-III; ●, 1.38 \mu M HC-II, 3.4 \mu M AT-III.

**FIG. 13.** Thrombin generation by varying factor VIIa-TF concentrations in the presence of 3.4 \mu M AT-III and 2.5 nM TFPI. Initiating factor VIIa-TF concentrations are 5 (●), 10 (▲), 25 (■), and 125 pM (○). Explosive thrombin generation initiated by factor VIIa-TF becomes a threshold event in the presence of AT-III and TFPI (compare 10 pM and 25 pM factor VIIa-TF).

**FIG. 14.** The maximal rate of thrombin formation in the presence of both AT-III (3.4 \mu M) and TFPI (2.5 nM) is shown in A as a function of the factor VIIa-TF concentration. The integrated area under the thrombin formation curves over the first 10 min of the reaction is shown in B. The data are obtained from the thrombin curves shown in Fig. 13.
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