

α -Thrombin within Fibrin Clots: Inactivation of Thrombin by Antithrombin-III

Michael C. Naski[§] and Jules A. Shafer[¶]

[§]Department of Biological Chemistry, the University of Michigan, Ann Arbor, Michigan 48109 and [¶]Merck Research Laboratories, WP16-101, Sumneytown Pike, West Point, Pennsylvania 19486

(Received 25.8.1992; accepted in revised form 4.1.1993 by Editor C.W. Francis)

Abstract To demonstrate that human α -thrombin is effectively inactivated by human antithrombin III (AT) during the production of a fibrin clot we measured the amount of α -thrombin activity which can be recovered from a clot generated from purified human proteins. We discovered that 0.05-0.07% of the original α -thrombin activity is recovered from a fibrin clot produced from a reaction mixture where the initial concentrations of AT and α -thrombin were chosen at a ratio (17.5) to allow complete conversion of fibrinogen to fibrin. These results indicated that α -thrombin is successfully inactivated by AT during the production of a fibrin clot. Further, when an amount of α -thrombin equal to that recovered from a fibrin clot is introduced into a solution of fibrinogen and AT identical to that utilized to produce the clot only 4% of the fibrinogen is converted to fibrin. These results suggest that i) when a fibrin clot is dissolved during fibrinolytic therapy little active α -thrombin should be released from the clot and ii) this amount of thrombin is insufficient to catalyze rethrombosis without proposing de novo production of thrombin. The action on factors XI, VIII, and V of the small amount of thrombin released upon thrombolysis, however, may provide the stimulus for de novo production of sufficient thrombin to catalyze rethrombosis.

Key Words: thrombin, antithrombin III, fibrin,
Abbreviations: AT, antithrombin III; tPA, tissue plasminogen activator; FPA, fibrinopeptide A; FPB, fibrinopeptide B; tos-GPR-pNA, N-p-Tosyl-Gly-Pro-Arg-p-nitroanilide; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; PEG, polyethylene glycol 6000; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis
Corresponding author: Dr. Jules A. Shafer, Merck Research Laboratories, WP16-101 Sumneytown Pike, West Point, PA 19486

The process of blood coagulation comprises a balanced set of reactions which compete to both activate and inactivate a number of serine proteases required for the production of a fibrin clot. During the final stages of the coagulation cascade the serine protease α -thrombin proteolytically cleaves fibrinopeptides A and B (FPA and FPB) from fibrinogen. The α -thrombin-catalyzed hydrolysis of FPA enables the resulting fibrin monomer to polymerize with additional fibrin units and thereby construct the insoluble fibrin clot (for review see ref 1). In competition with the proteolysis of fibrinogen, α -thrombin is inactivated by the protease inhibitors present in plasma. The plasma inhibitor antithrombin III (AT) is a major inhibitor of α -thrombin and inactivates α -thrombin by forming an irreversible complex with the active site of α -thrombin (2-3).

α -Thrombin binds to fibrin during the process of coagulation and it has been determined that the dissociation constant of the thrombin:fibrin complex ranges between 2 and 4 μ M (4-8). The magnitude of the dissociation constant suggests that α -thrombin should be extensively bound to the fibrin clot within a volume element of the vasculature where the majority of the fibrinogen present in plasma is converted to fibrin. It is reported that α -thrombin bound to a fibrin clot is resistant to inactivation by protease inhibitors such as AT(4, 9-11). Interestingly, it has been observed that there is a frequent occurrence of rethrombosis when thrombi in the coronary vasculature are dissolved during thrombolytic therapy with agents such as streptokinase or tissue plasminogen activator (12-14) and during this therapy there is a transient increase in thrombin activity as measured by an increase in plasma concentrations of FPA (17). Consequently, it has been hypothesized that when a clot is dissolved during thrombolytic therapy, catalytically active α -thrombin formerly bound to the clot may be released and thereby catalyze rethrombosis (9, 11, 15-17).

Evidence suggests that α -thrombin interacts with fibrin through a fibrin(ogen) binding exosite which is distinct from the active site of α -thrombin. Investigations have shown that α -thrombin which is modified at the active site is still competent to bind to fibrin.(4, 6-7, 18-23) suggesting that the active site is not required for the recognition of fibrin by α -thrombin. Additional studies have shown that peptides derived from hirudin, a potent inhibitor of α -thrombin, bind to the exosite domain of α -thrombin and block the interaction of α -thrombin with both fibrinogen and fibrin (24-26). Investigations of the influence of fibrin on the inactivation of α -thrombin by AT demonstrated that α -thrombin bound to fibrin is competent to react with AT (27-29). Cumulatively, these findings are consistent with the view that α -thrombin binds to fibrin through an exosite binding domain which leaves the active site of α -thrombin accessible to react with AT. Thus, during the production of a fibrin clot α -thrombin should be readily inactivated by inhibitors such as AT which react with the active site of α -thrombin.

The reactivity of fibrin-bound α -thrombin with AT predicts that i) thrombin should be inactivated by AT during the production of a fibrin clot and ii) dissolution of a fibrin clot during fibrinolytic therapy should not present a risk rethrombosis based solely on

the thrombin activity present in the clot. To determine whether α -thrombin is resistant to inactivation during the production of a fibrin clot we measured the amount of α -thrombin activity recovered from fibrin clots produced in the presence of AT using purified human proteins. We chose a model system consisting of purified components to enable us to determine i) the amount of active α -thrombin that is released following dissolution of a fibrin clot, and ii) the potential of this amount of thrombin has for catalyzing rethrombosis by its direct action on fibrinogen. A model system of purified proteins enabled us to directly measure the thrombin activity remaining in the clot without contributions to the activity from processes resulting in conversion of prothrombin to thrombin subsequent to clot lysis. Our results show that α -thrombin can be extensively inactivated by AT during the production of a fibrin clot and that following plasmin-mediated dissolution of a clot little catalytically active α -thrombin is recovered from the lysate. These results suggest that it is unlikely that the risk of rethrombosis upon lysis of a fibrin clot (12-14) is due solely to the direct action of the released thrombin on fibrinogen.

MATERIALS AND METHODS

Fibrinogen was purified from pooled outdated human plasma using a modification of the β -alanine precipitation procedure (30). The precipitated protein was dissolved in and dialyzed against 0.3 M NaCl, 0.05 M sodium phosphate, pH 7.5 and chromatographed through lysine-Sepharose (Sigma) and gelatin-agarose (Sigma) to remove plasminogen (31) and fibronectin. (32). The concentration of fibrinogen was determined in 0.3 M NaCl using an $E_{280}^{1\%} = 15.1$ and an $M_r = 340,000$ (33). Human α -thrombin was a gift from Dr. John W. Fenton II, New York State Department of Health. The thrombin was determined to be >95% α -thrombin based on SDS PAGE. The concentration of α -thrombin was determined from the pseudo first order rate constant for the release FPA from fibrinogen under conditions where the concentration of fibrinogen was $< K_m/10$. (30). Antithrombin III was a generous gift from Dr. Steven T. Olson, Henry Ford Hospital, Detroit, MI. AT concentrations were determined using an $E_{280}^{1\%} = 6.5$ and an $M_r = 58,000$ (34). Two-chain tissue plasminogen activator and plasminogen were purchased from American Diagnostica Inc. and Sigma respectively. N-p-Tosyl- Gly-Pro-Arg-p-nitroanilide (tos-GPR-pNA) was purchased from Sigma, dissolved in distilled water, and its concentration determined in 10 mM Tris, 10 mM HEPES, 0.1 M NaCl, pH 7.8 using an absorption coefficient at 342 nm of $8270 \text{ M}^{-1}\text{cm}^{-1}$ (35).

Assay of Thrombin Activity

The activity of α -thrombin was measured at 25°C in 40 mM sodium phosphate, 0.084 M NaCl, 0.1% PEG, pH 7.4. Initial rates of α -thrombin-catalyzed hydrolysis of tos-GPR-pNA were observed at 405 nm in a 1 ml sample containing 60 μ M tos-GPR-pNA, and 0.01 mg/ml aprotinin (Boehringer Mannheim). Initial rates were determined from the slope of the line for the appearance of absorbance at 405 nm vs the reaction time. The data for hydrolysis of tos-GPR-pNA were obtained prior to hydrolysis of 10% of the substrate initially present. The amount of α -thrombin was then determined by comparison of the measured initial rate to a standard curve of initial rates measured at known α -thrombin concentrations. The activity of α -thrombin was measured in both the supernatant solution containing free α -thrombin and the lysate solution containing the α -thrombin which was formerly bound to fibrin (the presence of fibrin fragments had no detectable effect on the thrombin-catalyzed cleavage of tos-GPR-pNA). Control samples were assayed to account for the small amount of substrate hydrolysis catalyzed by plasmin and t-PA. Care was taken to account for the loss of α -thrombin activity (through a pathway independent of AT) during the incubation required for lysis of the clot. Control experiments showed that the loss of α -thrombin activity behaved according to first order kinetics and that the rate was sensitive to both the buffer conditions and the concentration of fibrinogen present in the reaction mixture (data not shown). This rate was independent of the concentration of AT

Binding of Thrombin to Fibrin and Dissolution of Fibrin Clots

The binding of α -thrombin to fibrin was measured at 37°C in 40 mM sodium phosphate, 0.1% PEG, pH 7.4. Briefly, fibrinogen (4.1-12 μ M) was incubated at 37°C whereupon α -thrombin was added to the reaction mixture at a final concentration of 4.3 nM and allowed to react for one hour. The fibrinopeptides were completely released from fibrinogen during the incubation with α -thrombin. The fibrin clot was then collected on a glass rod and washed with distilled water (5 ml) over a 0.5 μ m Teflon membrane (Millipore) using a Büchner funnel. The clot was lysed at 37°C in a 0.5 ml mixture containing 3 μ M plasminogen, 9 nM tissue plasminogen activator (t-PA), 40 mM sodium phosphate, 0.084 M NaCl, 0.1% PEG, pH 7.4. Dissolution of the clot required 6 hours and was considered complete when the clot was no longer visible.

Measurement of the Recovery of Thrombin Activity from a Clot produced in the Presence of AT

Fibrinogen (7.5 μ M) was incubated at 37°C in the presence of 74-3000 nM AT whereupon α -thrombin was added to the mixture to give a final α -thrombin concentration of 4.3-1400 nM. The concentration of α -thrombin and AT were chosen to maintain a [AT]/[thrombin] ratio equal to 17.5. This ratio was chosen to ensure complete conversion of fibrinogen to fibrin yet still provide sufficient AT for inactivation of α -thrombin. An additional experiment was performed using 1.4 μ M α -thrombin and 3 μ M AT. The proteolytic release of fibrinopeptides was verified by HPLC determination of the peptides using previously reported methods (36). Following sufficient time to allow for complete inactivation (>95%) of α -thrombin by AT the clot was collected on a glass rod, lysed as described under "Binding of Thrombin to Fibrin and Dissolution of Fibrin Clots", and assessed for the presence of catalytically

active α -thrombin as described above. The time required for completion of the reactions between AT, α -thrombin, and fibrin(ogen) were determined using our previously published kinetic model for these reactions (29). This model predicts the time course for the reactions of α -thrombin with AT and fibrin(ogen) utilizing the known kinetic parameters for these reactions and initial concentrations of the reactants. The required incubations time for completion of the reactions of α -thrombin with AT and fibrin(ogen) ranged from 0.5 hours at the highest concentration of α -thrombin and AT to 3 hours at the lowest concentration of α -thrombin and AT.

Release of Fibrinopeptides from Fibrinogen

The release of fibrinopeptides were determined at 37°C in 40 mM sodium phosphate, 0.1% PEG, pH 7.4. α -Thrombin (0.002-56nM) was incubated with fibrinogen (7.5 μ M) and AT (74-3000 nM) until α -thrombin was completely inactivated by AT. The incubation time required for inactivation of α -thrombin by AT was determined using our previously published kinetic model for the reactions of α -thrombin with AT and fibrin(ogen) (29). Complete inactivation was verified by determination of the quantity of fibrinopeptide released at two different incubation times. Thereafter the proteins were precipitated with 14% HClO₄ and following centrifugation the fibrinopeptides were quantified by HPLC using methods previously reported (36).

RESULTS

The production of a fibrin clot yields an insoluble matrix that binds α -thrombin. To demonstrate the binding of α -thrombin to fibrin we incubated α -thrombin with several concentrations of fibrinogen. The mixture was incubated at 37°C to allow sufficient time for the α -thrombin-catalyzed release of fibrinopeptides and formation of the fibrin matrix. The fibrin clot was removed and the uncomplexed α -thrombin was determined from the activity of α -thrombin discovered in the supernatant solution after removal of the fibrin clot and the bound α -thrombin. Increasing concentrations of fibrinogen resulted in decreasing amounts of α -thrombin remaining in the supernatant solution or conversely increasing amounts of α -thrombin bound to the fibrin clot (Table 1). Independent measurements of both the α -thrombin in the supernatant solution and the α -thrombin bound to the clot following dissolution of the clot demonstrated that α -thrombin could be quantitatively recovered from the reaction mixtures (Table 1). Assuming that α -thrombin binds to fibrin with a stoichiometry of one molecule of α -thrombin bound per one fibrin monomer, the measurements of the α -thrombin recovered from the supernatant solution were consistent with a value for the dissociation constant of the thrombin:fibrin II complex equal to $1.9 \pm 0.2 \mu$ M.

TABLE I

Binding of Thrombin to Fibrin^a

| [Fibrinogen] (μ M) | Percent of α -Thrombin in the Supernatant | Percent of α -Thrombin Bound to the Clot |
|----------------------------|---|--|
| 4.1 | 30 | 70 |
| 4.8 | 25 | 75 |
| 7.6 | 22 | 78(70) ^b |
| 7.7 | 21 | 79(90) ^b |
| 8.5 | 17 | 83 |
| 12 | 12 | 88 |

^aThe binding of α -thrombin to fibrin was measured at 37°C in 0.04 M sodium phosphate, 0.1% PEG, pH 7.4. The percent of free α -thrombin was determined from the α -thrombin activity recovered from the supernatant solution following removal of the fibrin clot. The percent of bound α -thrombin was calculated from the α -thrombin activity recovered in the supernatant assuming complete recovery of α -thrombin activity. ^bThe values in parentheses represent the percent of α -thrombin recovered from a fibrin clot following dissolution of the clot by plasmin.

TABLE II

Recovery of α -Thrombin Activity from a Fibrin Clot Produced in the Presence of AT^a

| Initial Concentrations | | Percent of α -Thrombin Activity Recovered | |
|-------------------------------|--------------|---|------------|
| [α -thrombin] (nM) | [AT] (nM) | supernatant | lysed clot |
| 4.3 | 74 | 0.1 | 0.05 |
| 21.5 | 375 | 0.08 | 0.07 |
| 43 | 740 | 0.06 | 0.07 |
| 1400 | 3000 | 0.1 | 4.0 |

^aThe recovery of α -thrombin activity was determined after the reactions between the purified human proteins α -thrombin, fibrinogen and AT had ceased. α -Thrombin activity was measured both in the supernatant solution and the lysate solution following dissolution of the clot with plasmin as described under "Materials and Methods".

To determine the effectiveness of antithrombin III (AT) as an inhibitor of α -thrombin during the simultaneous production of a fibrin clot, we performed experiments where a fibrin clot was produced in the presence of AT. Using a

fibrinogen concentration equal to $7.5 \mu\text{M}$ which is equal to typical plasma concentrations of fibrinogen we wished to determine the amount of catalytically active thrombin that remains in the clot when the clot is generated in the presence of AT. α -Thrombin (4.3 nM) and AT (74 nM) concentrations were chosen such that the fibrinopeptides would be completely released from fibrinogen prior to inactivation of α -thrombin by AT. The second order rate constant for inactivation of α -thrombin by AT in the absence of fibrin ($1.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) predicts a half-life of $\sim 10 \text{ min}$ for this reaction in the absence of fibrinogen. To produce a fibrin clot similar to one that might occur at the site of a thrombus, we chose AT concentrations that would allow almost complete release of fibrinopeptides prior to the inactivation of thrombin. Reactions proceeded for sufficient time to allow the α -thrombin-catalyzed release of fibrinopeptides and inactivation of α -thrombin by AT. The clot generated by these reactions was assessed for catalytically active α -thrombin following dissolution of the clot by plasmin. We discovered that only 0.07% (Table II) of the initial α -thrombin activity was recovered from the clot under conditions where 70-90% of the total α -thrombin should be bound to fibrin. Also, as expected very little α -thrombin activity was recovered from the supernatant solution after removal of the fibrin clot. Similar experiments were performed varying the α -thrombin and AT concentrations 10-fold while maintaining a constant $[\text{AT}]/[\text{thrombin}]$ ratio (17.5). This ratio was chosen to produce a reaction mixture which was biased toward production of fibrin rather than inactivation of α -thrombin by AT (see discussion). Under these conditions similar fractions of the total thrombin activity were recovered from the clot after proteolytic dissolution of the clot with plasmin (Table II). Although it is generally considered that only a small fraction of the serum prothrombin is converted to thrombin during the production of a fibrin clot, we wished to determine the amount of thrombin activity that is recovered from a fibrin clot produced during conditions of maximal activation of thrombin where the AT concentration may become limiting. We reacted $1.4 \mu\text{M}$ thrombin, a typical plasma prothrombin concentration (37), with $3 \mu\text{M}$ AT, a typical plasma AT concentration (38), in the presence of $7.5 \mu\text{M}$ fibrinogen and found that 4% of the total thrombin activity was recovered from the fibrin clot.

We then wished to determine the amount of fibrinogen that is converted to fibrin under conditions which might represent the event where α -thrombin which previously had been bound to a fibrin clot is suddenly released into a solution containing fibrinogen and AT. In this experiment we used a concentration of α -thrombin equal to that which was recovered from the fibrin clot generated in the presence of AT. This amount of α -thrombin was then added to a solution containing AT and fibrinogen where the AT and fibrinogen concentrations were equal to those present prior to formation of the clot. When the $[\text{thrombin}]/[\text{AT}]$ ratio was equal to 17.5, 0.05-0.07% of the thrombin activity was recovered in the clot. When this amount of thrombin was reacted with fibrinogen and AT, we observed that 3-4% of the fibrinogen was converted to fibrin as measured by HPLC determination of the fibrinopeptides. During conditions of maximal thrombin activation ($[\text{AT}]/[\text{thrombin}] \sim 2$) we recovered 4% of the initial α -thrombin activity. When this amount of thrombin was reacted with AT and fibrinogen at their original concentrations, 67% of the fibrinogen was converted to

fibrin as assessed by the amount of FPA released prior to inactivation of thrombin by AT.

DISCUSSION

During the process of coagulation α -thrombin catalyzes the proteolysis of fibrinogen and binds to the fibrin clot which is produced. We have estimated a dissociation constant for the fibrin:thrombin complex equal to 1.9 μ M. This value is consistent with the values for the dissociation constant reported by others (4-8) where in some cases different classes of binding sites were observed. The binding of α -thrombin to fibrin is mediated by an exosite domain of α -thrombin. The exosite interaction anchors α -thrombin to fibrin leaving the active site of α -thrombin free to react with certain substrates and inhibitors. (27-29,39-40) Although α -thrombin complexed with fibrin is resistant to heparin-catalyzed acceleration of the reaction of between α -thrombin and AT, (10, 15-16, 28) AT can still effectively inactivate fibrin-bound α -thrombin. (27-28) Using a model system of purified human proteins we were able to determine the amount of thrombin activity remaining in a fibrin clot without contamination of the activity from processes resulting in conversion of prothrombin to thrombin. We demonstrated that when thrombin is present in a clotting reaction where the initial [AT]/[thrombin] ratio is equal to 17.5 only 0.05-0.07% of the initial α -thrombin activity is recovered from the resulting clot. Similar experiments performed under circumstances of maximal thrombin activation ([AT]/[thrombin] \sim 2) demonstrated 4% of the original α -thrombin activity is recovered from the clot. These experiments were performed under conditions where the reactions between α -thrombin, AT and fibrinogen were allowed to proceed to completion. We assume that the reactions which occur at the site of a thrombus also proceed to completion. However, if thrombolytic therapy is initiated very soon after initiation of the thrombotic event the quantity of catalytically active thrombin present within the clot may be greater than that which we have observed in our model system. Calculations based on the results of other investigators suggest that only a small fraction (<1%) of the initial α -thrombin activity is recovered from a fibrin clot produced from human plasma (16,41) and thrombin produced *in vivo*. (41) Francis et al. demonstrated that 10-15% of the total initial thrombin remains bound to a fibrin clot produced from plasma and only 4% of the bound quantity is enzymatically active. Similarly, Weitz et al. demonstrated that catalytically active thrombin remains bound to fibrin clots produced from plasma, but the quantity of catalytically active thrombin based on the rate of FPA release from fibrinogen is <1% of the thrombin initially present. Although thrombin bound to a washed fibrin clot when reintroduced into plasma is relatively protected from heparin-catalyzed inactivation of thrombin by AT (16), other results have shown that given sufficient time for the washed clot to equilibrate with the surrounding plasma milieu, thrombin is efficiently inactivated by AT (9). The small amount of active α -thrombin present within the clot may result from the release of active thrombin from a previously inactive thrombin-AT complex. Experimental procedures using a prolonged interval of clot washing (in an environment devoid of AT) may provide sufficient time for thrombin-

catalyzed cleavage of the P₁-P₁' peptide bond of AT and release of active α -thrombin and cleaved, inactive inhibitor (42-43).

To demonstrate that the small amount of α -thrombin recovered from the fibrin clot is insufficient to catalyze the proteolysis of fibrinogen, we determined the amount of fibrinopeptides that were generated by α -thrombin recovered from the clot. To simulate the reactions which may ensue when a fibrin clot is rapidly dissolved to release the bound α -thrombin, we reacted an amount of α -thrombin equal to that which was found to be catalytically active in a fibrin clot, with fibrinogen and AT at concentrations equal to those originally present when the clot was produced. When the clot was generated under conditions of a 17.5-fold molar excess of AT we observed that thrombin at a concentration equal to that recovered from the fibrin clot converted 3-4% of the fibrinogen to fibrin. This finding suggests that dissolution of a clot will not yield a significant amount of fibrin through the direct action of the released α -thrombin on fibrinogen. Although we observed much greater conversion of fibrinogen to fibrin (67%) during the experiment under conditions of maximal activation of thrombin ([AT]/[thrombin] \sim 2), this experiment is unlikely to represent the events as they occur *in vivo*. We suggest that the pathologic state present during disseminated intravascular coagulation (DIC) may more accurately reflect the amount of active thrombin present during coagulation *in vivo*. This condition represents a state where the activation of thrombin within the vasculature may equal that which occurs at the site of a thrombus. Measurements of the amount of thrombin generated (as measured with assays for the products of prothrombin activation) during DIC showed the production of 40-60 nM thrombin (44). This value is consistent with the predicted amount of α -thrombin required to produce a thrombus of a known composition of fibrin (29). Using the values of 60 nM α -thrombin and 3 μ M AT we then predict an [AT]/[thrombin] ratio equal to 50 during an aggressively prothrombotic state. This suggests that our reactions performed using only a 17.5 molar excess of AT are more biased towards thrombosis than the reactions which occur during DIC. Since the [AT]/[thrombin] ratio we have chosen (17.5) for our model coagulation system is smaller than the value we predict to occur during DIC, we are confident that we have not biased our system toward inactivation α -thrombin by utilizing too great a concentration of AT relative to that of α -thrombin.

Francis et al. and others (45) showed that following plasmin-mediated dissolution of a fibrin clot α -thrombin is extensively bound to the fibrin and fibrin fragments as demonstrated of comigration of thrombin with fibrin fragments during sucrose gradient centrifugation and size-exclusion chromatography. Although α -thrombin complexed with fibrin is able to process certain substrates such as small synthetic substrates and factor XIII as well as react with AT (27-29, 39-40), the complexed α -thrombin is unable to catalyze proteolysis of fibrinogen. α -Thrombin binds to fibrin through its exosite domain which is also required for the interaction of α -thrombin with fibrinogen (24,29). Therefore the binding of α -thrombin to fibrin and fibrin fragments through the exosite domain will block the interaction of α -thrombin with fibrinogen and thereby competitively inhibit production of fibrin. These findings

suggest that following dissolution of a fibrin clot, the processing of fibrinogen by the small amount of preexisting α -thrombin released from the clot will be diminished by i) inactivation of α -thrombin by AT and ii) competitive inhibition of the interaction between α -thrombin and fibrinogen due to the binding of α -thrombin to fibrin and fibrin fragments.

Following thrombolytic therapy an increase in thrombin activity is observed as measured by an increase in plasma concentrations of FPA (17,44). However, the source of the thrombin activity is uncertain. Our results demonstrate that during α -thrombin-catalyzed production of a fibrin clot, α -thrombin is extensively inactivated by AT. These results suggest that when a preexisting clot is dissolved during thrombolytic therapy, little active α -thrombin will be released from the clot. These considerations make it difficult to ascribe the frequent occurrence of rethrombosis to the direct action of the released thrombin on fibrinogen. There is evidence to suggest that the addition of streptokinase to plasma results in a small amount of activation of thrombin (46), which may in part account for the observed increase in thrombin activity following thrombolytic therapy. Additionally, the small amount of thrombin released from the preexisting clot or reexposure of a thrombogenic surface following dissolution of the clot may act as catalysts for de novo production of active thrombin in amounts exceeding that which is released during thrombolysis. Further studies are required to determine the extent to which de novo production of thrombin reflects the activation of factors XI, VIII, and V by the small amount of thrombin that is released upon thrombolysis.

Acknowledgments

This work was supported by National Institutes of Health Grant HL026345.

REFERENCES

1. SHAFER, J.A, and HIGGINS, D.L. Human fibrinogen. *Crit. Rev. Clin. Lab. Sci.* **26**:1-41, 1988
2. ROSENBERG, R.D. Chemistry of the hemostatic mechanism and its relationship to the action of heparin. *Fed. Proc.* **36**:10-18, 1977
3. ROSENBERG, R.D, and DAMUS, P.S. The purification and mechanism of action of human antithrombin-heparin cofactor. *J. Biol. Chem.* **248**:6490-6505, 1973
4. LIU, C.Y., NOSSEL, H.L., and KAPLAN, K.L. The binding of thrombin to fibrin. *J. Biol. Chem.* **254**: 10421-10425, 1979
5. LIU, C.Y., KAPLAN, K.L., MARKOWITZ, A.H., and NOSSEL, H.L. Thermodynamic characterization of thrombin binding by cross-linked and non-cross-linked fibrin in the presence and absence of Ca^{2+} . *J. Biol. Chem.* **255**: 7627-7630, 1980
6. KAMINSKI, M, and McDONAGH, J Studies on the mechanism of thrombin: Interaction with fibrin. *J. Biol. Chem.* **258**:10530-10535, 1983
7. KAMINSKI, M, and McDONAGH, J Inhibited thrombins: Interactions with fibrinogen and fibrin. *Biochem. J.* **242**:881-887, 1987
8. HOGG, P.J., and JACKSON, C.M. Heparin promotes the binding of thrombin to fibrin polymer. Quantitative characterization of a thrombin-fibrin polymer-heparin ternary complex. *J. Biol. Chem.* **265**:241-247, 1990

9. MIRSHAHI, M., SORIA, J., SORIA, C., FAIVRE, R., LU, H., COURTNEY, M., ROITSCH, C., TRIPIER, D., and CAEN, J.P. Evaluation of the inhibition by heparin and hirudin of the coagulation activation during r-tPA-induced thrombolysis. *Blood* 74:1025-1030, 1989
10. LINARDIC, C, and GREENBERG, C Inhibition of the anticoagulant properties of antithrombin III and heparin by fibrin. *Ann NY Acad. Sci.* 556:459, 1989
11. BADIMON, L., BADIMON, J.J., LASSILA, R., MERINO, A., CHESEBRO, J.H., and VALENTIN, F. Rethrombosis on an evolving thrombus is mediated by thrombus-bound thrombin that is not inhibited by systemic heparin. *Thromb. Haemostasis* 65:760, 1991
12. TOPOL, E.J., GEORGE, B.S., KEREIAKES, D.J., STUMP, D.C., CANDELA, R.J., ABBOTTSMITH, C.W., ARONSON, L., PICKEL, A., BOSWICK, J.M., LEE, K.L., ELLIS, S.G., and CALIFF, R.M. A randomized controlled trial of intravenous tissue plasminogen activator and early intravenous heparin in acute myocardial infarction. *Circulation* 79:281-286, 1989
13. YASUDA, T., GOLD, H.K., FALLON, J.T., LEINBACH, R.C., GUERRERO, J.L., SCUDDER, L.E., KANKE, M., SHEALY, D., ROSS, M.J., COLLEN, D., and COLLER, B.S. Monoclonal antibody against the platelet glycoprotein IIb/IIIa receptor prevents coronary artery reocclusion after reperfusion with recombinant tissue-type plasminogen activator in dogs. *J. Clin. Invest.* 81:1284-1291, 1988
14. WILLIAM, D.O., BORER, J., BRAUNWALD, E., CHESEBRO, J.H., COHEN, L.S., DALEN, J., DODGE, H.T., FRANCIS, C.K., KNATTERAD, G., LUDBROOK, P., MARKIS, J.E., MUELLER, H., NICKENS, P., PASSAMANI, E.R., POWERS, E.R., RAO, A.K., ROBERTS, R., ROSS, A., RYAN, T.J., SOBEL, B.E., WINNIFERD, M., ZARET, B., and co-investigators Intravenous recombinant tissue-type plasminogen activator in patients with acute myocardial infarction: a report from the NHLBI thrombolysis in myocardial infarction trial. *Circulation* 73:338-346, 1986
15. MASSEL, D.R., HUDOBA, M., and WEITZ, J.I. Clot-bound thrombin is protected from heparin inhibition- a potential mechanism for rethrombosis after lytic therapy. *Circ Suppl.* 80:1671, 1989
16. WEITZ, J.I., HUDOBA, M., MASSEL, D., MARAGANORE, J., and HIRSCH, J. Clot-bound thrombin is protected from inactivation by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. *J. Clin. Invest.* 86:385-391, 1990
17. OWEN, J., FRIEDMAN, K., GROSSMAN, B., WILKINS, C., BERKE, A., and POWERS, E. Thrombolytic therapy with tissue plasminogen activator or streptokinase induces transient thrombin activity. *Blood* 72:616-620, 1988
18. BERLINER, L.J., SUGAWARA, Y., and FENTON, J.W. II. Human alpha-thrombin binding to fibrin-Sepharose: Evidence for an anionic binding site. *Biochemistry* 24:7005-7009, 1985
19. WILNER, G.D., DANITZ, M.P., MUDD, M.S., HSIEH, K.H., and FENTON, J.W. Selective immobilization of alpha-thrombin by surface bound fibrin. *J. Lab. Clin. Med.* 97:403-411, 1981
20. FENTON, J.W. II, ZABINSKI, M.P., HSIEH, K., WILNER, G.D. Thrombin noncovalent-protein binding and fibrin(ogen) recognition. *Thromb. Haemostasis* 46:177, 1981
21. CHANG, T-L, FEINMAN, R.D., LANDIS, B.H., and FENTON, J.W. II Antithrombin reactions with alpha and gamma thrombins. *Biochemistry* 18:113-119, 1979
22. VALI, Z., and SCHERAGA, H The localization of the binding site on fibrin for the secondary binding site of thrombin. *Biochemistry* 27:1956-1963, 1988
23. KACZMREK, E., and McDONAGH, J. Thrombin binding to the Aa, Bb and gamma-chains of fibrinogen and to their remnants contained in fragment E. *J. Biol. Chem.* 263:13896-13900, 1988

24. NASKI, M.C., FENTON, J.W. II, MARAGANORE, J.M., OLSON, S.T., and SHAFER, J.A. The COOH-terminal domain of hirudin: An exosite-directed competitive inhibitor of the action of alpha-thrombin on fibrinogen. *J. Biol. Chem.* **265**:13484-13489, 1990
25. MAO, S.J., YATES, M.T., OWEN, T.J., and KRSTENANSKY, J.L. Interaction of hirudin with thrombin: Identification of a minimal binding domain of hirudin that inhibits clotting activity. *Biochemistry* **27**:8170-8173, 1988
26. MARAGANORE, J.M., CHAO, B., JOSEPH, M.L., JABLONSKI, J., and RAMACHANDRAN, K.L. Anticoagulant activity of synthetic Hirudin peptides. *J. Biol. Chem.* **264**:8692-8698, 1989
27. NASKI, M.C., and SHAFER, J.A. alpha-Thrombin-catalyzed hydrolysis of fibrin I: alternative binding modes and the accessibility of the active site in fibrin I-bound alpha-thrombin. *J. Biol. Chem.* **265**:1401-1407, 1990
28. HOGG, P.J., and JACKSON, C.M. Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III: Implications for heparin efficacy. *Proc. Natl. Acad. Sci. USA* **86**:3619-3623, 1989
29. NASKI, M.C., and SHAFER, J.A. A kinetic model for the alpha-thrombin-catalyzed conversion of plasma levels of fibrinogen to fibrin in the presence of antithrombin III. *J. Biol. Chem.* **266**:13003-13010, 1991
30. LEWIS, S.D., and SHAFER, J.A. A thrombin assay based upon the release of fibrinopeptide A from fibrinogen: Definition of a new thrombin unit. *Thromb. Res.* **35**:111-120, 1984
31. DEUTSCH, D.G., and MERTZ, E.T. Plasminogen: Purification from human plasma by affinity chromatography. *Science* **170**:1095-1096, 1970
32. ENGVALL, E., ROUSLAHTI, E., MILLER, E.J. Affinity of fibronectin to collagens of different types and to fibrinogen. *J. Exp. Med.* **147**:1584--1595, 1978
33. MIHALYI, E. Physicochemical studies of bovine fibrinogen IV. Ultraviolet absorption and its relation to the structure of the molecule. *Biochemistry* **7**:208-223, 1968
34. NORDENMAN, B., NYSTROM, C., and BJORK, I. The size and shape of human and bovine antithrombin III. *Eur. J. Biochem.* **78**:195-203, 1977
35. LOTTENBERG, R., and JACKSON, C.M. Solution composition dependent variation in the extinction coefficients for p-nitroaniline. *Biochim. Biophys. Acta* **742**:558-564, 1983
36. HIGGINS, D.L., and SHAFER, J.A. Fibrinogen petoskey, a dysfibrinogenemia characterized by replacement of Arg-A alpha by a histidyl residue. Evidence for thrombin-catalyzed hydrolysis at a histidyl residue. *J. Biol. Chem.* **256**:12013-12017, 1981
37. SUTTIE, J. and JACKSON, C. Prothrombin structure, activation and biosynthesis. *Physiological Reviews* **57**:1-70, 1977
38. CONRAD, J., BROSSTAD, LARSEN, M.L., SAMAMA, M., and ABILDGAARD, U. Molar antithrombin concentration in normal human plasma. *Haemostasis* **13**:363-368, 1983
39. NASKI, M.C., LORAND, L., and SHAFER, J.A. Characterization of the kinetic pathway for fibrin promotion of alpha-thrombin-catalyzed activation of plasma factor XIII. *Biochemistry* **30**:934-941, 1991
40. HOGG, P.J., and JACKSON, C.M. Formation of a ternary complex between thrombin, fibrin monomer, and heparin influences the action of thrombin on its substrates. *J. Biol. Chem.* **265**:248-255, 1990
41. FRANCIS, C.W., MARKHAM, R.E., BARLOW, G.H., FLORACK, T.M., DOBRZYNSKI, D.M., and MARDER, V.J. Thrombin activity of fibrin thrombi and soluble plasmic derivatives. *J. Lab. Clin. Med.* **102**:220-230, 1983

42. JESTY, J. The kinetics of formation and dissociation of the bovine thrombin-antithrombin III complex. *J. Biol. Chem.* 254:10044-10050, 1979
43. DANIELSSON, A., and BJORK, I. Slow, spontaneous dissociation of the antithrombin-thrombin complex produces a proteolytically modified form of the inhibitor. *FEBS Lett* 119:241-244, 1980
44. TEITEL, J.M., BAUER, K.A., LAU, H.K., and ROSENBERG, R.D. Studies of the prothrombin activation pathway utilizing radioimmunoassays for the F₂/F₁₊₂ fragments and thrombin-antithrombin complex. *Blood* 59:1086-1097, 1982
45. WEITZ, J.I., LESLIE, B., and HUDOBA, M. Thrombin remains bound to soluble fibrin degradation products and is partially protected from inhibition by heparin-antithrombin III. *Thromb. Haemostasis* 65:931, 1991
46. SEITZ, R., BLANKE, H., PRATORIUS, G., STRAUER, B.E., and EGBRING, R. Increased thrombin activity during thrombolysis. *Thromb Haemostasis* 59:541, 1988