Thrombin Binds to Soluble Fibrin Degradation Products
Where it Is Protected From Inhibition by Heparin-Antithrombin but Susceptible to Inactivation by Antithrombin-Independent Inhibitors

Jeffrey I. Weitz, MD; Beverly Leslie, BSc; Monika Hudoba, MD

Background—Thrombolytic therapy induces a procoagulant state characterized by elevated plasma levels of fibrinopeptide A (FPA), but the responsible mechanism is uncertain.

Methods and Results—Washed plasma clots were incubated in citrated plasma in the presence or absence of tissue plasminogen activator (t-PA), and FPA generation was monitored as an index of unopposed thrombin activity. FPA levels are almost twofold higher in the presence of t-PA than in its absence. This primarily reflects the action of thrombin bound to soluble fibrin degradation products because (a) there is progressive FPA generation even after clots are removed from t-PA–containing plasma, and (b) clot lysates produce concentration-dependent FPA generation when incubated in citrated plasma. Using thrombin-agarose affinity chromatography, (DD)E and fragment E but not D-dimer were identified as the thrombin-binding fibrin fragments, indicating that the thrombin-binding site is located within the E domain. Heparin inhibits thrombin bound to fibrin degradation products less effectively than free thrombin. In contrast, D-Phe-Pro-ArgCH2Cl, hirudin and hirugen inhibit free thrombin and thrombin bound to fibrin degradation products equally well.

Conclusions—Thrombin bound to soluble fibrin degradation products is primarily responsible for the increase in FPA levels that occurs when a clot undergoes t-PA–induced lysis. Like clot-bound thrombin, thrombin bound to fibrin derivatives is protected from inhibition by heparin but susceptible to inactivation by direct thrombin inhibitors. These findings help to explain the superiority of direct thrombin inhibitors over heparin as adjuncts to thrombolytic therapy.

Key Words: plasminogen activators ■ thrombolysis ■ thrombus ■ anticoagulants

Coronary thrombolysis is the treatment of choice for patients presenting early after the onset of symptoms of acute myocardial infarction. Thrombolytic therapy with either streptokinase or t-PA induces a procoagulant state characterized by elevated plasma levels of FPA consistent with increased thrombin activity.1–5 This procoagulant state may explain why reinfarction occurs in 3% to 6% of patients despite successful coronary thrombolysis.6 A number of mechanisms have been proposed to explain the procoagulant state induced by thrombolytic therapy. These include free plasmin formed as a result of the systemic lytic state, which can trigger the coagulation mechanism by activating contact factors,7 factor V,8 and possibly prothrombin.9,10 Another possibility is that thrombin bound to fibrin is progressively exposed as the clot undergoes lysis.11 Since fibrin-bound thrombin is protected from inactivation by fluid-phase inhibitors,12,13 it has the potential to locally activate platelets14 and accelerate coagulation.15 Alternatively, thrombin released from the lysing thrombus could induce a local procoagulant state.16 Finally, the increased FPA levels in patients treated with t-PA may not reflect thrombin activity because like thrombin, t-PA can also release FPA from fibrinogen.17 In this study we demonstrate a different mechanism. Thus we have shown that as a clot undergoes lysis, thrombin remains bound to soluble fibrin degradation products, where it still is protected from inactivation by fluid-phase inhibitors and hence has the potential to induce a systemic procoagulant state.

Methods

Reagents
Predominantly single-chain recombinant human t-PA (lot L9135A2) was obtained from Genentech, Inc, San Francisco. Human α-thrombin (sp act 2850 U/mg) was kindly provided by Dr J. Fenton II, New York State Department of Health, Albany, NY. PPACK was purchased from Hematologic Technologies, Inc, Essex Junction, Vt, and streptavidin-agarose was obtained from Sigma Chemical Co. Fragment E was purchased from Diagnostica Stago, Asnieres, France. Glu-plasminogen and a mouse monoclonal antibody against t-PA (PAM-2) were obtained from American Diagnostica, New York, NY. A mouse monoclonal antibody against D-dimer (DD) was generously provided by Dr D. Collen, University of Leuven, Belgium, whereas a monospecific rabbit antibody against fragment E was purchased from Sigma Chemical Co. Glu-plasminogen and a mouse monoclonal antibody against t-PA (PAM-2) were obtained from American Diagnostica, New York, NY. A mouse monoclonal antibody against D-dimer (DD) was generously provided by Dr D. Collen, University of Leuven, Belgium, whereas a monospecific rabbit antibody against fragment E was purchased from Diagnostica Stago. Standard heparin from porcine intestinal mucosa was obtained from Sigma Chemical Co, and PPACK was from

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Preparation of 125I-Labeled Fibrinogen

Fibrinogen was precipitated from barium sulfate–adsorbed plasma with 2 mol/L β-alanine as described in detail elsewhere. The isolated fibrinogen was then trace labeled with 125I to a specific activity of 100±5 μCi/mg.

Preparation of 125I-Labeled Cross-Linked Plasma Clots

Blood was collected from the antecubital veins of healthy volunteers into plastic syringes prefilled with 1/10 vol of 3.8% trisodium citrate. After thorough mixing with the anticoagulant, the red cells were sedimented by centrifugation at 1700g for 15 minutes at 4°C. The harvested platelet-poor plasma was supplemented with 125I-fibrinogen (≈120 000 cpm/mL) and 500-μL aliquots were transferred to polypropylene Eppendorf tubes. Labeled cross-linked fibrin clots were formed around wire hooks by the addition of CaCl₂ (final concentration, 25 mmol/L). The clots were aged for 60 minutes at 37°C with constant agitation and then washed six times with 1-ml aliquots of 0.1 mol/L NaCl buffered with 0.05 mol/L Tris-HCl, pH 7.4 (TBS) over the course of 18 hours to eliminate FPA trapped within the clots. The washed clots were then counted for radioactivity for 1 minute using a Clinigamma counter (LKB Instruments, Inc).

Clots formed in this fashion are cross-linked because they remain intact after 24 hours of incubation in 2% acetic acid. Further, SDS-PAGE analysis under reducing conditions of clots solubilized in SDS as described by Francis et al. demonstrates bands corresponding to the β-chains, γ-γ dimers, and α-polymers (data not shown). Non–cross-linked α- or γ-chains are not present, thus indicating complete cross-linking.

Clot-Induced FPA Generation in the Presence or Absence of t-PA

Washed 125I-labeled plasma clots were incubated with 1-ml aliquots of fresh citrated plasma for 90 minutes at 37°C in the presence or absence of 1 μg/mL t-PA, and clot lysis and FPA generation were monitored. As a control, citrated plasma was incubated without plasma clots for 90 minutes at 37°C in the presence or absence of 1 μg/mL t-PA. The extent of t-PA induced clot lysis was quantified (1) by monitoring the time course of release of 125I-labeled fibrin degradation products and (2) by removing the clots from the plasma at intervals and counting their residual radioactivity for 1 minute after the clots were briefly washed three times with 500-μL aliquots of TBS. To calculate the percentage clot lysis, the difference between the radioactivity originally incorporated into the clot and the residual radioactivity was calculated and expressed as a percentage of the original radioactivity.

In parallel, clot-induced FPA generation also was monitored. To accomplish this, 100-μL aliquots of plasma were removed at intervals, and unreacted fibrinogen was precipitated by the addition of 300 μL of chilled ethanol followed by centrifugation at 15 000g for 5 minutes. The ethanol supernatants were then evaporated to dryness in a Speed-Vac concentrator (Savant Instruments, Inc), reconstituted to original volume with distilled water, and assayed for FPA using a previously described radioimmunoassay. This assay utilizes antiserum R.2, which is specific for FPA and cross-reacts poorly with fibrinogen or larger FPA-containing fragments.

Preparation of Clot Lysates

Lysates of extensively washed 125I-labeled plasma clots were prepared as we have previously described with only minor modifications. Briefly, the clots were incubated for 60 minutes at 37°C in 500-μL aliquots of TBS containing 4 μg/mL t-PA, 1.7 μmol/L glu-plasminogen, and 0.02% Tween 80. At the end of the incubation period, the residual clots were removed and the clot lysates were then pooled and immunodepleted of t-PA by affinity chromatography. A mouse monoclonal antibody against the kringle-1 region of human t-PA (PAM-2, American Diagnostica) was coupled to cyanogen bromide activated CH Sepharose 4B (Pharmacia Fine Chemicals) at a concentration of 20 mg/mL. The clot lystate (10 mL) and anti-t-PA IgG coupled to CH Sepharose 4B (750 μL of a 50% suspension) were mixed in a tube and agitated for 1 hour at 23°C. After centrifugation at 10 000g for 5 minutes, the supernatants were carefully removed, and the immunodepletion procedure was then repeated for two additional cycles. The final material, which contained <50 ng of t-PA as measured antigenically using an enzyme-linked immunosorbent assay kit from American Diagnostica, was then concentrated 10-fold using an ultrafiltration cell (series 8050, Amicon Division, W.R. Grace & Co) fitted with a 5000-mw cutoff membrane (YM5 disk membrane, Amicon), and stored in aliquots at −70°C.

Clot Lysate-Induced FPA Generation in Plasma

To examine the ability of soluble fibrin degradation products to generate FPA, varying amounts of clot lysate were incubated in 500-μL aliquots of citrated plasma for 60 minutes at 37°C. At intervals, 100-μL aliquots were removed, and the unreacted fibrinogen was precipitated with 300 μL of chilled ethanol followed by centrifugation at 15 000g for 5 minutes. The ethanol supernatants were then evaporated to dryness, reconstituted to original volume with distilled water, and assayed for FPA.

Effect of Thrombin Inhibitors on FPA Generation in Plasma Induced by Fluid-Phase Thrombin and Clot Lysates

To compare the ability of various thrombin inhibitors to block clot lysate–induced FPA generation with their activity against FPA generation produced by fluid-phase thrombin, clot lysates (in amounts ranging from 2.5 to 10 μL) or human α-thrombin (in concentrations ranging from 0.2 to 4.0 nmol/L) were incubated with 500-μL aliquots of citrated plasma for 60 minutes at 37°C. At intervals, 100-μL aliquots of plasma were removed, and the unreacted fibrinogen was precipitated with 300 μL of chilled ethanol followed by centrifugation at 15 000g for 5 minutes. The ethanol supernatants were then evaporated to dryness, reconstituted to original volume with distilled water, and assayed for FPA. The percent inhibition of FPA generation produced by each concentration of thrombin inhibitor was then calculated.

Adsorption of 125I-Labeled Fibrin Degradation Products to Thrombin-Agarose

To identify those fibrin derivatives that bind thrombin, aliquots of the clots of 125I-labeled cross-linked fibrin clots were subjected to chromatography on thrombin-agarose, and the bound and unbound fractions were then isolated and characterized by SDS-PAGE and autoradiography. To couple thrombin to agarose, human α-thrombin (2 mg) was incubated for 30 minutes at 37°C with a 10-fold molar excess of biotinylated PPACK. The mixture was then dialyzed against TBS containing 6 mg/mL polyethylene glycol (PEG) to remove uncomplexed PPACK. After packing a column (5×1.2 cm) with 6 mL of streptavidin-agarose (50% suspension), which was then equilibrated with TBS containing 6 mg/mL PEG, the biotinylated PPACK-thrombin complexes were passed over the column. Based on the absorbance of the effluent at 280 nm, over 98% of the active

Selected Abbreviations and Acronyms

FPA = fibrinopeptide A
PPACK = D-Phe-Pro-ArgCH2Cl
t-PA = tissue plasminogen activator

Calbiochem Corp. Recombinant hirudin with a Val-Val amino-terminal sequence was purchased from American Diagnostica. The synthetic tyrosine–sulfated dodecapeptide known as hirudin, which is comprised of residues 53 to 64 of hirudin (H-peptide, BG8865), was generously provided by Dr J. Maraganore, Biogen Inc, Cambridge, Mass.

Preparation of 125I-Labeled Fibrinogen

Fibrinogen was precipitated from barium sulfate–adsorbed plasma with 2 mol/L β-alanine as described in detail elsewhere. The isolated fibrinogen was then trace labeled with 125I to a specific activity of 100±5 μCi/mg.
site-blocked thrombin bound to the column. After blocking unreacted streptavidin with 10 mL of TBS containing 1.2 mg/mL biotin, the column was extensively washed with 0.05 mol/L NaCl buffered with 0.05 mol/L Tris-HCl and containing 1 mg/mL PEG (TBS-PEG).

Aliquots of the lysate of 125I-labeled fibrin clots, suspended in TBS-PEG, were then subjected to affinity chromatography at 23°C on the thrombin–agarose column. 0.5–mL fractions were collected and their radioactivity and absorbance at 280 nm were determined. After extensive washing of the column with TBS-PEG, the bound material was eluted with 0.3 mol/L NaCl buffered with 0.05 mol/L HCl to pH 7.4 and containing 1 mg/mL PEG. Again, 0.5 mL fractions were collected, and their radioactivity and absorbance at 280 nm were determined. After confirming the protein concentrations using the method of Lowry, 25 the peak protein-containing fractions were pooled, and then concentrated using Centricon-10 ultracentrifugation cartridges (Amicon Division, W.R. Grace & Co).

Gel Filtration of Clot Lysates
125I-labeled fibrin degradation products eluted from thrombin–agarose were subjected to chromatography at 23°C on a column (90 x 1.6 cm) of Sephacryl-S300 HR (Pharmacia Fine Chemicals) using TBS at a rate of 50 mL/h. For preparative procedures, dextran blue (Sigma Chemical Co) was used to determine the void volume, and the column was calibrated with thyroglobulin, gamma-globulin, ovalbumin, myoglobin, and cyanocobalamin with molecular weights of 670 000, 158 000, 44 000, 17 000, and 1350 D, respectively. Fractions (2 mL) were collected and their radioactivity and absorbance at 280 nm were measured. After confirming the protein concentrations using the method of Lowry, 25 peak protein-containing fractions were pooled and concentrated using Centricon-10 ultrafiltration cartridges.

PAGE and Immunoblot Analysis of Fibrin Degradation Products
The fibrin degradation products that bind and do not bind to thrombin–agarose were characterized using a combination of PAGE and immunoblot analysis. Samples were diluted in an equal volume of 60 mmol/L Tris-HCl containing 0.001% bromphenol blue with or without 2% SDS and 5% glycerol. Two electrophoretic systems were used: a 7.5% polyacrylamide slab gel (4% stacking gel) containing 0.1% SDS and a 7.5% polyacrylamide gel (4% stacking gel) under nondissociating conditions according to the method of Davis. 27 The gels were either fixed in 40% methanol/10% acetic acid and stained with Coomassie blue or the separated proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were then probed with either a monoclonal antibody against DD (8D3) or with a monospecific antibody against fragment E as we have previously described. 25

Statistical Analyses
Where appropriate, means and 95% confidence intervals (CI) were calculated. Significance of differences was determined by one-way, two-way, or repeated-measures ANOVA, 28 and a value of P < .05 was considered statistically significant.

Results
Formation of 125I-Labeled Fibrin Clots
The recalcification of 500–μL aliquots of plasma containing 125I-labeled fibrinogen results in the formation of clots of standard size as determined by their radioactivity. More than 95% of the radiolabeled fibrinogen is incorporated into the clots so that each clot contains 57 215 ± 1380 cpm (mean ± SD).

Increased FPA Generation in the Presence of t-PA
When washed 125I-labeled fibrin clots are incubated in citrated plasma, there is time-dependent generation of FPA (Fig 1).

Mechanism for Increased Clot-Induced FPA Generation in the Presence of a Lysing Thrombus
To investigate the mechanism responsible for the increased FPA generation in the presence of a lysing thrombus, 125I-labeled fibrin clots were incubated for 2 hours at 37°C in 500–μL aliquots of plasma containing 1 μg/mL t-PA. At varying intervals, the clots were removed, and the time-course of clot lysis was monitored by measuring the release of 125I-labeled fibrin degradation products (Fig 2A), while clot-induced FPA generation was followed in parallel (Fig 2B). The concentration of released 125I-labeled fibrin degradation products depends on the duration of exposure of the clots to t-PA. Once the clots are removed from the t-PA–containing plasma however, release of 125I-labeled fibrin degradation products...
ceases (Fig 2A). In contrast, there is ongoing FPA generation after the clots are removed (Fig 2B).

These data were analyzed by first calculating the slope of the relationships between the levels of 125I-labeled fibrin degradation products or FPA and time from the points where the clots were removed from t-PA–containing plasma. Using one-way ANOVA, the mean slopes did not differ as a function of the time that the clots were removed from plasma. Whereas the mean slope for the time courses of 125I-labeled fibrin degradation product generation (Fig 2A) was 0.0078 (95% CI: −0.0049, 0.0051), a value by one-sample t test not significantly different from zero (P = .95), the mean slope for the time courses of FPA generation (Fig 2B) was 0.84 (95% CI: 0.73, 0.95); a value that was significantly different from zero by t test (P < .0001). The continuing FPA generation after clot removal excludes the possibility that clot-bound thrombin is responsible for this phenomenon, and suggests that a soluble factor also contributes.

To examine the possibility that thrombin bound to soluble fibrin degradation products is responsible, at least in part, for ongoing FPA generation after clots are removed from t-PA–containing plasma, varying amounts of clot lysate were incubated in plasma and the levels of FPA were measured. As illustrated in Fig 3A, with each concentration of lysate added there is a slow, progressive increase in FPA levels. In contrast, when free thrombin is incubated in plasma, FPA generation reaches a plateau in minutes as the thrombin is rapidly complexed to inhibitors (Fig 3B). Analysis of these data by repeated-measures ANOVA indicates a highly significant (P < .0001) interaction between lysate-induced FPA generation and time (Fig 3A). In contrast, with free thrombin (Fig 3B), there is no significant time effect (P = .98) once maximum FPA release has been achieved at 5 minutes. The slow, time-dependent increase in FPA levels produced by the lysates indicates that the thrombin associated with soluble fibrin degradation products is enzymatically active, and is able to cleave FPA from fibrinogen despite the presence of physiological concentrations of antiproteinases.

Comparison of the Activity of Thrombin Inhibitors Against Thrombin Bound to Fibrin Degradation Products With Their Activity Against Fluid-Phase Thrombin

To compare the ability of different thrombin inhibitors to inactivate thrombin bound to fibrin degradation products, varying amounts of clot lysate were incubated in plasma in the presence or absence of the inhibitors and FPA generation was determined. These results were then compared with the ability of the thrombin inhibitors to block FPA generation induced by varying concentrations of free thrombin. PPACK (Fig 4), hirugen (Fig 5), and hirudin (Fig 6) are equally effective inhibitors of free and bound thrombin because they block FPA generation mediated by free thrombin and thrombin bound to soluble fibrin degradation products to a similar extent. Thus two-way ANOVA indicate nonsignificant differences in the extents to which PPACK, hirugen, and hirudin inhibit FPA generation triggered by free thrombin and clot lysates, respectively (P = .75, .14, and .85, respectively). In contrast to direct thrombin inhibitors, heparin is less effective at inhibiting thrombin bound to fibrin degradation products than it is at blocking the fluid-phase enzyme (Fig 7). Thus, two-way analysis of variance shows highly significant (P < .0001) differences between its effect on free thrombin and thrombin bound to fibrin degradation products. For example, 0.1 U/mL of heparin produces 70% inhibition of FPA release mediated by fluid-phase thrombin but only inhibits thrombin bound to fibrin degradation products.
fibrin degradation products by 30%. A concentration of heparin of 0.5 U/mL totally inhibits FPA generation by fluid-phase thrombin. In contrast, this concentration of heparin only blocks lysate-induced FPA generation by 70%.

Adsorption of ¹²⁵I-Labeled Fibrin Degradation Products to Thrombin-Agarose
To isolate those soluble fibrin degradation products that bind thrombin, the adsorption of these derivatives to thrombin-agarose was examined. Approximately 60% of the ¹²⁵I-labeled fibrin degradation products in the crude clot lysates bound to thrombin-agarose (data not shown), and the bound and unbound fractions were analyzed using SDS-PAGE followed by autoradiography (Fig 8). In the material that binds to thrombin-agarose, two major bands are visualized at 195 000 D and 60 000 D, respectively. In contrast, only the ≈195 000 D band is seen when the material that does not bind to thrombin-agarose is analyzed.

Gel Filtration of Fibrin Degradation Products That Bind to Thrombin-Agarose
To further characterize the fibrin degradation products that bind and do not bind to thrombin-agarose, the material that bound to this absorbent was subjected to chromatography on Sephacryl S300 HR to separate the products according to their molecular weight. As illustrated in Fig 9, the major protein peak (peak A) has a molecular weight of ≈225 000 D, based on its elution profile, while the second smaller peak (peak B) has a molecular weight of ≈60 000 D. The peak protein-containing fractions from the gel filtration column were collected, pooled as indicated, and then further characterized by PAGE and immunoblot analysis.

Characterization of Fibrin Degradation Products
Those fibrin derivatives that bind to thrombin-agarose were identified using a combination of PAGE and immunoblot analysis. Electrophoresis was performed under both dissociating and nondissociating conditions. PAGE analysis under nondissociating conditions indicates that the peak eluting at ≈225 000 D (Fig 9, peak A) consists mainly of (DD)E complex because there is a 255 000 D band (Fig 10A, lane 2) that is recognized both by the antibody against DD (Fig 10B, lane 2) and by the antibody against fragment E (Fig 10C, lane 2). In contrast, in the presence of SDS, this material separates into two bands at ≈195 000 and 60 000 D, respectively (data not shown). The higher molecular weight material is recognized...
by the monoclonal antibody against DD, whereas the antibody against fragment E primarily reacts with the lower molecular weight band.

The lower molecular weight peak recovered from the gel filtration column (Fig 9, peak B) migrates as a major band at \( \approx 55,000 \) D (Fig 10A, lane 3), which is recognized by the antibody against fragment E (Fig 10C, lane 3) and not by the antibody against DD (Fig 10B, lane 3), indicating that this is fragment E.

The fibrin degradation products that do not bind to thrombin-agarose (Fig 8, lane 2) also were characterized by immunoblot analysis (data not shown). This material consists of DD because the 195,000 D band is recognized by the monoclonal antibody against DD. Little or no fragment E is present since a 60,000 D band is not seen in Fig 8, nor is a band of this molecular weight visualized on immunoblot analysis using the antibody against fragment E.

### Discussion

It is well established that thrombolytic therapy induces a procoagulant state characterized by elevated plasma levels of FPA consistent with increased thrombin activity.\(^1\)\(^-\)\(^4\) Using plasma levels of FPA as an index of unopposed thrombin activity, like Mirshahi and colleagues\(^1\)\(^1\) we have demonstrated that a lysing clot generates more FPA than an intact thrombus (Fig 1). This is not due to direct, t-PA–mediated release of FPA from fibrinogen because only small amounts of FPA are generated when plasma is incubated with t-PA in the absence of a clot (Fig 1). Furthermore, our observation that there is ongoing FPA generation after a clot is removed from t-PA–containing plasma (Fig 2) indicates that a soluble factor is a more important mediator of this phenomenon than the progressive exposure of clot-bound thrombin as has been proposed in the past.\(^1\)\(^1\) This concept is supported by the finding that clot lysates produce concentration-dependent generation of FPA when incubated in plasma (Fig 3).

The pattern of FPA generation produced by clot lysates (Fig 3A) is different from that seen with solution-phase thrombin (Fig 3B). Thus FPA release from fibrinogen reaches a plateau in minutes when free thrombin is added to plasma because the enzyme is rapidly inhibited by plasma antithrombins (Fig 3B). In contrast, the lysates produce slow progressive FPA generation throughout the incubation period (Fig 3A). These findings indicate that thrombin associated with the fibrin degradation products is not only enzymatically active but also is protected from inactivation by plasma antiproteinases. Like the time course of clot lysate-induced FPA generation, there is ongoing FPA generation when clots are removed from t-PA–containing plasma indicating that release of free thrombin from the degrading clot is not responsible for the increased FPA generation produced by a lysing thrombus. This concept is further supported by the observation that heparin is considerably less effective at inhibiting lysate-induced FPA generation than it is at blocking FPA release mediated by fluid-phase thrombin (Fig 7).

Thrombin binds to the E domain of the degradation products of cross-linked fibrin because (DD)E complex and...
fragment E bind to thrombin–agarose, whereas d-dimer does not (Fig 8). These findings indicate that like its interaction with fibrinogen, thrombin binds to a site within the N-terminal disulfide knot of fibrin. Our results are consistent with those of Meh et al.,29 who identified a low affinity thrombin binding site on fibrin fragment E. The amino-terminal β15 to 42 sequence appears to be an important component of this site because fibrin molecules lacking this sequence showed diminished binding of thrombin.29 However, the α-chain may also be involved because a synthetic analogue of fibrinogen residues α27 to 50 inhibits thrombin-induced clotting of fibrinogen,30 presumably by competing with fibrinogen for thrombin binding. In addition to the low affinity thrombin binding site on the E domain, Meh and colleagues29 also identified a high affinity thrombin binding site on the D-domain of γ-chains, γ-chain variants with highly anionic carboxyl-terminal sequences. Our observation that d-dimer does not bind to thrombin–agarose raises the possibility that the high affinity binding sites on the γ-chain of fibrin is released early during the course of plasmic-mediated degradation of fibrin. The thrombin binding site may be particularly susceptible to plasmic cleavage because of the Arg residue at its amino-terminal.29 Further support for the concept that thrombin binds to (DD)E comes from the studies of Francis and colleagues,31 who demonstrated that thrombin found in plasmic digests of cross-linked clots was associated with (DD)E and larger (DD)E-containing fragments. In keeping with our findings, these authors also showed that thrombin bound to these fragments is enzymatically active. However, their studies of thrombin activity were done in buffer systems, whereas ours were done in plasma. By performing the experiments in a plasma system, we have demonstrated that thrombin bound to fibrin degradation products is not only enzymatically active, but also is protected from inactivation by fluid-phase inhibitors. Just as thrombin bound to fibrin degradation products is protected from inactivation by circulating antithrombins, it also is less likely to be complexed by thrombomodulin lining the microcirculation.

Whereas rate-limited diffusion could explain why circulating antithrombins are unable to inactivate thrombin bound to fibrin within the interstices of an intact clot, this cannot account for their inability to inhibit thrombin bound to soluble fibrin fragments. Instead, our findings suggest that the active site of thrombin undergoes a conformational change when the enzyme binds to fibrin thereby limiting its reactivity with its naturally occurring macromolecular inhibitors. This allosteric change may explain why, Ro 46–6240, a small molecule that interacts with the active site of thrombin, appears to inhibit clot-bound thrombin to a greater extent than free thrombin.32 However, the conformational change induced by fibrin binding does not limit thrombin’s reactivity with all active-site inhibitors because PPACK inhibits FPA generation produced by clot lysates and free thrombin equally well (Fig 4).

Like thrombin bound to an intact fibrin clot, thrombin bound to soluble fibrin degradation products is relatively protected from inhibition by heparin (Fig 7). Thus, 0.1 U/mL heparin produces ≈70% inhibition of FPA release mediated by fluid-phase thrombin but only inhibits lysate-induced FPA generation by about 30%. Even when heparin is used in a concentration of 0.5 U/mL, which is above the therapeutic range of 0.2 U/mL to 0.4 U/mL,33 it only produces about 70% inhibition of lysate-induced FPA generation. At a heparin concentration of 1 U/mL, a level which may be achieved after a 5000 U bolus of heparin is given to patients,34 there still is incomplete inhibition of lysate-induced FPA generation. These findings may explain why FPA levels remain elevated in some patients given thrombolytic therapy, even in the face of therapeutic doses of heparin.5,35 In contrast, any free thrombin generated as a result of plasmic-mediated activation of coagulation would be readily inhibited by heparin (Fig 7) making this a less plausible explanation for the heparin-resistant increase in FPA levels in the setting of thrombolytic therapy.

Comparison of the results of this study with our previous work35 indicates that thrombin bound to soluble fibrin degradation products is less protected from inhibition by heparin than thrombin bound to intact fibrin clots. For example, 0.2 U/mL heparin, a concentration that totally blocks FPA release by fluid-phase thrombin, inhibits lysate- and clot-induced FPA generation by 52% and 20%, respectively. These findings are consistent with the results of Prager and colleagues,37 who demonstrated that FPA generation triggered by whole blood...
clots was more readily suppressed by heparin when the clots were partially degraded by t-PA than when they were intact.

To catalyze thrombin inhibition by antithrombin, heparin must bind to both thrombin and antithrombin. The relative inability of heparin to inactivate thrombin bound to intact clots, and to fibrin degradation products (Fig 7) suggests that the heparin binding site on thrombin is masked when the enzyme is bound to fibrin or its soluble fragments. In contrast to heparin, the direct thrombin inhibitors inactivate free thrombin and thrombin bound to fibrin degradation products equally well (Figs 4 to 6), suggesting that their sites of interaction remain accessible when the enzyme is bound to fibrin derivatives. It is well established that thrombin’s interaction with fibrin involves a site distinct from the catalytic center. Since PPACK binds to the active site of thrombin, wherein it alkylates the active center histidine, it is not surprising that it is an effective inhibitor of thrombin-bound to fibrin degradation products. In contrast to PPACK however, hirudin not only interacts with the active site of thrombin but also binds to the noncatalytic substrate-binding domain on thrombin known as anion-binding exosite 1, whereas hirugen interacts exclusively with exosite 1. If thrombin binds to fibrin or fibrin degradation products via exosite 1, the observation that hirudin and hirugen inhibit free thrombin and thrombin bound to soluble fibrin fragments equally well suggests that the affinity of these agents for exosite 1 on thrombin is greater than thrombin’s affinity for fibrin degradation products.

Hirudin inactivates free thrombin and thrombin bound to fibrin degradation products equally well (Fig 6). In contrast, we previously reported that hirudin is somewhat limited in its ability to inhibit thrombin bound to plasma clots. The limited ability of hirudin to inactivate clot-bound thrombin could reflect rate-limited diffusion of hirudin into the interstices of the clot. Thus with a molecular mass of 7000, hirudin may enter the clot less readily than PPACK whose molecular mass is about 500.

The finding that, like their superior activity against clot-bound thrombin, the direct thrombin inhibitors also are better than heparin at inactivating thrombin bound to fibrin degradation products (Figs 4 to 7) provides a plausible explanation for the observation that these agents have advantages over heparin when used as adjuncts to thrombolytic therapy in experimental animals or in humans. For example, in a rat thrombolysis model, hirulog was better than heparin at accelerating t-PA–induced thrombolysis and preventing thrombotic reocclusion. Similar results have also been obtained in humans. Thus when hirulog was compared with heparin as adjunctive therapy to streptokinase in patients with acute myocardial infarction, coronary artery patency rates at 90 and 120 minutes were significantly higher in hirulog-treated subjects than in those given heparin. Likewise, early patency rates also appear to be higher when hirudin was used in conjunction with t-PA than when heparin was given.

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References


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