Bivalent Binding to $\gamma_A/\gamma'$-Fibrin Engages Both Exosites of Thrombin and Protects It from Inhibition by the Antithrombin-Heparin Complex*

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Thrombin exosite 1 binds the predominant $\gamma_A/\gamma_A$-fibrin form with low affinity. A subpopulation of fibrin molecules, $\gamma_A/\gamma'$-fibrin, has an extended COOH terminus $\gamma'$-chain that binds exosite 2 of thrombin. Bivalent binding to $\gamma_A/\gamma'$-fibrin increases the affinity of thrombin 10-fold, as determined by surface plasmon resonance. Because of its higher affinity, thrombin dissociates 7-fold more slowly from $\gamma_A/\gamma'$-fibrin clots than from $\gamma_A/\gamma_A$-fibrin clots. After 24 h of washing, however, both $\gamma_A/\gamma'$- and $\gamma_A/\gamma_A$-fibrin clots generate fibrinopeptide A when incubated with fibrinogen, indicating the retention of active thrombin. Previous studies demonstrated that heparin heightens the affinity of thrombin 10-fold, as determined by surface plasmon resonance; because of its higher affinity, thrombin dissociates 7-fold more slowly from $\gamma_A/\gamma_A$-fibrin clots than from $\gamma_A/\gamma_A$-fibrin clots. This difference reflects bivalent binding of thrombin to $\gamma_A/\gamma'$-fibrin because (a) it is abolished by addition of a $\gamma'$-chain-directed antibody that blocks exosite 2-mediated binding of thrombin to the $\gamma'$-chain and (b) the dermatan sulfate-catalyzed rate of thrombin inhibition by heparin cofactor II also is lower with $\gamma_A/\gamma'$-fibrin than with $\gamma_A/\gamma_A$-fibrin clots. Thus, bivalent binding of thrombin to $\gamma_A/\gamma'$-fibrin protects thrombin from inhibition, raising the possibility that $\gamma_A/\gamma'$-fibrin serves as a reservoir of active thrombin that renders thrombin thrombogenic.

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2 The abbreviations used are: f., factor; Chz, chromozyme; DS, dermatan sulfate; PPR, D-Phe-Pro-Arg; GP, glycoprotein; HCII, heparin cofactor II; Ila, thrombin; RU, response unit; Req, RU equilibrium; SPR, surface plasmon resonance; $\tau_{1/2}$, half time of dissociation; YPR, D-Tyr-Pro-Arg.
the formation of a ternary heparin-thrombin-fibrin complex wherein thrombin binds $\gamma_A/\gamma^\prime$-fibrin via exosite 1 and heparin binds simultaneously to fibrin and to exosite 2 on thrombin, thereby bridging the thrombin to the fibrin (15, 16). Formation of this ternary heparin-thrombin-fibrin complex protects thrombin from inactivation by antithrombin and HCII. This protection reflects the inability of the serpin-bound heparin molecule to bridge to thrombin because exosite 2 is occupied by the fibrin-bound heparin (17). The extent of protection is greater for HCII than it is for antithrombin. This reflects the fact that, in addition to bridging of thrombin to HCII by heparin, the NH$_2$ terminus of HCII must also bind to exosite 1, which is already occupied. The phenomenon of thrombin protection when bound to fibrin may explain the limited capacity of heparin to block fibrin accretion in venous thrombosis models and the recurrent ischemic events that occur despite heparin treatment in patients with acute coronary syndromes (18, 19).

Both exosites of thrombin are engaged when the enzyme is incorporated within the ternary heparin-thrombin-fibrin complex. This bivalent binding protects thrombin from inhibition (17). Likewise, both exosites are also occupied in the high affinity interaction of thrombin with $\gamma_A/\gamma^\prime$-fibrin (14). It is currently unknown whether thrombin bound to $\gamma_A/\gamma^\prime$-fibrin is protected from inhibition by antithrombin and HCII. This is an important question because epidemiological studies suggest that elevated levels of $\gamma_A/\gamma^\prime$-fibrinogen are a risk factor for thrombosis (20, 21). To begin to address this issue, we (a) used surface plasmon resonance to better elucidate the mode of binding of thrombin to $\gamma_A/\gamma_A^\prime$- and $\gamma_A/\gamma^\prime$-fibrinogen, (b) examined the extent to which $\gamma_A/\gamma_A^\prime$- and $\gamma_A/\gamma^\prime$-fibrinogen sequester thrombin and modulate its clotting activity, and (c) compared the capacity of $\gamma_A/\gamma_A^\prime$ and $\gamma_A/\gamma^\prime$-fibrinogen to protect bound thrombin from inhibition by antithrombin and HCII.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Human $\alpha$-thrombin, $\gamma$-thrombin, and plasminogen-free fibrinogen were from Enzyme Research Laboratories (South Bend, IN). Heparin, chloramine T, and ovalbumin were from Sigma; Atroxin was from Centerchem Inc. (Norwalk, CT). Antithrombin, HCII, and a sheep antibody directed against human factor XIII were from Affinity Biologicals, Inc. (Ancaster, ON). The tyrosine-phosphorylated peptide corresponding to the COOH-terminal 20 residues of the $\gamma^\prime$-chain ($\gamma^\prime$-peptide), VRPEHAETEYDSLYPEDDL, and a rabbit polyclonal antibody directed against the $\gamma^\prime$-peptide were prepared by Bachem Bioscience, Inc. (King of Prussia, PA). Chromozyme-thrombin (Chz-Th) (tosyl-Gly-Pro-Arg $p$-nitroaniline acetate) was from Roche Applied Science. Dermatan sulfate (DS) was from Medelplanum Farmaceutici (Milan, Italy).

**Radiolabeling**—Thrombin was radiolabeled by reaction with $^{125}$I-labeled Tyr-Pro-Arg chloromethyl ketone (YPR) (22). YPR (Bachem Bioscience, Inc.) was first radiolabeled by mixing 20 mg/ml YPR, 1 mCi of Na$^{125}$I (Perkin-Elmer Life Sciences) 15 $\mu$L of 4 mg/ml chloramine T, and 100 $\mu$L of phosphate-buffered saline, pH 7.4, for 90 s. The labeling reaction was terminated by incubating for a further 60 s after addition of 25 $\mu$L of 6 mg/ml sodium metabisulfite. To this reaction mixture was added 100 $\mu$L of 3.2 mg/ml thrombin. After incubation for 30 min at room temperature, $^{125}$I-YPR-thrombin was separated from unincorporated YPR and $^{125}$I by size exclusion chromatography on a PD-10 column (GE Healthcare) equilibrated with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TS). Fractions of 0.5 ml were collected, and aliquots were counted for radioactivity in a $\gamma$-counter. $^{125}$I-YPR-thrombin concentration was 7.1 $\mu$M, as determined by absorbance at 280 nm (23), with a specific radioactivity of 680,000 counts/min/$\mu$g protein. D-Phe-Pro-Arg chloromethyl ketone (FPR) (EMD Chemicals, Inc., San Diego, CA) modified thrombin was prepared as described (23).

**Fibrinogen Preparation**—$\gamma_A/\gamma^\prime$- and $\gamma_A/\gamma^\prime$-fibrinogen were isolated by fractionation of fibrinogen on a DEAE-Sepharose column (14, 24) in the presence of 10 units/ml aprotinin. About 300 mg of fibrinogen was loaded onto a 2.5 × 20-cm DEAE-FS-Sepharose column (GE Healthcare) equilibrated with 39 mM Tris-phosphoric acid, pH 8.8 (buffer A). The column was developed with a concave gradient to 50% buffer B (500 mM Tris-phosphoric acid, pH 4) followed by a linear gradient to 100% B. Chromatography was performed using a high pressure liquid chromatography system (Beckman 32 Karat; Beckman-Coulter Inc., Fullerton, CA) at a flow rate of 5 ml/min. The two fractions of fibrinogen were pooled separately and precipitated with 50% ammonium sulfate. Fibrinogen pellets were washed twice with 25% ammonium sulfate and then resuspended in TS. The $\gamma_A/\gamma_A^\prime$-fibrinogen pool was diluted to 10 mg/ml and applied to a sheep anti-human f.XIIa column. The eluate was again ammonium sulfate-precipitated and dissolved in TS. Preparations were verified to be free of f.XIIa activity by examining thrombin-clotted samples for $\gamma-\gamma$ dimers on SDS-PAGE (25).

**MATERIALS AND METHODS**

**Surface Plasmon Resonance**—Fibrinogen ($\gamma_A/\gamma^\prime$ or $\gamma_A/\gamma_A^\prime$) or ovalbumin was bound to separate flow cells of a CM5 chip in a BIAcore 1000 using an amine coupling kit (BIAcore; GE Healthcare). Injection was continued until 7000 response units (RU) of fibrinogen or 2500 RU of ovalbumin was adsorbed. Flow cells were treated with 1 M ethanolamine and then washed with 20 mM Hepes-OH, pH 7.4, 150 mM NaCl (HBS) buffer containing 2 mM CaCl$_2$ and 0.005% Tween 20 at a flow rate of 20 $\mu$l/min. Flow cells were then treated with two injections of 300 $\mu$l of 100 $\mu$g/ml thrombin at 5 $\mu$l/min to ensure complete conversion of fibrinogen to fibrin. Using saturating quantities of the $\gamma^\prime$-peptide-directed antibody, 30% of the immobilized fibrin was determined to be in an accessible orientation. For binding studies, 20-$\mu$l aliquots of FPR-thrombin (0–5000 nM) were injected into the flow cells, followed by a 1-min wash to monitor dissociation. No regeneration of the flow cells between injections was necessary. Peak RU values (Req) were determined for each thrombin concentration and were corrected for the RU values with ovalbumin. Req values were converted to mass of FPR-thrombin, using the relation 1 pg = 1 RU (BIAtechnology Handbook; BIAcore). Moles of FPR-thrombin bound/mole of fibrin were calculated and plotted versus moles-free FPR-thrombin, and the data were analyzed by nonlinear regression of a one- or two-site rectangular hyperbola using TableCurve.
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(Jandel Scientific, San Rafael, CA). The results are reported as means \(\pm\) S.E. of two determinations.

The ability of the \(\gamma^-\)-peptide to compete with \(\gamma\)-thrombin binding to \(\gamma_A/\gamma^-\)-fibrin also was assessed by surface plasmon resonance. A CM5 chip with adsorbed \(\gamma_A/\gamma^-\)-fibrin was prepared as described above. Aliquots of 5 \(\mu\)M \(\gamma\)-thrombin containing 0–25 \(\mu\)M \(\gamma^-\)-peptide were injected, and Req values were determined.

\textit{Inhibition Kinetics—}Thrombin inhibition was determined in a continuous assay by monitoring residual thrombin activity within fibrin clots. Samples containing 400 nM antithrombin or HCII, 2.7 \(\mu\)M \(\gamma_A/\gamma^-\) or \(\gamma_A/\gamma^-\)-fibrinogen, 400 \(\mu\)M Chz-Th, and 1 \(\mu\)g/ml heparin or 5 \(\mu\)g/ml DS were prepared in a multwell plate. Clotting was initiated by the addition of 2.5 units/ml Atroxin and 2.2 \(\mathrm{nM}\) thrombin. Atroxin was used in addition to thrombin to ensure that both forms of fibrinogen clotted at a similar and rapid rate. The reactions were monitored at 405 and 450 nm in a plate reader (Molecular Devices, Sunnyvale, CA). Clot formation occurred within 45 s. Absorbance values subsequent to this were analyzed with respect to time (\(t\)) by nonlinear regression analysis of the equation \(A = V_o(k_1 \times (1 - \exp(-k_2 \times t))) + A_o\) to obtain \(k_1\), the apparent, pseudo-first-order rate constant of inhibition (26), where \(V_o\) is the initial reaction rate and \(A_o\) is the initial absorbance at 405 nm. The \(k_1\) value was multiplied by \((1 + [\text{Chz-Th}]/K_m)\) to correct for competition of the chromogenic substrate in the continuous assay, using a \(K_m\) of 15 \(\mu\)M. The apparent second-order rate constant (\(k_{\text{app}}\)) was obtained by dividing \(k_1\) by the inhibitor concentration.

\textit{Thrombin Dissociation from Intact Fibrin Clots—}Stock solutions consisting of 3 \(\mu\)M \(\gamma_A/\gamma^-\), \(\gamma_A/\gamma^-\), or unfractionated fibrinogen in TS containing 2 mM CaCl\(_2\), 0.005% Tween 20 (TS-TwCa), and 50,000 counts/min of \(^{125}\text{I}\)-YPR-thrombin were prepared. 125-\(\mu\)l aliquots were added to microcentrifuge tubes, and clots were formed around truncated plastic inoculation loops (Bac-Loop; Thermo-Fisher Scientific, Waltham, MA) by incubation with 100 \(\mathrm{nM}\) thrombin for 30 min at 37 °C. The clots were then removed from the tubes and washed three times with TS for 5 min. After quantifying radioactivity with a \(\gamma^-\)-counter, clots were suspended in 5 ml of TS-TwCa in 50-ml conical tubes and incubated with gentle agitation for 24 h at 23 °C. A parallel series of clots were incubated in TS-TwCa containing 2 mM NaCl to account for nonspecific binding. At intervals, clots were removed into 0.5 ml of the bathing buffer and the radioactivity was determined. The fraction of \(^{125}\text{I}\)-YPR-thrombin remaining in the clot at each time point was calculated as a percent of the initial amount, and time courses were fit to a two-phase exponential decay curve with zero end point to determine the rates of dissociation. The two phases reflect rapid dissociation of the unbound, fluid fraction of thrombin and the slower dissociation of the fibrin-bound fraction.

The residual thrombin activity of the washed clots was determined by measuring their capacity to release fibrinopeptide A from fibrinogen. After incubation for 24 h, clots were washed twice for 5 min in TSTwCa and their radioactivity was determined. Each clot was then placed in a tube containing 500 \(\mu\)l of a 3-\(\mu\)M solution of unfractionated fibrinogen in TS-TwCa buffer and incubated at 37 °C for 30 min. At intervals, 50-\(\mu\)l aliquots were removed into tubes containing 150 \(\mu\)l of cold 100% ethanol. After vortexing, the tubes were maintained on ice until processed. Fibrinogen was pelleted by centrifugation (13,000 \(\times\) g for 4 min), and the supernatants were removed and dried in a Speedvac (Thermo-Fisher Scientific) overnight. The samples were then reconstituted to their original volume with water and assayed for fibrinopeptide A by radioimmunoassay, as previously described (27).

\textbf{RESULTS}

\textit{Thrombin Binding to \(\gamma_A/\gamma^-\) and \(\gamma_A/\gamma^-\)-Fibrin—}To better elucidate the nature of the enhanced affinity of thrombin for \(\gamma_A/\gamma^-\)-fibrin, surface plasmon resonance (SPR) was used. \(\gamma_A/\gamma^-\) or \(\gamma_A/\gamma^-\)-fibrinogen was immobilized in separate flow cells of a CM5 sensor chip. Flow cells were treated with 100 \(\mathrm{nM}\) thrombin to convert fibrinogen to fibrin. Ovalbumin adsorbed to a separate flow cell served as a control. Successively higher concentrations of FPR-\(\alpha\)-thrombin or FPR-\(\gamma\)-thrombin were injected into the flow cells, followed by a dissociation step after each run. After the experiment, Req values were determined by the instrument software. Values were corrected for background signal in the ovalbumin flow cell, and the data were plotted against the input thrombin concentration (Fig. 1). Prior determination of the amount of fibrinogen adsorbed with the \(\gamma^-\)-peptide-directed antibody permitted calculation of stoichiometries of interaction. The binding of \(\alpha\)-thrombin to \(\gamma_A/\gamma^-\)-fibrin occurred via a single site with a \(K_d\) of 3.4 ± 0.5 \(\mu\)M and a molar stoichiometry of 2.9 ± 0.5 thrombin/fibrin. This \(K_d\) value is comparable with the value of 2.3 \(\mu\)M obtained for binding of thrombin to intact \(\gamma_A/\gamma^-\)-fibrin clots (14). The stoichiometry likely reflects the dimeric nature of the fibrin molecule. In contrast, binding of \(\alpha\)-thrombin to immobilized \(\gamma_A/\gamma^-\)-fibrin was mediated by two sites with \(K_d\) values of 11 ± 0.8 and 1100 ± 290 nm, respectively. These values are in agreement with \(K_d\) values of 100 and 1500 nm reported for the respective high and low affinity sites on intact \(\gamma_A/\gamma^-\)-fibrin clots (14). The molar stoichiometry of the higher affinity site was 0.5 ± 0.1 thrombin/fibrin, whereas that of the lower affinity site was 3.4 ± 0.5. The lower stoichiometry of the higher affinity binding site may reflect the presence of only one \(\gamma^-\)-chain in the \(\gamma_A/\gamma^-\)-fibrin molecule.

\(\gamma\)-thrombin was used in place of \(\alpha\)-thrombin to reveal the role of exosite 1 in binding fibrin. For \(\gamma\)-thrombin binding to \(\gamma_A/\gamma^-\)-fibrin, a single binding site with a \(K_d\) of 9 \(\mu\)M and a molar stoichiometry of 1 was observed (Fig. 1). No binding of \(\gamma\)-thrombin to \(\gamma_A/\gamma^-\)-fibrin was detected. In a subsequent experiment, a synthetic peptide analog of the COOH-terminal sequence of the \(\gamma^-\)-chain abrogated binding of \(\gamma\)-thrombin to \(\gamma_A/\gamma^-\)-fibrin (Fig. 2). The \(K_d\) value of the \(\gamma^-\)-peptide for \(\gamma\)-thrombin determined from this experiment is 3.8 \(\mu\)M, comparable with the value of 1.0 \(\mu\)M obtained in direct binding experiments (14). These data confirm that thrombin binding to the \(\gamma^-\)-chain of fibrin is mediated solely by exosite 2.

Although the binding parameters determined by SPR are comparable with those obtained in fibrin clots, fibrin on the SPR biosensor chips is likely monomeric, whereas that in intact clots is polymeric. This is relevant to the interaction of thrombin with \(\gamma_A/\gamma^-\)-fibrin because it is hypothesized that the higher affinity binding results from interaction with two adjacent fibrin monomers within the protofibril (8). Fibrin monomers would not be expected to polymerize when adsorbed to the
were then washed with HBS. The peak RU values (thrombin titration with increasing concentrations (0–10 M) of FPR-α-thrombin (FPR-IIa) were injected at a flow rate of 20 μl/min for 1 min, and the cells were then washed with HBS. The inset shows the sensorgram for FPR-α-thrombin titration with γA/γ′-fibrin and ovalbumin flow cells, respectively. Peak RU values (Req), after correction for background, were determined and plotted against thrombin concentration. The data were analyzed by nonlinear regression of a two-site binding equation to determine the binding parameters (line 1 in Fig. B). A data set for titration of γA/γ′-fibrin (closed symbols) or γA/γ′-fibrin (open symbols) with FPR-α-thrombin (circles) or FPR-γ-thrombin (squares) are shown. Lines represent nonlinear regression analyses.

carboxyldextran surface of the biosensor chip. However, the carboxyldextran matrix may serve as a spacer that renders bound fibrin molecules sufficiently mobile such that thrombin can bridge individual fibrin monomers. Thrombin-mediated bridging would remain sensitive to exosite antagonists, consistent with the results obtained here. Further support for this type of interaction comes from the observation that antibodies, because of their bivalent nature, have been observed to bridge immobilized antigen in SPR experiments (28). Thus, the SPR data presented here are consistent with those observed using fibrin clots (14).

Dissociation of Thrombin from Fibrin Clots—Both intact clot and SPR analyses reveal that γA/γ′-fibrin has higher affinity for α-thrombin than γA/γ′-fibrin. However, it is unknown whether this phenomenon translates into greater retention of thrombin by γA/γ′-fibrin clots than by γA/γ′-fibrin clots. To address this, fibrin clots formed around plastic loops were immersed in buffer, and dissociation of 125I-YPR-thrombin from the clots was monitored over time (Fig. 3). Intact, three-dimensional clots were used in these experiments to model dissociation of thrombin from thrombi. Control experiments, performed in the presence of 2 M NaCl, demonstrated that there was minimal nonspecific binding of 125I-YPR-α-thrombin to the fibrin clots (open symbols). Dissociation of 125I-YPR-thrombin from γA/γ′-fibrin clots was rapid and reached 83% after 24 h. In contrast, only 25% of 125I-YPR-thrombin dissociated from γA/γ′-fibrin clots in the same time period. Retention of fibrin binding ligands in clots is determined by two parameters, dif-

FIGURE 1. Binding of thrombin to fibrin measured using SPR. A, γA/γ′- or γA/γ′-fibrinogen was adsorbed to separate flow cells of a CMS Biacore chip to ~7000 RU. As a control, ovalbumin was adsorbed to a separate flow cell. Flow cells were treated with 100 nM thrombin to convert the fibrinogen to fibrin and washed with HBS. Increasing concentrations (0–10 M) of FPR-α-thrombin (FPR-IIa) were injected at a flow rate of 20 μl/min for 1 min, and the cells were then washed with HBS. The inset shows the sensorgram for FPR-α-thrombin titration with γA/γ′-fibrin and ovalbumin flow cells, respectively. Peak RU values (Req), after correction for background, were determined and plotted against thrombin concentration. The data were analyzed by nonlinear regression of a two-site binding equation to determine the binding parameters (line 1 in Fig. B). A data set for titration of γA/γ′-fibrin (closed symbols) or γA/γ′-fibrin (open symbols) with FPR-α-thrombin (circles) or FPR-γ-thrombin (squares) are shown. Lines represent nonlinear regression analyses.

FIGURE 3. Dissociation of 125I-YPR-α-thrombin from γA/γ′- or unfractionated fibrin clots. 125 μl of 3 μM unfractionated- (circles), γA/γ′- (squares), or γA/γ′- (triangles) fibrinogen was clotted around plastic loops by the addition of 100 nM α-thrombin and 2 mM CaCl2, in the presence of 50,000 counts/min 125I-YPR-α-thrombin. After washing, clots were placed in 50-ml conical tubes containing 5 ml of TSTw, and the radioactivity in the clots was determined at intervals. The fraction of 125I-YPR-α-thrombin remaining in the clots was calculated as a percent of the total and plotted versus time (closed symbols). Another series of clots was incubated in the presence of 2 M NaCl to account for nonspecific binding (open symbols). Lines represent nonlinear regression analysis of the data by two-component exponential decay. Data represent the mean and standard error of three experiments.
fusion in the fluid phase and binding with the fibrin phase (29). Therefore, the data were analyzed using a two-phase exponential decay model (lines), where the rates reflect macroscopic dissociation from the intact clot. The first phase demonstrated a rapid rate of thrombin dissociation from all three forms of fibrin with a half-life \( t_{1/2} \) of 1–2 h. This rate was comparable with that obtained in the presence of 2 M NaCl and reflects macroscopic dissociation of the unbound fraction of thrombin from the fluid phase of the clot. The second phase reflects retarded diffusion due to thrombin adsorption by fibrin. This phase yielded a slower rate of dissociation with a \( t_{1/2} \) of thrombin dissociation from \( \gamma_A/\gamma_A \)-fibrin clots of 13.9 h. The \( t_{1/2} \) value of the slow phase of thrombin dissociation was 3.3-fold longer from unfractionated fibrin clots and 7.1-fold longer from \( \gamma_A/\gamma' \)-fibrin clots. It is notable that thrombin dissociation from clots prepared from unfractionated fibrinogen, which contain only \( \sim 10\% \) \( \gamma_A/\gamma' \)-fibrin, more closely resembled that from \( \gamma_A/\gamma' \)-fibrin clots. These data reveal that \( \gamma_A/\gamma' \)-fibrin clots retain thrombin more avidly than do \( \gamma_A/\gamma_A \)-fibrin clots and that the presence of even small amounts of \( \gamma_A/\gamma' \)-fibrin slows the rate of dissociation of thrombin.

Although \( \gamma_A/\gamma' \)-fibrin clots retain more thrombin, both exosites are engaged in its interaction with this form of fibrin. Consequently, it is unclear whether thrombin bound to \( \gamma_A/\gamma' \)-fibrin clots retains catalytic activity against macromolecular substrates. To address this, thrombin activity of clots that had been washed for 24 h was determined by immersing them in fibrinogen-containing buffer for 30 min and monitoring fibrinopeptide A generation over this time period. Fibrinogen cleavage, rather than chromogenic activity, was determined so that the availability of exosite 1 of clot-associated thrombin could be assessed. Although \( \gamma_A/\gamma' \)-fibrin clots retained more thrombin than \( \gamma_A/\gamma_A \)-fibrin clots, the extent of fibrinopeptide A cleavage was similar with the two types of clots (Fig. 4). This likely reflects the fact that fibrin-bound thrombin is unable to cleave fibrinogen. Therefore, by virtue of the lower affinity interaction, thrombin associated with \( \gamma_A/\gamma_A \)-fibrin is more accessible to fibrinogen than that associated with \( \gamma_A/\gamma' \)-fibrin. Consequently, thrombin associated with unfractionated fibrin clots had greater fibrinogen cleaving activity than that associated with either individual form of fibrin because unfractionated fibrin clots contain more thrombin than \( \gamma_A/\gamma_A \)-fibrin clots. These data demonstrate that thrombin bound to fibrin clots remains active but that higher affinity binding to \( \gamma_A/\gamma' \)-fibrin clots reduces the capacity of thrombin to release fibrinopeptide A from fibrinogen.

**Protection of Thrombin from Inhibition by Binding to \( \gamma_A/\gamma' \)-Fibrin**—Fibrin-bound thrombin is resistant to heparin-catalyzed inactivation by antithrombin and HCII (16, 30). Protection is a consequence of formation of the ternary complex in which fibrin-bound heparin binds exosite 2 and impairs the ability of antithrombin-bound heparin to bridge to thrombin. Because the \( \gamma' \)-chain also occupies exosite 2 with high affinity, we hypothesized that thrombin bound to \( \gamma_A/\gamma' \)-fibrin would demonstrate greater protection from inhibition by antithrombin or HCII than thrombin bound to \( \gamma_A/\gamma_A \)-fibrin. Inhibition experiments were done using clots prepared from the two forms of fibrinogen. Clotting was initiated with Atroxin to ensure a uniform rate of clot formation. Control experiments verified that thrombin binds comparably to clots prepared with Atroxin as it does to those made with thrombin (not shown). The residual chromogenic activity of 2.2 nm thrombin against Chz-Th was monitored continuously and used to calculate the apparent second-order rate constant of inhibition under pseudo-first-order conditions. The apparent heparin-catalyzed rate of thrombin inhibition by antithrombin was \( 1.1 \times 10^8 \) \( \text{M}^{-1}\text{min}^{-1} \) in the absence of fibrin (Fig. 5A), which is comparable with the rate of \( 3.0 \times 10^8 \) \( \text{M}^{-1}\text{min}^{-1} \) obtained using a discontinuous inhibition assay (16). In the presence of \( \gamma_A/\gamma_A \)-fibrin, the rate was reduced 11-fold, confirming the protection observed previously. In the presence of \( \gamma_A/\gamma' \)-fibrin, the rate of inhibition was reduced 55-fold, revealing 5-fold greater protection than that afforded by \( \gamma_A/\gamma_A \)-fibrin. The experiment was repeated in the presence of the \( \gamma' \)-peptide-directed antibody that reduces thrombin binding to \( \gamma_A/\gamma' \)-fibrin to that obtained with \( \gamma_A/\gamma_A \)-fibrin. In the presence of the antibody, the rate of inhibition with \( \gamma_A/\gamma' \)-fibrin was similar to that with \( \gamma_A/\gamma_A \)-fibrin. Thus, the additional interaction of thrombin with the \( \gamma' \)-chain affords thrombin more protection from inhibition by the heparin-antithrombin complex. Similar results in the presence of heparin were obtained when HCII was substituted for antithrombin (not shown).

Interpretation of these results could be compromised by the fact that in \( \gamma_A/\gamma' \)-fibrin clots thrombin still has the capacity to form a ternary heparin-thrombin-fibrin complex that could compete with the \( \gamma' \)-chain for thrombin exosite 2 binding. To address this possibility, the experiment was repeated using DS and HCII in place of heparin plus antithrombin. Although DS binds weakly to exosite 2, it does not bind to fibrin. Consequently, DS does not promote ternary complex formation (31). The DS-catalyzed rate of thrombin inhibition by HCII in the
absence of fibrin was $1.3 \times 10^8 \text{ M}^{-1}\text{min}^{-1}$ (Fig. 5B). In the presence of $\gamma_A/\gamma_A$-fibrin, the rate decreased ~5-fold. A 27-fold lower rate was observed with $\gamma_A/\gamma_Y$-fibrin clots. This reduced rate was abrogated when the $\gamma_Y$-peptide-directed antibody was added. Thus, $\gamma_A/\gamma_Y$-fibrin affords thrombin greater protection from inhibition by either antithrombin or HCII than does $\gamma_A/\gamma_Y$-fibrin. Furthermore, the enhanced protection with $\gamma_A/\gamma_Y$-fibrin is independent of formation of the ternary heparin-thrombin-fibrin complex.

**DISCUSSION**

This study (a) confirms that $\gamma_A/\gamma_Y$-fibrin binds thrombin with higher affinity than $\gamma_A/\gamma_A$-fibrin, (b) demonstrates that thrombin bound to $\gamma_A/\gamma_Y$-fibrin is protected from inhibition by antithrombin to a greater extent than thrombin bound to $\gamma_A/\gamma_A$-fibrin, and (c) shows that $\gamma_A/\gamma_Y$-fibrin serves as a reservoir of active thrombin, which may contribute to the prothrombotic nature of thrombi. The data also reveal the distinct mechanisms by which $\gamma_A/\gamma_Y$- and $\gamma_A/\gamma_A$-fibrin protect thrombin from inhibition.

Previous work suggested that the mechanism by which thrombin within the ternary heparin-thrombin-fibrin complex is protected from inhibition reflects the inability of antithrombin-bound heparin to access exosite 2 of thrombin, thereby precluding essential bridging of enzyme and inhibitor by heparin (16). In the current study, we observed that both exosites of thrombin are ligated when bound to $\gamma_A/\gamma_Y$-fibrin and that the resultant protection exceeds that provided by $\gamma_A/\gamma_A$-fibrin clots. This conclusion was validated in two ways. First, the addition of a $\gamma_Y$-peptide-directed antibody reduced the extent of protection of thrombin from inhibition by the heparin-antithrombin complex afforded by $\gamma_A/\gamma_Y$-fibrin clots to a level similar to that of $\gamma_A/\gamma_A$-fibrin clots. These results demonstrate that the enhanced protection of thrombin provided by $\gamma_A/\gamma_Y$-fibrin reflects the presence of the $\gamma_Y$-sequence. Second, the enhanced protection of thrombin by $\gamma_A/\gamma_Y$-fibrin is not the result of increased formation of the ternary heparin-thrombin-fibrin complex, because $\gamma_A/\gamma_Y$-fibrin clots still afford thrombin more protection from inhibition by HCII than $\gamma_A/\gamma_A$-fibrin clots when heparin is replaced with DS, a glycosaminoglycan that does not bind fibrin. These results confirm that the protection of thrombin from inhibition by the heparin-antithrombin or heparin-HCII complex that occurs either by formation of the ternary heparin-thrombin-fibrin complex on $\gamma_A/\gamma_A$-fibrin or via bivalent binding of thrombin to $\gamma_A/\gamma_Y$-fibrin reflects impaired access of catalytic heparin, bound to antithrombin or to HCII, to exosite 2 on thrombin (8).

As a consequence of its higher affinity for $\gamma_A/\gamma_Y$-fibrin, more thrombin is sequestered in $\gamma_A/\gamma_Y$-fibrin clots than in $\gamma_A/\gamma_A$-fibrin clots. Thus, $\gamma_A/\gamma_Y$-fibrin clots may serve as a reservoir of active thrombin, which could render these clots thrombogenic. Such a phenomenon could explain the observed correlation between elevated levels of $\gamma_A/\gamma_Y$-fibrinogen and coronary artery disease (20). Recently, this correlation has been questioned (32, 33). It has been suggested, in fact, that $\gamma_A/\gamma_Y$-fibrin may play an anticoagulant role by sequestering thrombin and limiting its capacity to participate in coagulation or to activate platelets (33, 34). The fibrinopeptide A-generating activity of thrombin in washed fibrin clots was examined to verify that clot-associated thrombin retained coagulant activity. The activity of thrombin associated with $\gamma_A/\gamma_Y$-fibrin clots was comparable with that bound to $\gamma_A/\gamma_A$-fibrin clots, despite the fact that $\gamma_A/\gamma_Y$-fibrin clots contained more thrombin. Therefore, thrombin associated with both forms of fibrin clots retains catalytic activity, although that associated with $\gamma_A/\gamma_Y$-fibrin has reduced activity compared with that associated with $\gamma_A/\gamma_A$-fibrin. These findings are consistent with the observation that thrombin-induced fibrinopeptide release from $\gamma_A/\gamma_Y$-fibrinogen is slower than that from $\gamma_A/\gamma_A$-fibrinogen (35). Although these data support the concept that $\gamma_A/\gamma_Y$-fibrin plays an anticoagulant role as antithrombin I, this may be countered by the fact that $\gamma_A/\gamma_Y$-fibrin clots endow thrombin with a survival advantage that prolongs its procoagulant activity. Such a concept is supported by the observations that (a) clot-associated thrombin retains clotting activity in
plasma, even in the presence of heparin (27), and (b) venous and arterial thrombi collected from autopsy specimens harbor active thrombin that can be inhibited with hirudin (36). Taken together, these findings support the concept that fibrin-bound thrombin contributes to the thrombogenic potential of thrombi (27, 37).

In addition to activation of platelets and upstream coagulation factors, another potential consequence of enhanced binding of thrombin to \( \gamma_A/\gamma' \)-fibrinogen is alteration of fibrin structure. Fibers formed from \( \gamma_A/\gamma' \)-fibrinogen are thinner and display greater branching than those formed from \( \gamma_A/\gamma \)- or unfractionated fibrinogen (35, 38). In addition to thrombin, \( \gamma_A/\gamma' \)-fibrin also binds FXIII with higher affinity than \( \gamma_A/\gamma \)-fibrin (24), which may impair cross-linking (35). Enhanced binding of thrombin and FXIII may explain why clots prepared from \( \gamma_A/\gamma' \)-fibrinogen differ from those formed from \( \gamma_A/\gamma \)-fibrin. Altered clot structure may thus render \( \gamma_A/\gamma' \)-fibrin clots more resistant to fibrinolysis (35, 39). Therefore, enhanced binding of thrombin represents only one mechanism by which the level of \( \gamma_A/\gamma' \)-fibrinogen may influence the risk of thrombosis.

Crystallographic studies have revealed the nature of individual interactions at exosites 1 and 2 with fibrinogen fragment E and the \( \gamma' \)-peptide, respectively (40, 41). When thrombin is bound to \( \gamma_A/\gamma' \)-fibrin, both of its exosites are ligated simultaneously. Similar bivalent binding of thrombin occurs when it interacts with chondroitin sulfate-containing thrombomodulin, bothrojaracin, HCII, or GP1bα. Dual exosite occupation likely represents a mechanism by which the affinity of thrombin binding can be heightened and thrombin activity can be modulated. Thrombomodulin redirects the focus of thrombin to anticoagulation by providing a nascent substrate binding site for protein C or to attenuation of fibrinolysis by promoting thrombin-activable fibrinolysis inhibitor activation (42). Bothrojaracin is a high affinity thrombin inhibitor found in Bothrops venom that binds both exosites (43). HCII uses a double bridging mechanism to achieve effective rates of inhibition, binding exosite 1 via its NH₄-terminal tail and exosite 2 via heparin (31). GP I bα utilizes exosite 2 to tether thrombin to platelets, orienting it such that it can effectively activate the thrombin receptor (6). Furthermore, crystallographic studies demonstrate that GP I bα can interact with both exosites of thrombin (44, 45). Fibrin, either via \( \gamma_A/\gamma' \)-fibrin alone, or via \( \gamma_A/\gamma \)-fibrin with heparin, protects thrombin from inactivation by the heparin-antithrombin or heparin-HCII complex. All of these examples represent efforts to uniquely modulate thrombin activity in various environments. Interactions that utilize both exosites serve to retain and restrain thrombin activity subsequent to coagulation. In contrast, most procoagulant activities of thrombin, such as activation of f.V, f.VIII, and fibrinogen, typically involve only exosite 1. Ligation of a single exosite results in a lower affinity interaction that permits efficient substrate turnover. These observations demonstrate that selective utilization of exosites by substrates and ligands plays an important role in regulating thrombin activity in hemostasis (46).

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