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Modes and consequences of thrombin's interaction with fibrin

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Abstract

Thrombin mediates the balance between coagulant and fibrinolytic forces and has numerous cellular effects. This intricate balance is maintained by biochemical mechanisms that regulate thrombin activity. Disruption of this balance could lead to bleeding or thrombosis. Once thrombin is generated, two major mechanisms regulate its activity. By binding fibrin, thrombin's activity is localized to the thrombus, a process that limits its systemic procoagulant effects. Thrombin that escapes into the circulation is efficiently inactivated by plasma inhibitors, such as antithrombin, or is sequestered by thrombomodulin on the endothelium. Although thrombin's interaction with fibrin limits its systemic effects, fibrin-bound thrombin resists inactivation and can produce a local procoagulant stimulus that triggers thrombus growth. Direct thrombin inhibitors were developed, at least in part, to target fibrin-bound thrombin. These agents are finding their niche for the prevention and treatment of venous and arterial thrombosis. The mechanisms by which thrombin binds fibrin are reviewed in this paper. As well, the potential pathological consequences of thrombin's interaction with fibrin are discussed.

Keywords: Thrombin; Fibrinogen; Fibrin; Thrombosis; Heparin; Exosite

Thrombin is the ultimate product of the coagulation system and serves coagulant, procoagulant, and anticoagulant functions. Because thrombin plays such a pivotal role in hemostasis, its level must be tightly controlled. Generation of thrombin is regulated by both positive and negative feedback reactions in the coagulation cascade, many of which are performed by thrombin itself. Elimination of thrombin results from interaction with a number of plasma protease inhibitors. Because of the diverse array of primary and secondary targets, the specificity of thrombin is subject to strict regulation. Much of this control is mediated by unique structural domains in thrombin that confer functional adaptations. Two domains that reside adjacent to the active site of the enzyme have important regulatory functions. These domains, termed exosites, are composed of numerous basic residues and serve electrostatic steering roles guiding reactants to the active site. The exosites also mediate noncatalytic binding interactions with various ligands and cofactors. One such interaction involves binding of thrombin to fibrin after thrombin-mediated release of the fibrinopeptides. Consequently, fibrin, which serves as a potential reservoir of active thrombin, has historically been termed antithrombin I. Thrombin that binds to fibrin is protected from inhibition and may have activity that persists after the soluble fraction has been inactivated. Thus, fibrin-bound thrombin may continue to exert hemostatic or procoagulant effects. This paper will review the modes of interaction of thrombin with fibrin and the potential consequences of these interactions.

1. Thrombin structure

Thrombin belongs to the chymotrypsin family of serine proteases and is homologous with other clotting enzymes such as factors (f.) VIIa, IXa, and Xa [1,2]. Members of this family are distinguished by numerous short amino acid insertions in the prototypical chymotrypsin sequence [3,4].

Abbreviations: AT, antithrombin; IIa, thrombin; f., factor; FPR, Phe–Pro–Arg chloromethyl ketone; K_d, dissociation constant; RA-thrombin, R93A R97A R101A thrombin.

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Because the insertions are unique, it is hypothesized that these variable regions contribute to the distinct specificity demonstrated by individual proteases [3]. Two of the insertions in thrombin occur as loops that bracket the upper and lower opening to the active site cleft (Fig. 1). The 60- and 149-loops physically restrict access to the active site pocket and stabilize interactions with appropriate substrates, thereby serving as regulators of thrombin specificity [1,4]. Also unique to each protease are the inserts that contribute to the regulatory exosites. In thrombin, exosite 1 contributes to fibrinogen binding. In contrast, the corresponding domains in f.IXa and f.Xa comprise Ca^{2+} binding sites. Exosite 2 in all three proteases mediates glycosaminoglycan binding. Therefore, the exosites represent ligand binding sites that influence enzyme specificity.

Exosite 1 of thrombin is a regulator of thrombin specificity [1,5]. Initially termed the fibrinogen recognition site, exosite 1 was subsequently recognized to interact with numerous thrombin substrates, including f.VIII, f.V, and the thrombin receptor. Other interactions with exosite 1 mediate anticoagulant functions. These include heparin cofactor II, a thrombin inhibitor, and thrombomodulin, the cofactor responsible for promoting activation of protein C and thrombin-activable fibrinolysis inhibitor. With the exception of thrombomodulin, interactions at exosite 1 are envisioned to direct or steer the reactive domain of the substrate to the active site of thrombin. Thrombomodulin acts as a cofactor in protein C activation by serving as a template that binds both enzyme and substrate. By occupying exosite 1, thrombomodulin blocks access of fibrinogen and factors V and VIII [6]. Thrombomodulin also provides a new exosite that binds protein C and directs it to the active site of thrombin [7].

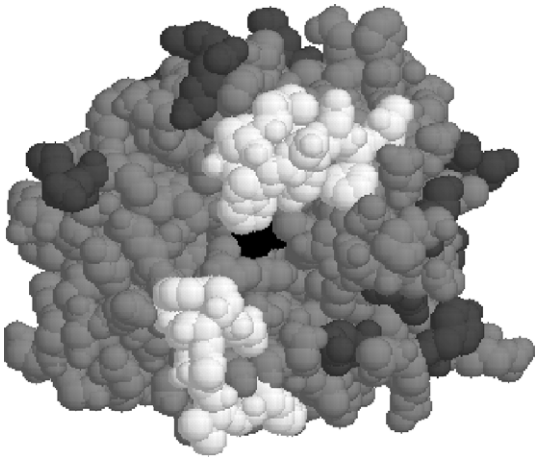

Fig. 1. Model of thrombin structure. A model of Phe–Pro–Arg thrombin based on the crystallographic data of structure 1PPB from the Protein Database (www.rcsb.org/pdb/) is shown in spacefill format. The B-chain is shown in gray in standard orientation with active site S195 (chymotrypsin numbering) in the center colored black. The 60-(Y60a, P60b, P60c, W60d) and 149-loops (E146–K149e), located above and below the active site, respectively, are shown in white. Residues contributing to exosites are colored dark gray, with exosite 1 to the right of the active site and exosite 2 to the left [24]. Exosite 1 residues include F34, K36, R67, R73, Y76, R77a, K81, K109, and K110, whereas those in exosite 2 include R93, R97, R126, K169, R173, K236, and K240. The active site inhibitor Phe–Pro–Arg choromethyl ketone was omitted for clarity. The structure was created using Protein Explorer (molvis.sdsc.edu/protexpl/frntdoor.htm).

Exosite 2 differs from exosite 1 because it plays a bridging role in thrombin. One of its primary ligands is thought to be heparan sulfate. This naturally occurring glycosaminoglycan and heparin, its medicinal equivalent, promote thrombin inhibition by antithrombin by acting as a template onto which both enzyme and inhibitor bind [8]. This serves to localize the reactants, effecting a three orders of magnitude increase in the second order rate constant of thrombin inhibition. Heparin cofactor II utilizes a similar mechanism to inhibit thrombin using either heparan sulfate or dermatan sulfate, another glycosaminoglycan, as catalysts. Exosite 2 is also proposed to interact with factors V and VIII to promote cofactor activation and glycoprotein Ib to promote platelet activation [5,9–11]. Another polypeptide, prothrombin fragment 2, binds exosite 2, likely reflecting the intramolecular interaction in prothrombin [12].

In addition to serving as binding sites, both exosites may exert allosteric effects on the active site of thrombin. Ligands that bind exosite 1 alter the environment of the active site as demonstrated using active site-tethered fluorophores. This effect has been observed with thrombomodulin, derivatives of the thrombin inhibitor hirudin, and thrombin-binding nucleotide aptamers [13–16]. These ligands also influence the catalytic properties of thrombin with peptidyl substrates [17–19]. Similar responses are elicited by ligands that bind to exosite 2, such as heparin, prothrombin fragment 2, and exosite 2-directed aptamers [13,16,20,21]. There is also evidence that the exosites are in direct communication because occupation of one site influences binding characteristics at the other exosite [17].

Thrombin's essential and diverse role in hemostasis is reaffirmed as new interactions and regulatory reactions are described. Because of its position in the terminal stage of the coagulation pathway and its critical roles in feedback processes, thrombin is regarded as the pivotal mediator of hemostasis [22]. Consequently, molecules that influence the activation, activity, and inhibition of thrombin have a major effect on hemostasis. Because fibrinogen is a thrombin substrate that continues to bind thrombin after conversion to fibrin, the interactions between fibrin and thrombin are of considerable scientific interest. These interactions will be explored below.

2. Fibrinogen and fibrin binding sites

Thrombin binding domains on each of the Aα- and Bβ-chains have traditionally been recognized as the sites at which thrombin exosite 1 interacts with fibrinogen [23]. These sites align the active site of thrombin with the scissile bonds of fibrinogen prior to cleavage of fibrinopeptides A and B. The location of these thrombin-binding sites on fibrinogen has been identified by peptide, mutagenesis,

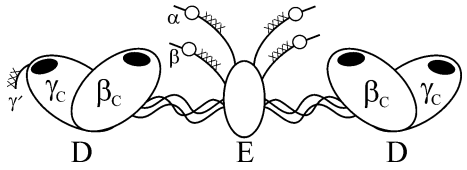

Fig. 2. Model of fibrinogen structure. The major domains of γ_A/γ' fibrinogen are illustrated in schematic form. The globular D domains, composed of β_C- and γ_C-chains, are connected by the coiled coil region to the central globular E domain, whereas the α_C-chains are omitted for clarity. The NH_2 termini of the α- and β-chains, with fibrinopeptides intact and masking the polymerization knobs (white circles), are shown projecting out of the E domain. Adjacent to the knobs are the putative thrombin binding sites (XXXX). The COOH terminus of the γ′-chain, with its thrombin binding domain, is found on one D domain. Polymerization holes are located on the D domains, where they are represented as black ovals.

crystallography, and modeling studies [23,24]. Residues ~20–50 of the Aα- and Bβ-chains, which are proximal to the fibrinopeptide cleavage sites, are proposed to serve as thrombin binding domains (Fig. 2). These regions remain part of the fibrin molecule after release of the fibrinopeptides and thus satisfy the observation that fibrin binds thrombin. Confirming this concept, thrombin binds comparably to fibrinogen or fibrin immobilized on agarose [25]. Additional support comes from the observation that the K_d for thrombin binding to fibrin of ~2 μM is comparable to the K_m for fibrinopeptide cleavage [26]. Because thrombin has comparable affinity for both fibrinogen and fibrin, thrombin binding is not dependent on fibrin polymerization. Therefore, polymer-dependent mechanisms, such as conformational changes or amalgamation of partial sites on adjacent monomers, do not contribute to thrombin binding. The segments of the α- and β-chains to which thrombin bind are not resolved in crystallographic structures of fibrinogen likely because the NH_2-termini are flexible [27]. This mobility facilitates the capture of thrombin and provides the nascent α- and β-chain knobs with sufficient flexibility to locate their respective pockets in adjacent fibrin monomers, thereby promoting polymerization.

The relative contribution of each chain of fibrinogen to thrombin binding is unknown. However, removal of the β15–42 sequence with a snake venom protease considerably reduces fibrin's affinity for thrombin [28–30]. This suggests either that the α-chain site has lower affinity for thrombin, or that the α- and β-chains act in concert for optimal binding. Crystallographic analysis of chicken fibrinogen has revealed a negatively charged domain comprised of residues α35–40 and β68–71, supporting the latter model where both chains contribute to the binding site [27]. Involvement of the β68–71 region is highlighted by studies with dysfibrinogen Naples I (Bβ A68T), which exhibits reduced thrombin affinity and impaired clot formation [31]. Alternatively, if thrombin binds to the α- and β-chains independently then it could be hypothesized that, because fibrinopeptide A is released far more efficiently than fibrinopeptide B [32], binding of thrombin to the α-chain is favoured. Thus, identification of the domains on fibrin to which thrombin binds must await higher resolution structural and mutagenesis studies.

A binding site independent of the α- and β-chains occurs at the COOH-terminus of a variant γ-chain form. About 10% of fibrinogen molecules are synthesized with a 20-residue replacement of the terminal 4 residues of the γ-chain as a result of alternate transcriptional processing [33]. This γ′-chain usually occurs as a γ_A/γ' heterodimer with the native γ_A-chain and is separable from the more abundant γ_A/γ_A fibrinogen by ion exchange chromatography [33]. Binding studies demonstrate that γ_A/γ' fibrin binds thrombin with about 10-fold higher affinity than γ_A/γ_A fibrin [16,29]. Thus, Scatchard analysis of the data depicting thrombin binding to γ_A/γ_A or γ_A/γ' fibrin demonstrate single- or two-site binding, respectively (Fig. 3). The K_d value for thrombin's interaction with γ_A/γ_A fibrin is 2.3 μM, whereas K_d values for high and low affinity binding to γ_A/γ' fibrin are 0.1 and 1.5 μM, respectively. It has subsequently been shown that peptides derived from the γ′-chain COOH terminus inhibit the high-affinity interaction of thrombin with γ_A/γ' fibrin [16,34]. Studies have delineated residues in the γ′ extension that contribute to thrombin binding. Whereas there is no consensus thrombin binding sequence, the γ′ extension is negatively charged and contains proline and sulfated tyrosine residues analogous to other ligands that bind exosite 1 [24,34,35].

Therefore, the current understanding is that thrombin binding to fibrin is a remnant of substrate binding on the α- and β-chains. The small fraction of fibrin containing the γ′-chain binds thrombin with higher affinity. The mode by which thrombin binds and the subsequent consequences will be discussed below.

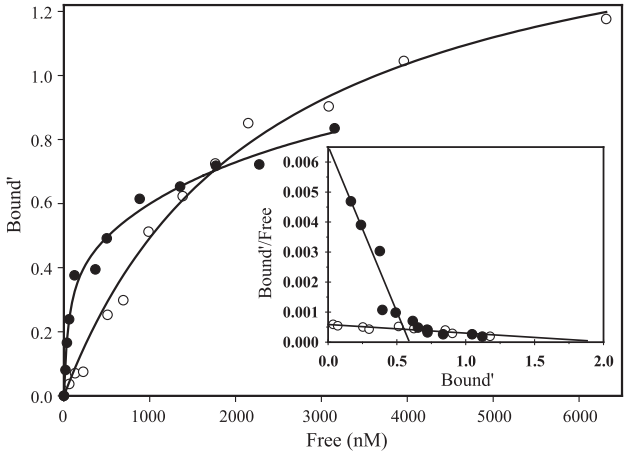

Fig. 3. Thrombin binding to γ_A/γ_A or γ_A/γ' fibrin. 2 μM γ_A/γ_A (open symbols) or γ_A/γ' (closed symbols) fibrinogen and 2 mM $CaCl_2$ was clotted with 10 nM active thrombin in the presence of ^{125}I-FPR-thrombin in concentrations ranging from 0 to 8 μM. Clot supernatants were counted for radioactivity to quantify unbound ^{125}I-FPR-thrombin. The amount of ^{125}I-FPR-thrombin bound to fibrin was divided by the fibrin concentration (bound′) and plotted versus the concentration of free ^{125}I-FPR-thrombin. These data were analyzed by nonlinear regression (line) to obtain K_d values (Table 1). From these data, Scatchard plots of bound′/free versus bound′ were also prepared (inset).

3. Role of thrombin exosites

Evidence that thrombin exosite 1 is involved in fibrin binding comes from studies demonstrating that γ-thrombin and hirudin-inactivated α-thrombin have reduced fibrin affinity [36]. γ-Thrombin is a proteolytic derivative of native α-thrombin that has exosite 1 inactivated, but exosite 2 and the active site remain intact. Hirudin is a leech salivary protein that binds thrombin at exosite 1 and the active site. That thrombin's binding to fibrin is independent of its active site was previously demonstrated with thrombin derivatives that had their active site occupied by covalent inhibitors [36,37]. Subsequently, it has been shown that ligands that specifically bind to exosite 1 block thrombin binding to fibrin. These include peptides derived from hirudin or heparin cofactor II and exosite 1-directed aptamers [16,29]. Additionally, a variant thrombin with an impaired exosite 2 binds fibrin with normal affinity [16]. This variant, designated RA-thrombin (R93A, R97A, R101A), has 20-fold reduced affinity for heparin, but normal fibrinogen clotting activity [38]. Site-directed mutagenesis has identified numerous residues within exosite 1 which contribute to fibrin binding [39,40]. As expected, these residues also are required for fibrinogen clotting activity. Although many of the residues are charged, their roles are not limited to ionic interactions with ligands. It is proposed that two ion pairs in exosite 1 serve to hold Tyr76 in the proper orientation for interaction with a hydrophobic stretch found on many ligands [24]. Consistent with these models, crystal structures of thrombin with hirudin peptides reveal that hydrophobic interactions predominantly stabilize the interactions [41,42]. Other basic residues contribute to the overall positive charge of the exosite that attracts negatively charged domains on the ligand. Although the ligands that interact with exosite 1 have similar amino acid compositions, no consensus sequence is discernable, making it likely that each ligand interacts with a unique repertoire of residues on thrombin [34,39].

The K_d for thrombin binding to fibrin is ~2–5 μM [43]. However, early reports also observed a higher affinity component to the interaction [37]. When the binding of thrombin with γ_A/γ_A and γ_A/γ' fibrin was studied systematically, the high-affinity interaction was observed only with γ_A/γ' fibrin clots (Fig. 3) [29]. It was presumed that the γ′-chain bound to exosite 1 because peptide analogues of the γ′ sequence inhibited thrombin binding to γ_A/γ_A fibrin [34]. However, when binding of the γ′-peptide to thrombin was measured directly, the interaction was mediated by exosite 2 [16,35]. This is confirmed by the inability of the γ′-peptide to bind RA-thrombin, the exosite 2 variant with reduced heparin affinity [16]. Furthermore, using exosite-directed aptamers, only the exosite 2-binding aptamer displaced the γ′-peptide from thrombin. To confirm the role of exosite 2 directly, fibrin binding studies were performed with native α-thrombin, γ-thrombin, and RA-thrombin. As expected, α-thrombin bound γ_A/γ_A fibrin with low affinity and γ_A/γ' fibrin with high affinity (Table 1). RA-thrombin, with an impaired exosite 2, bound both forms of fibrin with comparable low affinity. γ-Thrombin predictably did not bind to γ_A/γ_A fibrin but bound to γ_A/γ' fibrin, albeit with low affinity. Thus, even without exosite 1, γ-thrombin was able to bind fibrin via exosite 2 when the γ′-sequence was present. This interaction could be inhibited completely with the γ′-peptide [16]. These results revealed that both thrombin exosites contributed to binding at unique binding sites.

Closer inspection of the data further revealed that the high-affinity interaction required the presence of both exosites on thrombin as well as both thrombin binding sites on γ_A/γ' fibrin. Thus, thrombin binding to γ_A/γ' fibrin via exosite 2 alone (γ-thrombin) yielded only a low affinity interaction. This was also true of the interaction of des β15–42 γ_A/γ' fibrin with α-thrombin. These results suggest that high-affinity interaction of thrombin with fibrin results from a binary interaction of thrombin with distinct sites on fibrin. Evidence for such a cooperative interaction was observed previously with Naples I γ_A/γ' fibrinogen [44]. Therefore, thrombin's high-affinity interaction with γ_A/γ' fibrin requires simultaneous ligation of both thrombin exosites 1 and 2 by the α/β NH_2-termini and γ′ sequences, respectively. This concomitant occupation of both exosites of thrombin is structurally analogous to the ternary thrombin–fibrin–heparin complexes that augment thrombin binding to fibrin [43,45].

Contribution of exosite 2 to fibrin binding would thus appear to be limited to the minor population of γ_A/γ' fibrinogen. However, exosite 2 also indirectly modulates exosite 1-mediated binding. Conflicting results have been obtained in studies aimed at determining whether there is an allosteric linkage between the two exosites on thrombin [17,46]. Fibrin binding studies provide affirmative evidence for such a linkage. Exosite 2-binding prothrombin fragment 2 reduced thrombin binding to γ_A/γ_A fibrin, an interaction

Table 1
Interaction of α-IIa, γ-IIa or RA-IIa with γ_A/γ_A, γ_A/γ', des β15–42 γ_A/γ_A or des β15–42 γ_A/γ' fibrin

Thrombin	K_d (μM)			
	Native fibrin		des β15–42 fibrin	
	γ_A/γ_A	γ_A/γ'	γ_A/γ_A	γ_A/γ'
α-IIa	2.25±0.05	0.08±0.03	7.67±0.09	3.51±0.07
RA-IIa	3.11±0.02	2.02±0.03	11.72±0.05	10.93±0.03
γ-IIa	no binding	5.54±0.02	no binding	5.86±0.02

A series of tubes containing 0–10 μM γ_A/γ_A, γ_A/γ', des Bβ1–42 γ_A/γ_A or des Bβ1–42 γ_A/γ' were incubated with 30 nM α-IIa, γ-IIa or RA-IIa in the presence of 2 mM $CaCl_2$. Atroxin (5% vol/vol) was used to induce clotting when γ-IIa was used. Binding was quantified by thrombin chromogenic activity in the clot supernatants. Data were analyzed by nonlinear regression analysis to determine the K_d values. Each value represents the mean of three experiments, each done in duplicate, ±standard error. Reproduced with permission [16].

mediated exclusively by exosite 1 (Fig. 4). Although prothrombin fragment 2 reduced thrombin binding to fibrin in a concentration-dependent and saturable manner, inhibition was incomplete, suggesting that prothrombin fragment 2 exerted its effect by altering the affinity of exosite 1 for fibrin. Similar results were obtained with other ligands of exosite 2, the γ′-peptide and an exosite 2-directed aptamer [16]. These observations lend support to the allosteric linkage proposal because this experiment utilizes fibrin, a native macromolecular ligand for exosite 1. In contrast, previous experiments used fluorescent peptide ligands. These results also suggest that ligands directed to exosite 2 could act to disrupt thrombin binding to fibrin, much as hirudin$^{54–65}$ impairs thrombin binding to fibrin by competing for exosite 1 [19,47].

Because fibrin binding is an indicator of exosite function, binding studies were performed with prothrombin activation intermediates to examine how progressive development of exosite 1 correlates with fibrin binding. Experiments examined the ability of prothrombin activation intermediates to reduce ^{125}I-FPR-α-thrombin binding to unfractionated fibrin. In separate experiments, ^{125}I-FPR-thrombin bound to fibrin with a K_d of 1.5 μM and binding was inhibited by hirudin$^{54–65}$ (not shown). As anticipated, prothrombin had no effect on thrombin binding (Fig. 5). Prethrombin 1, an inactive intermediate missing the fragment 1 domain, also was unable to compete for thrombin binding. However, active site blocked-meizothrombin des F1, the activated form of prethrombin 1, was as effective as unlabelled thrombin as an inhibitor of binding. Likewise, prethrombin 2, the unactivated conformer of thrombin, also was an effective competitor of thrombin binding to fibrin. These results confirm that exosite 1 is functional in activated derivatives of prothrombin, meizothrombin desF1 and thrombin, consistent with studies using hirudin$^{54–65}$ as a probe of exosite 1 [20]. In contrast, divergent results were obtained with inactive intermediates. Prethrombins 1 and 2 demonstrated similar, intermediate affinity for hirudin$^{54–65}$ relative to those of prothrombin and thrombin [20], whereas with fibrin, prethrombin 1 did not bind but prethrombin 2 did. The similar affinity of prethrombin 2 and thrombin in the fibrin binding experiments suggests that exosite 1 is fully organized without the requirement of activation. These discrepant results may reflect differences in the mode of interaction of hirudin and fibrin with exosite 1, consistent with the proposal that each ligand that binds exosite 1 exploits unique interactions [34].

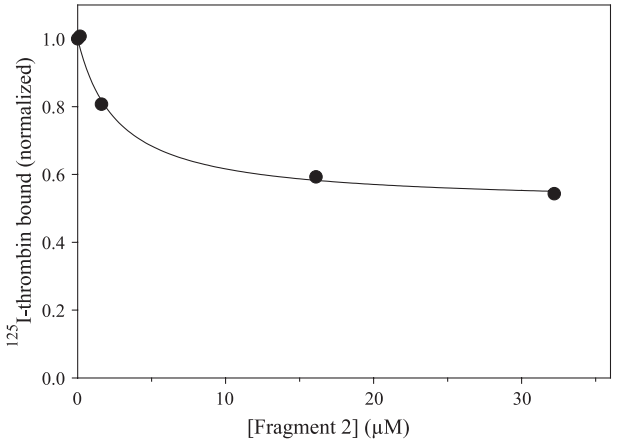

Fig. 4. Influence of exosite 2 ligands on thrombin binding to γ_A/γ_A fibrin. A series of tubes containing 0.7 μM γ_A/γ_A fibrinogen, 2 mM $CaCl_2$, and 0.02 μM ^{125}I-thrombin was prepared and the contents were clotted with 10 nM thrombin in the presence of varying concentrations of prothrombin fragment 2. The fraction of ^{125}I-thrombin bound was calculated for each condition and plotted versus the concentration of prothrombin fragment 2.

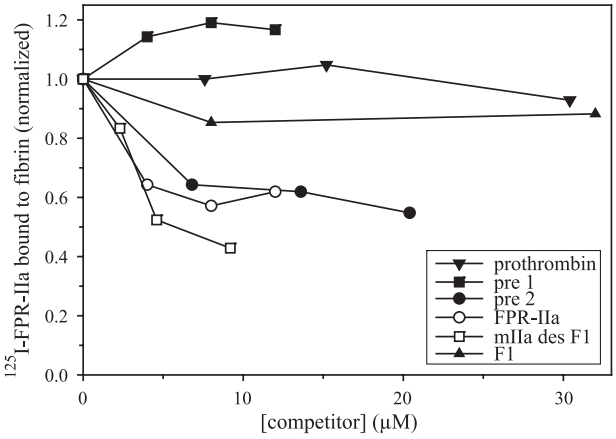

Fig. 5. Capacity of prothrombin activation intermediates to compete with α-thrombin for fibrin binding. Varying concentrations of prothrombin activation intermediates (prothrombin, prethrombin 1, dEGR-chloromethyl ketone inhibited meizothrombin desF1, prethrombin 2, FPR-thrombin, prothrombin fragment 1) were added to a series of tubes containing 3 μM unfractionated fibrinogen, 2 mM $CaCl_2$, and 0.1 μM ^{125}I-FPR-thrombin. The fibrinogen was clotted with 10 nM thrombin and clots were subsequently removed by centrifugation. The fraction of ^{125}I-FPR-thrombin bound to fibrin compared to the amount of thrombin bound to fibrin in the absence of competitor was determined and plotted versus the concentration of the competitor.

4. Consequences of thrombin binding to fibrin

The physiological significance of binding of thrombin to fibrin is revealed in certain pathological states. Fibrinogen variants with reduced thrombin affinity, such as Naples I or New York I, elicit thrombotic complications in affected subjects. This is thought to result from a deficiency of antithrombin I. Thus, patients with these dysfibrinogenemias have reduced capacity to limit the systemic procoagulant activity of thrombin by sequestering it within fibrin clots. In contrast, clot-associated thrombin is also considered prothrombotic [48,49]. These seemingly paradoxical conditions both result from the persistence of thrombin activity, either systemically or locally. It is hypothesized that localized thrombin activity, which may contribute to thrombus growth, plays an important part in the pathogenesis of acute coronary syndromes [50].

Thrombi have long been considered a source of thrombin [51]. Recently, analyses of pathological specimens have directly confirmed the presence of active thrombin in thrombi [52]. Histological assays demonstrated that this residual thrombin pool retained its ability to cleave fibrinogen. These results affirm findings with fibrin clots immersed in plasma [47,53], and suggest that fibrin-bound thrombin is resistant to inhibition by antithrombin. They also suggest that fibrin-bound thrombin retains the ability to cleave fibrinogen, a finding that has been confirmed in plasma and with purified fibrinogen [26,53]. Adding to its prothrombotic potential, clot-associated thrombin is also capable of activating platelets and factors V, VIII, XI, and XIII in vitro [54,55]. It is recognized that other clot-associated components, such as tissue factor, factor Xa, and platelets, contribute to the procoagulant activity of thrombi [48,49]. Therefore, although thrombin may not be the sole trigger of this activity, it represents the ultimate effector and thus remains an important target for new antithrombotic drugs. Thrombotic risk associated with thrombin binding to fibrin may be exacerbated by the elevated affinity of thrombin for γ_A/γ' fibrin. This may be a factor in the observed association between elevated γ_A/γ' fibrinogen levels and coronary artery disease [56].

The persistence of thrombin in thrombi results from an impairment in the susceptibility of fibrin-bound thrombin to inhibition by natural anticoagulant mechanisms. Thus, one consequence of thrombin binding to fibrin is its protection from inhibition by the serpins antithrombin and heparin cofactor II. Protection of thrombin is greatest in the presence of heparin. This occurs because heparin binds both thrombin and fibrin causing thrombin to be bound more avidly by the clot [43,45]. In this ternary complex, exosite 2 is more effectively engaged with heparin and is therefore inaccessible to heparin–antithrombin complexes (Fig. 6). Protection is enhanced with heparin cofactor II because this serpin also requires access to exosite 1 to effect inhibition [45,57].

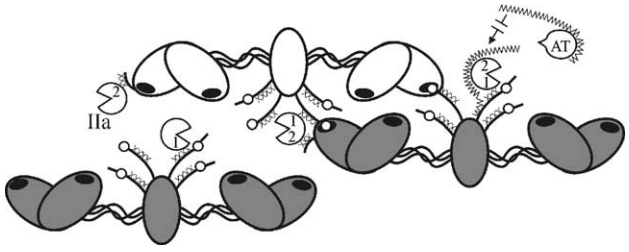

Fig. 6. Model of thrombin's modes of interaction with fibrin. Individual fibrinogen molecules, depicted as described in Fig. 2, are shown with or without shading for clarity. Two molecules, both missing fibrinopeptides A, are shown in polymerized association with reciprocal A/a interactions (open circles and black ovals). Thrombin molecules (IIa) are modeled as circles with openings for the active site and with bound exosites 1 or 2 labeled. In the diagram, separate thrombin molecules are bound to fibrin via either exosite 1 (α-chain) or exosite 2 (γ′-chain). The thrombin molecule in the center is bound via both exosites 1 and 2 to α- and γ′-chains simultaneously. The thrombin molecule on the right is bound via both exosites in a ternary complex with fibrin and heparin (jagged line) that results from thrombin–fibrin, fibrin–heparin and heparin–thrombin binary interactions. Thrombin within this complex is resistant to inhibition by heparin–antithrombin (AT) because heparin is unable to access exosite 2 on thrombin.

Because the ternary thrombin–fibrin–heparin complex is formed by three binary interactions, the heparin–fibrin interaction is also a key contributor to the protection of thrombin from inhibition. Heparin binds fibrin with a K_d value of 0.1–0.5 μM [57–59]. The heparin binding site appears to be located close to the NH_2-terminus of the β-chain, although interaction with distal sites has also been reported [59,60]. The proximity of the heparin and thrombin binding sites on the β-chain of fibrin is consistent with the formation of the ternary thrombin–fibrin–heparin complex. Supporting this concept, studies have demonstrated that heparin promotes thrombin binding to agarose-immobilized fibrinogen [45,57,58]. Because heparin has a heterogeneous composition, different chain lengths have been examined for their ability to bind fibrin and promote thrombin binding. Low molecular weight heparin fractions (<11 kDa) cause considerably less ternary complex formation and protection of thrombin from inhibition than unfractionated heparin (~18 kDa) [57,58]. These studies add support to the ternary complex model of protection of thrombin from inhibition and reveal new avenues for development of antithrombotic agents.

5. Summary and future developments

Recent reports have revealed that the interaction of thrombin with the γ′-chain of fibrin is mediated by exosite 2 [16,35]. This adds a new mode of interaction to the well-described exosite 1-mediated binding of thrombin to the α/β-chains of fibrin [23,29]. To determine the contribution of the different modes of interaction to the observed affinity of thrombin for fibrin, each interaction was quantified separately using fibrin derived from fibrinogen fractions differing in the presence of the γ′-chain and thrombin exosite variants [16]. It was revealed that, in isolation, exosite 1 and exosite 2 each bound fibrin with moderate affinity (K_d values 2–10 μM). High-affinity binding (K_d 0.1 μM) was observed only when both exosite 1- and 2-mediated interactions occurred. These observations suggest that (a) both exosites of thrombin must be bound simultaneously by fibrin, and (b) that the γ′-chain COOH terminus must be in proximity to the NH_2-termini of the α- and β-chains. This model can be accommodated in the polymerized structure of fibrin. Because of the half-staggered alignment of monomers, the central E domain is ligated to two COOH-terminal D domains by knob–hole interactions. This positions the COOH-terminus of the γ′-chain close to the NH_2 termini of the α- and β-chains (Fig. 6) [61]. Furthermore, all the termini extend beyond their respective globular domains, a feature that endows them with a high degree of flexibility and range of motion. The proximity of α/β and γ′ binding sites in polymerized fibrin could facilitate binding of thrombin via both exosites simultaneously (Fig. 6). This

dual ligation of both exosites of thrombin by γ_A/γ' fibrin is analogous to the ternary thrombin–fibrin–heparin complex that protects thrombin from inhibition by heparin–antithrombin complexes. Therefore, high-affinity binding of thrombin to γ_A/γ' fibrin provides another mechanism by which fibrin clots may serve as a reservoir of active thrombin.

Because a fraction of thrombin that is generated in response to vascular injury remains active within the confines of a clot, thrombin is able to maintain local procoagulant activity. Although heparin is an effective therapeutic agent, it has limitations in the clinical setting of thrombosis. Elucidation of the mechanism of binding of thrombin to fibrin and subsequent protection from inhibition has led to the development and refinement of more specific thrombin inhibitors. Direct thrombin inhibitors, agents that target the active site or exosite 1 of thrombin, are receiving increasing attention as antithrombotic agents [50,62]. The list includes bivalirudin, a polypeptide inhibitor modeled after hirudin, and active site-directed synthetic molecules, such as argatroban and melagatran [63]. Another approach to target fibrin-bound thrombin is to use glycosaminoglycans that resist ternary complex formation. These include low molecular weight heparin derivatives that retain catalytic function with antithrombin but have reduced thrombin affinity. Another glycosaminoglycan, dermatan sulfate, which promotes thrombin inhibition by heparin cofactor II but does not bind fibrin is also under investigation [57].

Development of direct thrombin inhibitors has benefited from an understanding of how thrombin interacts with fibrin. Inhibitors derived from hirudin likely displace thrombin from fibrin, in addition to occupying its active site. Therefore, the specialized functions that have evolved for thrombin's exosites can be exploited to counter pathological consequences. Greater knowledge of the mechanism of interaction of thrombin with fibrin will reveal new insights into the roles of the exosites in mediating interaction with other reactants such as factors V, VIII, and XI and platelets. This will permit development of new and improved antithrombotic agents.

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