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Identification and Characterization of the Thrombin Binding Sites on Fibrin\*

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# Abstract

Thrombin binds to fibrin at two classes of non-substrate sites, one of high affinity and the other of low affinity. We investigated the location of these thrombin binding sites by assessing the binding of thrombin to fibrin lacking or containing γ′ chains, which are fibrinogen γ chain variants that contain a highly anionic carboxyl-terminal sequence. We found the high affinity thrombin binding site to be located exclusively in D domains on γ′ chains (*Ka*, 4.9 × 106−1; *n*, 1.05 per γ′ chain), whereas the low affinity thrombin binding site was in the fibrin E domain (*Ka*, 0.29 × 106−1; *n*, 1.69 per molecule). The amino-terminal β15-42 fibrin sequence is an important constituent of low affinity binding, since thrombin binding at this site is greatly diminished in fibrin molecules lacking this sequence. The tyrosine-sulfated, thrombin exosite-binding hirudin peptide, S-Hir53-64 (hirugen), inhibited both low and high affinity thrombin binding to fibrin (IC50 1.4 and 3.0 μ, respectively). The presence of the high affinity γ′ chain site on fibrinogen molecules did not inhibit fibrinogen conversion to fibrin as assessed by thrombin time measurements, and thrombin exosite binding to fibrin at either site did not inhibit its catalytic activity toward a small thrombin substrate, S-2238. We infer from these findings that there are two low affinity non-substrate thrombin binding sites, one in each half of the dimeric fibrin E domain, and that they may represent a residual aspect of thrombin binding and cleavage of its substrate fibrinogen. The high affinity thrombin binding site on γ′ chains is a constitutive feature of fibrin as well as fibrinogen.

# INTRODUCTION

Thrombin binds to its substrate fibrinogen in the central amino-terminal region and cleaves fibrinopeptides A and B from the Aα and Bβ chains, respectively, converting fibrinogen to fibrin. The thrombin-fibrinogen binding interaction is mediated through an anion-binding fibrinogen recognition exosite in thrombin (1, 2, 3) that is situated in an extended patch of positively charged residues in the region of the thrombin loop segment centered around Lys70-Glu80 (4). The exosite also binds to heparin cofactor II (5), the platelet or endothelial cell thrombin receptor (6), thrombomodulin (7, 8), GPIbα1 (9), as well as to a strongly anionic sequence in the carboxyl-terminal region of the leech thrombin inhibitor, hirudin (10, 11, 12, 13, 14, 15).

In addition to binding to fibrinogen at its substrate site, thrombin binds to fibrin at a “non-substrate” site(s) (1, 2, 16, 17, 18). It is commonly believed that non-substrate binding takes place at the same location as fibrinogen substrate binding, namely the central E domain. As determined from binding experiments with 125I-thrombin by Liu *et al.* (19), two classes of non-substrate sites exist in fibrin, one of “high” affinity (*Ka*, ∼6 × 105−1) and the other of “low” affinity (*Ka*, ∼7 × 104−1). Hogg and Jackson (20) also found two classes of sites in fibrin with affinity constants of 3.3 × 106 and 3.0 × 104, respectively. It has been inferred from available information that all non-substrate thrombin binding, especially that of high affinity, is in the E domain (2), although to our knowledge this subject has not been specifically addressed.

Human fibrinogen is chromatographically separable into two major components (“peak 1” and “peak 2”), which differ with respect to the structure of their γ chains (21). Dimeric peak 1 fibrinogen molecules each contain two γA chains (γ1-411V), whereas peak 2 fibrinogen molecules, which amount to ∼15% of the total fibrinogen population (22), have one γA and one γ′ chain (γ1-427L) (23, 24). Similar γ chain variants have been identified in rodent (25, 26) and bovine2 fibrinogens and may exist in other animal species as well (27). In humans, γ′ chains arise through alternative processing of the primary mRNA transcript (28) and differ structurally in their COOH-terminal sequences in that γA chain residues 408-411 are replaced in γ′ chains by an anionic 20 amino acid sequence (24, 29). In rats (25, 30) and cows2γA408-411 is replaced by a shorter but homologous sequence (Table I). The rat and human γ′ chains are tyrosine-sulfated at γ′418 (31, 32) and also at γ′422 in humans.2

Table I. Carboxyl-terminal sequences of γ chains and hirudin

Homologous positions are bolded.

|  |  |
| --- | --- |
| **Chain (position)** | **Amino acid sequence** |
| Human γA (408-411) | A G D V |
| Human γ′ (408-427) | **V** R P **E** **H** P A **E** T **E** **Y** E S L Y P E D D L |
| Rat γ′ (408-419) | **V** S V **E** **H** E V **D** V **E Y** P |
| Bovine γ′ (408-419) | **V** R V **E** **H** H V **E** I **E Y** D |
| Hirudin (53-65) | N G D F E E I P E **E Y** L Q |

γA and γ′ chains are functionally equivalent with respect to factor XIIIa-catalyzed cross-linking (23), but unlike the γA chain, γ′ chains lack the complete platelet binding sequence, γA400-411, and therefore do not support ADP-induced fibrinogen binding or platelet aggregation (33, 34, 35). Our group has recently presented evidence that plasma factor XIII binds specifically to γ′ chains (36), but little else is known about its functions. In this report we present compelling evidence that the anionic carboxyl-terminal γ′ chain sequence situated in the fibrin D domain constitutes the high affinity thrombin binding site, which is itself separate and distinct from the low affinity thrombin binding sites that reside in the central E domain.

# MATERIALS AND METHODS

Human fibrinogen fraction I-2 was isolated from normal citrated plasma by glycine precipitation (37) and separated into peaks 1 and 2 fibrinogen by anion exchange chromatography on DEAE-cellulose (36). Des-Bβ1-42 fibrinogen was produced from peak 1 or peak 2 fibrinogen by digestion with *Crotalus atrox* protease III (38). Fibrinogen concentrations were determined spectrophotometrically at 280 nm using an absorbance coefficient of 1.51 ml mg−1 cm−1 (22). Molecular weights of 340,000 and 325,000 were used for fibrinogen and des Bβ1-42 fibrinogen, respectively (38, 39).

Fibrin-Sepharose was prepared by coupling CNBr-activated Sepharose with peak 2 fibrinogen and then converting the resin-bound fibrinogen to fibrin in the presence of thrombin (2 units/ml) for 16 h at 4°C as described by Heene and Mathias (40). The fibrin-Sepharose was washed with 1.0 NaCl, 50 m HEPES pH 7.4 buffer, followed by 100 m NaCl, 50 m HEPES pH 7.4 buffer containing 50 m CaCl2 and 2 m phenylmethylsulfonyl fluoride.

Human α-thrombin (specific activity, 3.04 units/μg) was obtained from Enzyme Research Laboratories, Inc., South Bend, IN. A molecular weight of 36,500 and an absorbance coefficient of 1.83 ml mg−1 cm−1 were used for calculating thrombin concentrations (41). PPACK-thrombin was prepared by adding a 5-fold molar excess of PPACK (Calbiochem) to α-thrombin and after dialysis the mixture was labeled with 125I (42). The labeled protein was separated from free iodine by affinity chromatography on peak 2 fibrin-Sepharose CL-4B that had been equilibrated with 50 m HEPES, 100 m NaCl, pH 7.4, buffer containing 0.01% (w/v) PEG 8000. Elution of thrombin was achieved with HEPES buffer, pH 7.4, containing either 500 m NaCl or 40 m CaCl2.

Factor XIII (1.95 units/μg) was prepared from pooled human plasma (43) and the activity assayed by the method of Loewy *et al.* (44). Factor XIII (500 units/ml) in 100 m NaCl, 50 m HEPES, pH 7.4, was activated to XIIIa in the presence of 500 μ dithiothreitol and 10 m CaCl2 by incubation with thrombin (10 units/ml, final) for 30 min at 37°C (45).

Thrombin-fibrin binding experiments were performed using a modification of the method reported by Liu *et al.* (19). Fibrin monomer solutions were prepared from fibrinogen clotted at 1 mg/ml in 60 m NaH2PO4 buffer, pH 6.4, with thrombin (1 unit/ml, final) for 2 h at room temperature. The clots were synerized and dissolved in 20 m acetic acid to >10 mg/ml fibrin and repolymerized in a 10-fold excess of 100 m NaCl, 50 m Tris, pH 7.4, buffer containing 40 m CaCl2 and 2 m *N*-ethylmaleimide. These clots were synerized and dissolved in 20 m acetic acid to a 10 mg/ml stock solution. Clots containing 0.5 or 1 nmol of fibrin were formed by adding a fibrin monomer solution to a 100 m NaCl, 50 m HEPES, 0.01% (w/v) PEG 8000, pH 7.4, buffer containing varying amounts of 125I-labeled PPACK-thrombin and incubated for 2 h at room temperature. Clot-bound thrombin was separated from free thrombin by syneresis of the clot. The final concentration of reactants in the clotting mixture were fibrin, 2.5 μ, 125I-PPACK-thrombin, 0-37.5 μ, in a final volume of 200 or 400 μl. For clotting mixtures containing des-Bβ1-42 fibrin, which polymerizes slowly and incompletely, full clot recovery (>95%) was assured by cross-linking the fibrin with factor XIIIa (25 units/ml) for 2 h at room temperature. After the incubation period, tubes were centrifuged and thrombin-bound clots separated from free thrombin by syneresis. The distribution of thrombin bound to the clot and free in solution was determined by radioactivity counting in a Packard Multi-prias 4 γ counter. The amount of thrombin trapped in the clot was estimated from the radioactive counts that were retained in cross-linked clots of peak 1 or des-Bβ1-42 peak 1 fibrin in the presence of 25 μ S-Hir53-64, which had been added to block thrombin exosite binding to fibrin.

The binding data were graphed as Scatchard plots (46). Data indicating a two-component system were deconvoluted by the method of Klotz and Hunston (47). It was not technically feasible to reach thrombin concentrations which saturated the low affinity site in samples of peak 2 fibrin that contained high levels of the high affinity component. In these experiments, the low affinity component was defined by peak 1 (γA,γA) fibrin values and was used for correcting high affinity values (47). High affinity thrombin binding to des Bβ1-42 peak 2 fibrin was not significantly affected by a low affinity binding component, and these data were therefore not corrected. The level of thrombin entrapment in the clots (≤4% of total counts) did not significantly effect binding parameters, and therefore no corrections were applied to the data.

Competitive binding experiments involving thrombin anionic exosite binding were performed with the sulfated hirudin peptide, S-Hir 53-64, which was a generous gift from Dr. John Maraganore of Biogen Inc., Cambridge, MA. Hirugen at concentrations up to 40 μ was added to 125I-PPACK-thrombin (1 μ) and 0.5 nmol of fibrin at a final volume of 200 μl as described above for thrombin binding measurements. Peptide concentrations were estimated spectrophotometrically at 215 nm using an absorbance coefficient of 15.0 ml mg−1 cm−1 (48).

A Fibrometer Precision Coagulation Timer (BBL Microbiology Systems) was used to determine the thrombin time for the conversion of fibrinogen (1 mg/ml final) to fibrin in 50 m Tris, 100 m NaCl, pH 7.4 at 37°C at a thrombin level of 0.6 unit/ml. Hydrolysis of S-2238 (H–phenylalanyl–pipecolyl–arginine-*p*-nitroanilide dihydrochloride; Chromogenix, Mölndal, Sweden) by thrombin (3.2 n) in 0.10 NaCl, 0.05 Tris, pH 7.5 buffer, was monitored at 405 nm at room temperature. Samples contained S-2238 (50 μ), with or without peak 1 fibrin (1 μ), or peak 2 fibrin (1 μ). The hydrolysis rate was estimated from the increase in absorbance at 405 nm during the first 3 min of the reaction.

# RESULTS

## Thrombin Binding to Fibrin

In our studies of thrombin binding to fibrin we found it useful as a general condition to covalently cross-link the fibrin polymer in the presence of factor XIIIa during the binding experiment in order to assure complete fibrin recovery (>95%). This procedure was particularly useful for recovering des Bβ1-42 fibrin clots, which polymerize slowly and incompletely in the absence of cross-linking (49). There were no significant differences in thrombin binding behavior to cross-linked and non-cross-linked fibrin (Fig. 1), confirming the findings of Liu *et al.* (50). Thrombin entrapment in the clot, as assessed in the presence of 25 μ S-Hir53-64, was ≤4% of the total counts and did not significantly change any of the calculated binding parameters.

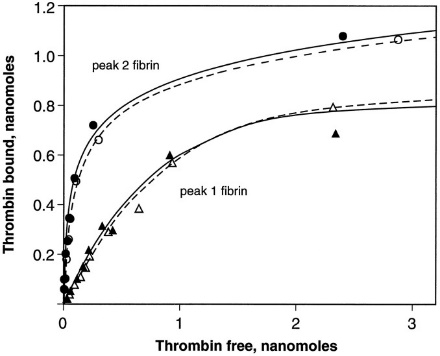


Fig. 1. **125I-PPACK-thrombin binding to factor XIIIa-cross-linked (•; –) and non-cross-linked (∘; - - - -) peak 1 or peak 2 fibrin.** The curves represent the mean values of three to six separate experiments for each condition. The regression coefficients for the curves ranged from 0.91-0.98.

## Low and High Affinity Binding Sites

Our previous study with des-Bβ1-42 fibrin had indicated that the β15-42 sequence was a component of the non-substrate thrombin binding site in the fibrin E domain (49). To extend those observations we carried out a systematic study of non-substrate thrombin binding to several fibrin preparations that differed with respect to their γ chain composition, their Bβ1-42 content, or both. Fraction I-2 fibrin, which has ∼15%γ′-containing molecules (22), was studied first (Fig. 2). As assessed from the Scatchard plot, our results correspond to those reported by Liu *et al.* (19), who studied a similar fibrinogen subfraction. The data indicate two classes of binding sites, one of high affinity (*Ka*, 5.5 × 106−1) and the other of low affinity (*Ka*, 0.45 × 106−1) (Table II).

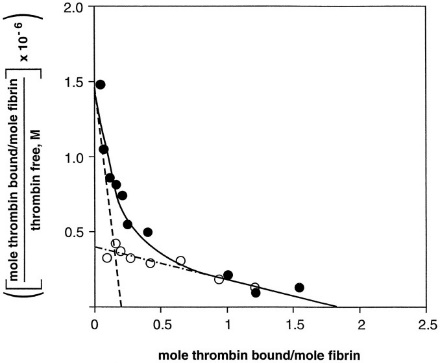


Fig. 2. **Scatchard analysis of the binding of** **125I-PPACK-thrombin to fraction.** I-2 fibrin (•) peak 1 fibrin (∘). The *dashed line* represents the slope of the high affinity binding component in fraction I-2 fibrin. The *broken line* represents the slope of the low affinity component in peak 1 fibrin.

Table II..

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fibrin fraction** | ***n*** | **High affinity site****a** |  | ***n*** | **Low affinity site****b** |  |
|  |  | ***Ka*× 10−6± S.D.** | **No. sites/mol ± S.D.** |  | ***Ka*× 10−6± S.D.** | **No. sites/mol ± S.D.** |
|  |  | *m−1* |  |  | *m−1* |  |
| Fraction I-2 | 4 | 5.5 ± 1.3 | 0.22 ± 0.02 | 4 | 0.45 ± 0.06 | 1.60 ± 0.20 |
| Peak 1 | 8 | 0 | 0 | 8 | 0.21 ± 0.05 | 1.80 ± 0.23 |
| Peak 2 | 6 | 5.6 ± 0.8 | 0.83 ± 0.12 | 3 | Indeterminate | Indeterminate |
| Des-Bβ1-42, Peak 1 | 4 | 0 | 0 | 4 | 0.11 ± 0.03 | 1.66 ± 0.34 |
| Des-Bβ1-42, Peak 2 | 6 | 4.2 ± 0.8 | 0.78 ± 0.11 | 6 | Indeterminate | Indeterminate |

aThe mean *Ka* for the high affinity site based on all determinations (*n* = 16) is 4.9 ± 1.2 × 106−1, with 1.05 ± 0.27 thrombin binding sites per γ′ chain.

bThe mean *Ka* for the low affinity site based on all determinations (*n* = 12) is 0.29 ± 0.14 × 106−1, with 1.69 ± 0.25 thrombin binding sites per fibrin molecule.

Studies of thrombin binding to peak 1 fibrin, which contains only γA chains, indicated a single class of binding site with a *Ka* of 0.21 × 106−1, corresponding to the low affinity site in fraction I-2 fibrin, and having a binding stoichiometry of 1.80 per molecule of fibrin (Fig. 2). Parallel analysis of thrombin binding to peak 2 fibrin demonstrated that high affinity binding dominated the Scatchard plot and that there were 0.83 high affinity sites per fibrin molecule (Fig. 3), a stoichiometry that corresponds well to the γ′ chain content in peak 2 fibrinogen preparations (48%γ′, 52%γA) (51). Low affinity binding in peak 2 fibrin was too low for accurate quantitation, but was in the same range as was found for peak 1 or fraction I-2 fibrin.

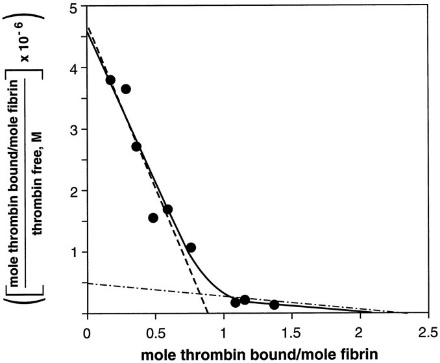


Fig. 3. **Scatchard analysis of the binding of** **125I-PPACK-thrombin to peak 2 fibrin.** The *dashed line* represents the slope of the high affinity component in peak 2 fibrin. The low affinity component, determined from the low affinity binding component of peak 1 fibrin, is represented by the *broken line*.

There was a marked reduction of low affinity binding to des-Bβ1-42 peak 2 fibrin (Fig. 4), and therefore no corrections to the high affinity values were applied for the presence of a low affinity component. In the case of des-Bβ1-42 peak 1 fibrin, which lacks a high affinity binding site, reduced levels of low affinity thrombin binding were found (Fig. 4) and exceeded the amount that could be attributed to entrapment alone. The estimated *Ka* (0.11 × 106−1) was 38% of that found for peak 1 or fraction I-2 fibrin, but the stoichiometry was the same (*i.e.* 1.66 sites per molecule).

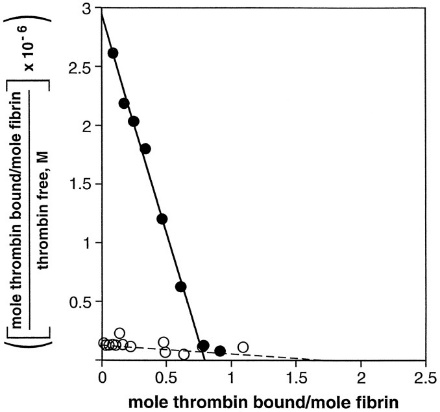


Fig. 4. **Scatchard analysis of the binding of** **125I-PPACK-thrombin to des-Bβ1-42 peak 2 fibrin (•) or des-Bβ1-42 peak 1 fibrin (∘).** The *solid line* represents the high affinity component. The low affinity component is represented by the *broken line*.

## Thrombin Exosite-binding Peptide

To provide additional evidence that the γ′ sequence contains the high affinity site for thrombin exosite binding, we evaluated thrombin binding in the presence of S-Hir53-64, a well characterized thrombin exosite binding peptide, to des-Bβ1-42 peak 2 (high affinity) or peak 1 (low affinity) fibrin. S-Hir53-64 was an effective competitive inhibitor of thrombin binding to fibrin with an IC50 of 3.0 μ for high affinity thrombin binding and 1.4 μ for low affinity binding (Fig. 5), thus indicating that both classes of sites bind thrombin through its exosite.

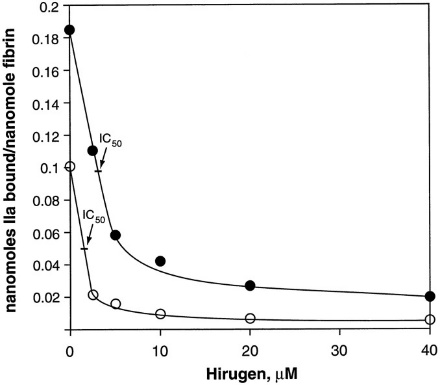


Fig. 5. **S-Hir53-64** **(hirugen) inhibition of** **125I-PPACK-thrombin binding to des-Bβ1-42 peak 2 fibrin (•) or peak 1 fibrin (∘).** The calculated IC50 for each curve is indicated.

## Fibrinogen to Fibrin Conversion and S-2238 Hydrolysis

The mean thrombin times for peak 1 and peak 2 fibrinogens were 20.5 ± 0.5 and 20.4 ± 0.5 s (*n* = 5), respectively, indicating that the presence of the γ′ sequence had no measurable effect on thrombin substrate cleavage of fibrinogen. Hydrolysis of S-2238 was not inhibited by the presence of peak 1 fibrin or peak 2 fibrin in the hydrolysis mixture (Table III).

Table III. Thrombin Hydrolysis of S-2238

|  |  |  |
| --- | --- | --- |
| **Substrate** | ***n*** | **Initial rate, Δ*A*405 nm/min** |
| S-2238 alone | 8 | 0.12 ± 0.01 |
| S-2238 + peak 1 fibrin | 8 | 0.13 ± 0.02 |
| S-2238 + peak 2 fibrin | 9 | 0.11 ± 0.01 |

# DISCUSSION

These present experiments show that there is a unique high affinity non-substrate binding site for thrombin in the carboxyl-terminal region of the γ′ chain and a low affinity class of binding site in the amino-terminal region of fibrin, the latter contained in part within the Bβ1-42 sequence. In studies of fraction I-2 fibrinogen, which contains approximately 8%γ′ chains, we detected the same two classes of binding sites that were identified by Liu *et al.* (19). The binding affinities we determined were about 10-fold higher for high affinity binding and 4-fold higher for low affinity binding (Table II). In peak 1 fibrin (γA,γA) only the low affinity binding component was observed, whereas with peak 2 fibrin (γ′,γA), there was increased high affinity thrombin binding corresponding to the increased content of γ′ chains. Overall, high affinity binding stoichiometry corresponds well to the content of γ′ chains, with one thrombin per γ′ chain.

Although the existence and structure of the γ′ chain has been known for many years (21, 23, 51), its role as the high affinity non-substrate thrombin binding site in fibrin has been overlooked for several reasons. First, it has been generally assumed that the entire thrombin binding site in fibrin was a residual of the substrate recognition site in fibrinogen. Thus, knowledge that there were two classes of binding sites in fibrin, coupled with the observation that high affinity thrombin binding was only a minor component of the total binding reaction in fraction I-2 fibrin (19, 20), evidently did not raise suspicion of another possible thrombin-binding location. Second, most investigations on this subject have involved only central E domain structures (17, 18, 52, 53) or in addition, plasmic D fragments (17, 18) from which the γ′ sequence had most likely been cleaved (54) or which had a low content of γ′-containing molecules to begin with (*i.e.* fraction I-2) (19, 55). Studies of thrombin binding to immobilized fibrin (1, 16, 56) or to a modified fibrin clot (des-Bβ1-42 fibrin) (49) could not have distinguished the specific location of any binding site.

We would therefore revise the current belief that all non-substrate thrombin binding takes place in the fibrin E domain, to stipulate that only low affinity thrombin binding takes place in this region. We would concur, however, with the notion that thrombin binding in the E domain is likely to represent a residual aspect of the site that participated in fibrinogen substrate recognition. Scatchard analyses indicated a stoichiometry of 1.69 thrombin molecules per fibrin molecule, suggesting that there are two low affinity sites in each dimeric fibrin molecule, corresponding to a fibrinogen substrate recognition site for each pair of fibrinopeptides (FPA, FPB). Whether recognition site binding is the same for FPA and FPB cleavage has yet to be determined.

Unlike the high affinity binding site in the γ′ chain, formation of the low affinity site in the E domain is not restricted to a single peptide sequence. Consistent with a previous report (49), our current data suggest that the β15-42 sequence contributes significantly to non-substrate binding and that ∼60% of low affinity binding is lost by removal of this sequence. Other evidence suggests that the fibrin Aα27-50 sequence contributes as well to low affinity thrombin binding (18, 53). The γ chains in the E domain have also been proposed as contributors to the thrombin binding site (17, 18), but the evidence for this is not well substantiated.

Fibrinogens New York I (des-Bβ9-72) and Naples I (Bβ A68 T) are dysfibrinogenemias, which have been characterized as having impaired thrombin binding (57, 58), presumably related to a defective amino-terminal substrate or non-substrate binding site. A recent study of recombinant γA-type Bβ A68 T fibrinogen has reaffirmed the importance of Bβ68 alanine in thrombin-mediated cleavage of Naples I fibrinogen (59). In the case of New York I, which is heterozygotic, thrombin binding to fibrin was 50% of normal, but there was no evidence to suggest a high affinity thrombin binding component (57). Similarly, thrombin binding to homozygous Naples I fibrin was reported to be absent (58), and thus there was also no collateral evidence for high affinity thrombin binding to the presumably normal Naples I γ′ chain. However, in another report on this same family, thrombin binding to fibrin from a homozygous proband was reduced to only one-third of normal (60). The available data derived from studies on Naples I fibrin do not permit an unambiguous distinction to be made as to the presence or absence of a high affinity binding component, although we would have expected only low affinity binding to have been affected.

Direct measurements of thrombin binding to substrate fibrinogen molecules have not been reported, owing to the fact that thrombin binding to its substrate is accompanied by concomitant conversion of fibrinogen to fibrin. Instead, estimation of substrate binding affinities have been made from kinetic experiments involving peptide A release from fibrinogen peptides or fibrinogen itself. The *Km* estimated from such studies is 6-11 μ (61, 62, 63, 64), and the *Kd* derived from similar kinetic studies was 1.3-2.6 μ (65, 66). Our results suggest that the high affinity non-substrate site has a significantly higher affinity for thrombin exosite binding (*Kd*, 0.26 μ) than that estimated from the *Km* or the *Kd* derived for the substrate site (61, 62, 63, 64, 65, 66). Nevertheless, the γ′ site itself in fibrinogen is not an effective competitor for thrombin binding and cleavage at the fibrinogen substrate site, as assessed by our thrombin time measurements in this study and in another (22). It therefore seems likely that the substrate binding site itself will prove to have a higher binding affinity for thrombin than has been estimated previously from *Km* measurements, by analogy with hirudin, which has a higher binding affinity for thrombin as a bivalent molecule than does its COOH-terminal exosite binding sequence alone.

The physiological role that the γ′ sequence plays in modulating thrombin function still remains to be determined. It is very likely that the measurable thrombin clotting activity found in fibrin and fibrin degradation products (67, 68, 69, 70) is attributable to non-substrate binding at the γ′ site, or the low affinity site, or at both sites. In light of our present findings, it will be important to study the relationship between thrombin binding to γ′-containing fibrin and thrombin activation of coagulation factors such as factors V, VIII, or XIII or cellular receptors such as those on platelets and endothelial cells.

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