Identification and Characterization of the Thrombin Binding Sites on Fibrin*

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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 carboxy-terminal sequence. We found the high affinity thrombin binding site to be located exclusively in D domain on γ' chains (Kθ, 4.9 × 10⁶ M⁻¹; 1.05 πM per γ' chain), whereas the low affinity thrombin binding site was in the fibrin E domain (Kθ, 0.29 × 10⁶ M⁻¹; 1.69 M 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 μM, 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.

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1 The abbreviations used are: GP Ibα, glycoprotein Ibα; PPACK, D-Phe-Pro-Arg chloromethyl ketone; S-2238, H-D-phenylalanyl-L-pipercollyl-t-arginine-p-nitroanilide dihydrochloride; PEG 8000, polyethylene glycol, average molecular weight 8000; S-Hir53–64, sulfated carboxy-terminal residues 53–64 of hirudin; des-Bβ1–42 fibrinogen, fibrinogen from which the amino-terminal 42 residues of the Bβ chain have been cleaved; β15–42, amino-terminal fibrin β chain sequence.

‡ A. Henschen, personal communication.
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TABLE I

Carboxyl-terminal sequences of γ chains and hirudin

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

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-BγI–II 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-BγI–II fibrinogen, respectively (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 M NaCl, 50 mM HEPES pH 7.4 buffer, followed by 100 mM NaCl, 50 mM HEPES pH 7.4 buffer containing 50 mM CaCl₂, and 2 mM 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 1 fibrin-Sepharose CL-4B that had been equilibrated with 50 mM HEPES, 100 mM 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 mM NaCl or 40 mM 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 mM NaCl, 50 mM HEPES, pH 7.4, was activated to XIIIa in the presence of 500 µM dithiothreitol and 10 mM 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 mM 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 mM acetic acid to >10 mg/ml fibrin and repolymerized in a 10-fold excess of 100 mM NaCl, 50 mM Tris, pH 7.4, buffer containing 40 mM CaCl₂ and 2 mM N-ethylmaleimide. These clots were synerized and dissolved in 20 mM acetic acid to a 10 mg/ml stock solution. Clots containing 0.5 or 1 n mole of fibrin were formed by adding a fibrin monomer solution to a 100 mM NaCl, 50 mM HEPES, 0.01% (w/v) PEG 8000, pH 7.4, buffer containing varying amounts of 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 was fibrin, 2.5 µM ¹²⁵I-PPACK-thrombin, 0–37.5 µM, and in a final volume of 200 or 400 µl. For clotting mixtures containing des-BγI–II 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-BγI–II peak 1 fibrin in the presence of 25 µM 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 (γAγA) fibrin values and was used for correcting high affinity values (47). High affinity thrombin binding to des-BγI–II 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 (5% of total counts) did not significantly affect 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-Hir53–64, which was a generous gift from Dr. John Marzogaro of Biogen Inc., Cambridge, MA. Hirugen at concentrations up to 40 µM was added to ¹²⁵I-PPACK-thrombin (1 µM) and 0.5 µmol 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 Finometer 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 mM Tris, 100 mM NaCl, pH 7.4 at 37°C at a thrombin level of 0.6 unit/ml. Hydrolysis of S-2238 (H-d-phenylalanyl-l-pipeeryl-l-arginine-p-nitroanilide dihydrochloride; Chromogenix, Mölndal, Sweden) by thrombin (3.2 nM) in 0.10 M NaCl, 0.05 M Tris, pH 7.5 buffer, was monitored at 405 nm at room temperature. Samples contained S-2238 (50 µM), with or without peak 1 fibrin (1 µM), or peak 2 fibrin (1 µM). 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 polymerization (>95%). This procedure was particularly useful for recording des-BγI–II fibrin clotting, which polymerizes 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 µM S-Hir53–64, was ≤4% of the total counts and did not significantly change any of the calculated binding parameters.

Low and High Affinity Binding Sites—Our previous study with des-BγI–II fibrin had indicated that the βγ5–42 sequence was a component of the non-substrate thrombin binding site in the fibrin E domain (49). To extend these 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 BγI–II 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 x 10⁶ M⁻¹) and the other of low affinity (Kₐ 0.45 x 10⁶ M⁻¹) (Table II). Studies of thrombin binding to peak 1 fibrin, which contains only γA chains, indicated a single class of binding site with a Kₐ of 21 x 10⁶ M⁻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

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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.

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 Kd (0.11 × 10^6 M⁻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).

**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 μM for high affinity thrombin binding and 1.4 μM for low affinity binding (Fig. 5), thus indicating that both classes of sites bind thrombin through its exosite.

**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).

**DISCUSSION**

These present experiments show that there is a unique high affinity non-substrate binding site for thrombin in the carboxy-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 over-

![Fig. 1](http://www.jbc.org/)

**FIG. 1.** ¹²⁵I-PPACK-thrombin binding to factor XIIIa-cross-linked (●; --) and non-cross-linked (○; ---) peak 1 or peak 2 fibrin. The broken line represents the slope of the low affinity binding component in peak 1 fibrin.

![Fig. 2](http://www.jbc.org/)

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

ᵃ The mean Kₐ for the high affinity site based on all determinations (n = 16) is 4.9 ± 1.2 × 10⁶ M⁻¹, with 1.05 ± 0.27 thrombin binding sites per γ chain.
ᵇ The mean Kₐ for the low affinity site based on all determinations (n = 12) is 0.29 ± 0.14 × 10⁶ M⁻¹, with 1.69 ± 0.25 thrombin binding sites per fibrin molecule.
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 ϵ 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 ϵ 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 ϵ 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 con-
comitant 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 $K_m$ estimated from such studies is $6–11 \mu M$ ($61–64$), and the $K_d$ derived from similar kinetic studies was $1.3–2.6 \mu M$ ($65, 66$). Our results suggest that the high affinity non-substrate site has a significantly higher affinity for thrombin exosite binding ($K_d$, $0.26 \mu M$) than that estimated from the $K_m$ or the $K_d$ derived for the substrate site ($61–66$). Nevertheless, the $\gamma$ 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 $K_m$ 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 $\gamma$ 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 ($67–70$) is attributable to non-substrate binding at the $\gamma$ 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 $\gamma$-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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