The Fibrinogen Sequences That Interact With Thrombin

By Cameron G. Binnie and Susan T. Lord

The serine protease thrombin interacts with a variety of substrates and as a consequence is involved in a number of physiologic processes. Fibrinogen is a particularly interesting substrate of thrombin for a number of reasons. First, thrombin cleaves not just one but four peptide bonds in fibrinogen, apparently in four successive steps. As a result of these proteolytic cleavages, the soluble fibrinogen molecules are converted into an insoluble fibrous polymer. In addition, the affinity of the enzyme for the final product, fibrin, is similar to that for the substrate, fibrinogen. Active thrombin is thus bound by the fibrin clot in a form largely resistant to inactivation by thrombin inhibitors, a situation that has important clinical consequences and complicates clinical anticoagulant therapy.

This review focuses on the specific sequences of fibrinogen and fibrin that interact with thrombin. As illustrated in recent x-ray crystal structures of thrombin, the thrombin active site can be considered as two separate domains: a catalytic site that includes the Asp-His-Ser catalytic residues, the specificity pocket and an apolar pocket; and an extended fibrinogen recognition site (FRS). The fibrinogen regions responsible for binding to the catalytic site and the FRS will be examined and the properties of fibrin-bound thrombin discussed.

BACKGROUND

Fibrinogen is a dimeric glycoprotein, with each monomer composed of three polypeptides: the Aα (610 residues), the Bβ (461 residues), and the γ (411 residues) chains. The six chains are connected by 29 disulfide bonds. Although the complete primary structure of the protein is known, high resolution structural analysis has not been achieved as the available crystals of fibrinogen diffract poorly. In electron micrographs, fibrinogen has an elongated, trinodular structure. However, the available crystals of fibrinogen diffract poorly. In electron micrographs, fibrinogen has an elongated, trinodular structure. The central globular domain contains the amino terminal disulfide knot, or NDSK. NDSK, which is composed of Aα 1-51, Bβ 1-118, and γ 1-78, represents the central globular domain along with some residues from the spacer arms and contains the fibrinopeptides cleaved by thrombin. NDSK is a simple competitive inhibitor of thrombin, which illustrates its involvement in thrombin binding. Furthermore, active site inhibited thrombin binds to NDSK-agarose but not to C-terminal fragments.

Thrombin cleaves Aα from fibrinogen before fibrin monomers that spontaneously polymerize to form a fibrin clot.

THE AMINO TERMINAL DISULFIDE KNOT

It is the central domain of fibrinogen (Fig 1) that binds to thrombin. Chemical cleavage of fibrinogen with cyanogen bromide generates a number of fragments, including one called the amino terminal disulfide knot, or NDSK. NDSK, which is composed of Aα 1-51, Bβ 1-118, and γ 1-78, represents the central globular domain along with some residues from the spacer arms and contains the fibrinopeptides cleaved by thrombin. NDSK is a simple competitive inhibitor of thrombin, which illustrates its involvement in thrombin binding. Furthermore, active site inhibited thrombin binds to NDSK-agarose but not to C-terminal fragments.

Thrombin cleaves Aα from fibrinogen almost as efficiently as from fibrinogen, as the Michaelis constant (Km) for Aα cleavage from fibrinogen is similar.

The NDSK-derived fragment Aα 1-51 is a good substrate for thrombin, which suggests that much of the information necessary for Aα cleavage is present in this sequence.

Residues that identify Aα Arg-16-Gly-17 as a target for thrombin cleavage are found, not surprisingly, within Aα itself and the sequence of Aα is shown in Fig 2. High resolution structures of Aα in thrombin-peptide complexes have been determined in solution by NMR and in crystals by x-ray analysis.

NMR spectra of synthetic peptides in the presence of thrombin show that the first five residues of the Aα chain do not interact with thrombin, whereas residues 7-16 form a compact structure, with some residues interacting directly with thrombin. The crystal structure of the synthetic peptide Aα 7-16 bound to thrombin has illustrated the precise requirements for fibrinopeptide recognition, i.e., a short α-helical segment, a chain reversal at Ala-10, and multiple main chain hydrogen bonds within residues 7-12, such that the sidechains of Phe-8, Leu-9, and Val-15 form a hydrophobic group that binds to the thrombin apolar pocket. The side chain of Arg-16 is inserted into the specificity (P1) pocket of thrombin.

Within the sequence of fibrinopeptide A there are both critical (nonvariable) residues and residues that can be varied without cost. As previously stated, the first five residues do not interact with thrombin and this is reflected in the...
Thrombin, a sphere of diameter 45 Å, has been drawn alongside. The trinodular structure of fibrinogen as modeled from electron micrographs of negatively stained specimens. The dimensions of each domain have been drawn to scale. The central domain, otherwise referred to as the amino terminal disulfide knot, contains the fibrinopeptides that are cleaved by thrombin and is also the region of fibrinogen that binds thrombin. The outer nodules are composed of two globular domains, the Bγ chain carboxy terminus (inner) and the γ chain carboxy terminus (outer). The Aα chain carboxy termini have not been drawn. For comparison, thrombin, a sphere of diameter 45 Å, has been drawn alongside.

By contrast, when fpA sequences from many mammalian species are aligned, any interspecies differences within Gly-6 to Arg-16 are quite conservative, suggesting that this region is critical to thrombin binding. When Asp-7 is replaced by Asn, as in the genetic variant fibrinogen Lille I, fibrinopeptide cleavage is reduced. However, substitution of Asp-7 with Ala had no detectable effect on the rate or specificity of fpA cleavage from an Aα 1-50 β-galactosidase fusion protein. Also, analysis of fpA release from Asp-7→Asn synthetic peptides by kinetic methods or analysis of the structure of the substituted peptide by NMR failed to show differences within the native sequence. Thus, the importance of Asp-7 in thrombin interaction remains uncertain. By contrast, several lines of evidence show that Phe-8 is extremely important. Phe-8 is highly conserved in the fpA sequence of many mammalian species. Peptide mapping studies showed that peptides containing Phe-8 were considerably better substrates than those in which it was absent. In fact, when Phe-8 is replaced by tyrosine in an Aα 1-50 fusion protein, thrombin does not cleave fpA at all. The role of this residue is illustrated by the crystal structure, in which the Phe-8 sidechain projects into the apolar pocket next to the catalytic site and provides binding affinity through hydrophobic interactions. The phenyl ring participates as a hydrophobic cluster consisting of residues from both fpA and thrombin. This interaction apparently provides binding energy and properly orients the scissile bond for hydrolysis.

Gly-12 is another nonvariable residue. The Gly-12→Val substitution present in fibrinogen Rouen I probably disrupts the compact structure necessary for fpA binding, and presumably alters the positioning of the Arg-Gly bond in the active site, resulting in markedly slower cleavage. This reduced cleavage rate was also exhibited in a fusion protein with a Gly-12→Val substitution. Similarly, a Gly-13→Val substitution in the fusion protein reduced the cleavage rate, indicating that this substitution also alters the structure and affects positioning of the scissile bond. In contrast, the substitution Gly-14→Val had little effect on the rate of fpA cleavage, as might be expected from the x-ray data.

Arg-16, which is the site of cleavage, is critically important. If replaced by His, as in fibrinogen Pekotsky I, thrombin will cleave at this residue, although considerably more slowly. The delayed release from this variant can be explained by the poor fit of histidine to the P1 specificity pocket of thrombin. With the substitution Arg-16→Leu, a variant fusion protein was cleaved by thrombin at Arg-19 instead of Arg-16. Upon extended digestion with high concentrations of thrombin, cleavage at Arg-19 occurs in normal fibrinogen after release of fpA, releasing the tripeptide ACT 17-20, may bind to thrombin, as presented in a hypothetical model. Variant fibrinogens have been identified with substitutions of residues 18 and 19. However, analysis of these variants show that it is generally the subsequent step of fibrin polymerization that is defective, rather than fpA release rate. For instance, Pro-18→Leu (Kyoto II) does not alter the rate of fpA release.
(Detroit)38 has fpA release that is normal.39 Nevertheless, Arg-19 → Gly (Aarhus I,40 Mannheim I41) did have reduced fpA release. In Fig 2, the critical and variable residues within the fpA sequence are illustrated.

**FIBRINOPEPTIDE B**

Fibrinopeptide B cleavage has not been as well studied as fpA. The NDSK-derived peptide Bb 1-118, in contrast to the case for the Aa chain fragment Aa 1-51, is a poor substrate for thrombin, suggesting that these residues are not sufficient for efficient cleavage or have an altered, suboptimal conformation in this fragment. A comparison of the sequence of fpB with fpA shows little similarity (Fig 2), an indication that the structure of the thrombin-bound fpB is different from fpA. Furthermore, mammalian fpB sequences are much less alike than fpA.23 No structural data is available for the fpB-thrombin complex; however, it has been suggested that the side chains of Phe-10 and Phe-11 bind to the apolar site in the opposite direction to fpA.*3 Support for the importance of Phe-11 comes from the observation that in mammalian species where Phe-11 is replaced, a hydrophobic residue, either Leu or Val, is almost invariably present. Variant fibrinogens that alter fpB cleavage have only been detected at the point of cleavage: Arg-14 (Ulmorden)45 and Gly-15 (Ise).46 However, variants that impair fpB cleavage may go undetected because removal of fpA (by the enzyme Reptilase) is sufficient to form a clot that is indistinguishable from that formed by thrombin.45

**BINDING TO THE FRS**

Although the fibrinogen sequences that bind to thrombin's catalytic site are well defined, there is less agreement about the fibrinogen sequence or sequences that bind to the FRS of thrombin. It is clear that fibrinogen and fibrin bind to a site distinct from the catalytic site because thrombin that is chemically blocked at the catalytic site with D-Phe-Pro-Arg-chloromethylketone (PPACK) binds to both fibrinogen-agarose and fibrin-agarose.46 Also, fibrin-bound thrombin remains catalytically active against small peptide substrates.57,48 It is a commonly held assumption that thrombin binds to fibrinogen in a manner similar to fibrin. In other words, the interactions that promote thrombin binding to fibrinogen as a substrate are those that anchor thrombin to fibrin as a product, with the obvious caveat that in fibrin the fibrinopeptides are no longer present. Support for this hypothesis comes from the similar affinity of thrombin for fibrin (Kd = 2 to 10 μM)47-51 to that for fibrinogen (Km = 8 to 10 μM).52

Clues to the location of the FRS binding residues have come from peptide studies. Although the fibrinogen fragment Aa 1-51 is a good substrate for thrombin, the rate of cleavage at Arg-16 is markedly reduced in a smaller fragment, Aa 1-23.53,54 The removed residues, 24-51, do not occupy the catalytic site, so the increased rate of cleavage of the larger peptide may result from binding to another site on thrombin, such as the FRS. (Alternatively, residues 24-51 may stabilize an optimal substrate conformation within 7-16, thus enhancing catalysis.55) Affinity chromatography of proteolytic fragments of fibrinogen and fibrin on active site inhibited thrombin-agarose identified the segment Aa 17-78 as a region that binds to thrombin, whereas a peptide analogous to Aa 27-50, but with the four cysteines replaced by alanine, inhibits clotting of fibrinogen by thrombin and also binds to PPACK-thrombin.56 From these studies it is clear that an FRS binding site is present within the common sequence, Aa 27-50.

Insight into the FRS domain and the sequences that bind to it has also come from the crystal structure of thrombin complexed to the inhibitor hirudin.57,58 Hirudin, a 65 residue polypeptide of known sequence,59 binds with its hydrophobic amino terminus at the catalytic site and its negatively charged carboxy terminus at the FRS. Examination of the hirudin carboxy terminal sequence reveals a number of negatively charged residues interspersed with hydrophobic residues. In the thrombin-hirudin crystal structure, hirudin C-terminal residues Asp-55, Glu-57, Glu-58, and Gln-65 participate in polar interactions with thrombin residues, whereas multiple nonpolar interactions also contribute to inhibitor-FRS binding. Although the structural data show several apparently critical interactions, analyses of individual hirudin residues by site-directed mutagenesis indicate that substitution of individual residues results in unexpectedly small changes in the affinity for thrombin.60,61 Thus, although individual residues make small contributions, the sum of these numerous interactions results in the significant binding affinity of the hirudin-FRS interaction.61

In similar site-directed mutagenesis experiments, we also found only small changes in the affinity of Aa 1-50-β-galactosidase fusion proteins with single amino acid substitutions (Fig 3). Substitutions of anionic residues with glycine caused a small decrease in binding affinity for thrombin, less than would be expected if these charged groups were critical for interaction with the thrombin FRS. Single substitutions of hydrophobic residues also caused small changes. The largest decrease in binding was seen with doubly substituted proteins. Thus, our results mirror those observed with hirudin. One final point: the Aa 1-50-β-galactosidase fusion protein has an affinity for thrombin, Residue number | Inhibition constant
--- | ---
33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | (μM)

Trp-Pro-Phe-Cys-Ser-Asp-Glu-Asp-Trp | 9 ± 2
Ser | Leu | Gly | Gly | Gly | Gly | Ser | Ser | Ala | Ser | 35 ± 4

Fig 3. Inhibition constants of Aa 1-50-β-galactosidase fusion proteins for human α-thrombin (mean ± SD, n = 4) were determined as previously described.28,29 The residue numbers correspond to the Aa chain of fibrinogen with the native sequence listed in the first row.
measured as $K_r$ (6 to 9 $\mu$mol/L), that is similar to the affinity of thrombin for fibrinogen. Substitutions in the region $\alpha_\alpha$ 7-16 decreased the rate of cleavage of the fibrinopeptide, but did not affect the binding affinity for thrombin. This is clear evidence that while substitutions within the affinity of thrombin for fibrinogen. Substitutions in the region $\alpha_\alpha$ 7-16 decreased the rate of cleavage of the fibrinopeptide region alter the rate of cleavage, a region outside the fibrinopeptide region also provides a site of interaction and this site can accommodate $\alpha_\alpha$ residues 17-50 in the fusion protein.

Some evidence points to the $\beta\beta$ chain as an additional site of thrombin FRS binding. Des-$\beta\beta$ 15-42 fibrin, which lacks both fibrinopeptides and the additional segment $\beta\beta$ 15-42, has severely diminished thrombin binding. The loss of thrombin binding suggests that the segment $\beta\beta$ 15-42 binds to the thrombin FRS. The sequence $\beta\beta$ 15-42 has also been shown to be essential for endothelial cell spreading on fibrin, although these investigators reported thrombin binding to des-$\beta\beta$ 15-42 fibrin that was equal to normal fibrin. Fibrinogen New York I has a deletion of residues $\beta\beta$ 9-72, and the fibrin clot has dramatically reduced thrombin binding. The deletion of such a large segment with concomitant loss of two interchain disulfide bonds within the NDSK may alter the conformation of the other chains, so a more convincing example is provided by fibrinogen Naples I, which has the single substitution $\beta\beta$ Ala-68 $\rightarrow$ Thr. When fibrinogen Naples from a homoygous patient is clotted by thrombin, both fpA and fpB release is reduced and both after considerable lag periods. Binding of thrombin to the $\alpha$ chain is also greatly reduced, being less than 10% of normal. The observation that not only is clot-bound thrombin reduced but also the cleavage rate of both fibrinopeptides is reduced emphasizes how important FRS binding is for normal fibrinopeptide cleavage, further supporting the assumption that thrombin-fibrinogen and thrombin-fibrin interactions are the same.

One study suggests that all three chains bind to thrombin, as thrombin bound to all three chains when the chains were immobilized to agarose. However, the affinity for the isolated chains was weak and abolished at high ionic strength.

The most attractive conclusion to be drawn from this information is that the nonsubstrate binding site is composed from both the $\alpha\alpha$ and $\beta\beta$ chains or perhaps all three chains. It is well known that the three chains of fibrinogen are evolutionarily related. A number of charged residues and hydrophobic residues that are conserved among chains may all participate in the FRS binding site. The NDSK sequences that have been discussed here as possible residues important for thrombin binding are highlighted in Fig 4.

If the binding site exists on a combination of more than one chain, then the approach of investigating isolated chain segments will not be fruitful. More promising is an approach investigating substitutions within the whole molecule. Congenital dysfibrinogenemias are one source of ready-made variant fibrinogens, and as the molecular basis of these dysfibrinogenemias is expanded, it is expected that these variant fibrinogens will be a source of great information. Another avenue that holds great promise is the generation of recombinant fibrinogens with designed substitutions, an approach recently initiated in our laboratory.

COAGULANT PROPERTIES OF CLOT-BOUND THROMBIN

Active thrombin is bound by the clot during clot formation, apparently in two orientations. In one orientation, both catalytic site and FRS are occupied by the fibrin; in the other, only the FRS is occupied, so that the catalytic site is accessible to other substrates. The fibrin clot thus acts as a hemostatic mechanism to ensure that clot formation is localized and thrombin is prevented from spreading to the general circulation. The importance of this mechanism is shown by the dysfibrinogenemias Naples I and New York I, whose patients have recurrent thromboses correlated with the inability of the clot to bind thrombin.

Bound thrombin also is well positioned to activate factor XIII. In the last step of clot formation, the transglutaminase factor XIII a covalently links fibrin molecules by introducing covalent bonds between $\gamma$ chains and between the $\alpha$ chains. In fibrin, the monomers overlap in a half staggered manner so that the outer globular domains (to which factor XIII binds in circulating fibrinogen) of two monomers interact with the NDSK (which binds thrombin) of a third monomer. This formation of a thrombin-fibrin-factor XIII ternary complex greatly enhances factor XIII activation.

A fibrin clot acts as a reservoir for enzymatically active thrombin and represents a localized thrombogenic surface. This clot-bound thrombin is resistant to inactivation by circulating thrombin inhibitors. During fibrinolysis, plasmin digests the fibrin clot into soluble fibrin degradation products but does not inactivate thrombin. Thrombin is thus released into the circulation complexed to macromolecular fibrin degradation products. As anticoagulant therapy using heparin is not effective in preventing rethrombosis in patients undergoing thrombolytic therapy, and as the thrombin complexed to fibrin degradation products is not inhibited by heparin-antithrombin III, it appears that the clot-bound thrombin exposed during thrombolytic therapy may mediate rethrombosis.
inhibitor against clot-bound thrombin, and may be effective in preventing rethrombosis during thrombolytic therapy.

**MOLECULAR MECHANISM**

The clotting of fibrinogen by thrombin is unusually complex because of the multistep reaction sequence. It is not known if thrombin dissociates between steps or if a single thrombin molecule binds to one molecule of fibrinogen, cleaves the first Aα Arg-16-Gly-17 bond, then the second, then successively the two Bβ Arg-14-Gly-15 bonds, and remains bound after polymerization. As currently depicted, the symmetric molecular models predict that each fibrinogen molecule contains two equivalent sites for thrombin binding, one on each half of the molecule. This model favors thrombin dissociation between steps as it infers that thrombin binds and cleaves each half of the molecule separately. Perhaps the symmetry is misleading. In an alternative model, residues at the dimer interface may form a single domain that binds to the thrombin FRS site with the catalytic site accessible for hydrolysis of each of the four scissile bonds. After cleavage of the fibrinopeptides, thrombin remains bound by this single domain. These speculations show that although the recent high resolution structural analyses of thrombin have provided important information relevant to the molecular basis for thrombin activity and specificity, there are many questions that remain unanswered when considering the details for thrombin cleavage of fibrinogen.

**ACKNOWLEDGMENT**

We are grateful to Dr Oleg Gorkun for helpful discussions and to Dr Gilbert White for critical review of this manuscript.

**REFERENCES**


35. Soria J, Soria C, Samama M, Henschens A, Southan C: Special report on fibrinogen Metz characterized by an amino acid substitution of Aa arginine-16 to cysteine which forms an extra interchain disulfide bridge between the two Aa chains. J Biol Chem 258:9276, 1983


50. Mihalyi E: Clotting of bovine fibrinogen. Kinetic analysis of the release of fibrinopeptides by thrombin and of the calcium uptake upon clotting at high fibrinogen concentrations. Biochemistry 27:976, 1988


68. Kaczmarek E, McDonagh J: Thrombin binding to the αα, ββ- and γ-chains of fibrinogen and to their remnants contained in fragment E. J Biol Chem 263:13896, 1988


72. Bonnie CG, Hettasch J, Strickland E, Lord ST: Characterization-
tion of purified recombinant fibrinogen: Partial phosphorylation of fibrinopeptide A. Biochemistry 32:107, 1993


The fibrinogen sequences that interact with thrombin

CG Binnie and ST Lord