Three-dimensional Modeling of Thrombin-Fibrinogen Interaction*

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Three-dimensional models of thrombin complexed with large fragments of the fibrinogen $\alpha$ and $\beta$ chains are presented. The models are consistent with the results of recent mutagenesis studies of thrombin and with the information available on naturally occurring fibrinogen variants. Thrombin recognizes fibrinogen with an extended binding surface, key elements of which are Tyr$^{76}$ in exosite I, located about 20 Å away from the active site, and the aryl binding site located in close proximity to the catalytic triad. A highly conserved aromatic-Pro-aromatic triplet motif is identified in the primed site region of fibrinogen and other natural substrates of thrombin. The role of this triplet, based on the three-dimensional models, is to correctly orient the substrate for optimal bridge binding to exosite I and the active site. The three-dimensional models suggest a possible pattern of recognition by thrombin that applies generally to other natural substrates.

Thrombin plays multiple functional roles in the blood by interacting with a variety of proteins. The procoagulant role of thrombin unfolds upon interaction with fibrinogen and culminates in the formation of fibrin intermediates that polymerize into a blood clot. The kinetic steps defining this interaction and the mechanism of fibrin polymerization have been characterized in great detail (1). The determinants of fibrinogen recognition by thrombin have also been elucidated by site-directed mutagenesis (2, 3) and provide an important data base of information that complements clinical findings of naturally occurring fibrinogen variants that are associated with bleeding phenotypes.

The thrombin-fibrinogen interaction has enjoyed renewed interest in recent years after the landmark solution of the crystal structure of fibrinogen derivatives (4–7). These structures have offered clues about the molecular events underlying the end-to-end assembly of fibrin monomers generated after the thrombin-induced release of FpA$^1$ from the $\alpha$ chain, the subsequent formation of fibrin protofibrils, and the thrombin-induced release of FpB from the $\beta$ chain (4). Information about the structural epithet of thrombin recognizing fibrinogen has also been obtained. Fragments of FpA covalently bound to the active site Ser$^{195}$ of thrombin have been reported by x-ray crystallography (8, 9) and NMR spectroscopy (10). These studies have delineated the interactions made by FpA with the active site moiety of thrombin.

It has long been known, however, that regions of thrombin distant from the active site are involved in fibrinogen recognition (11). Specifically, exosite I, which is located about 20 Å away from the active site, contains residues that are critical for fibrinogen binding. Macromolecular bridge binding of fibrinogen to exosite I and the active site confers high specificity to the interaction and serves as a binding mode exploited by other natural substrates like the G-protein-coupled thrombin receptors (3). Recent Ala-scanning mutagenesis studies of thrombin have identified a hot spot in exosite I, centered near Tyr$^{76}$, which stores much of the energy responsible for the interaction with fibrinogen (2, 3). Unfortunately, the available crystal structure of the thrombin-fibrinogen complex does not provide information about the contacts made by the natural substrate with exosite I. In the case of the interaction of thrombin with the $\beta$ chain of fibrinogen that is responsible for the release of FpB and profibril polymerization, no structural information is currently available.

This study fills an important structure-function gap in our understanding of the thrombin-fibrinogen interaction. Here we report about three-dimensional models of thrombin complexed with large fragments of the $\alpha$ and $\beta$ chains of fibrinogen following a strategy recently used in the case of thrombin interaction with the G-protein-coupled receptors PAR1, PAR3, and PAR4 (3). The models are highly consistent with available data on site-directed and naturally occurring mutants of thrombin and fibrinogen.

MATERIALS AND METHODS

The N-terminal fragments of the fibrinogen $\alpha$-(20–62) and $\beta$-(31–72) chains were built, capped, and completed for missing hydrogens (pH 7.0) with Biopolymer and InsightII (Accelrys, San Diego, CA) using the human sequences P02671, P02675, and P02679 from the SwissProt sequence data base (12). First, 500 models were produced with Modeler (13) by comparative modeling of every substrate on the surface of thrombin using several available crystal structures of peptide-thrombin complexes as templates. Substrate conformation was then optimized from the best model by simulated annealing during molecular dynamic processes with Discover (Accelrys).

The fibrinogen fragment $\alpha$-(26–35) was threaded over the ACFPLAEFAQGVR peptide from the x-ray crystal structures 1BR and 1UCY (9, 14). Residue Arg$^{25}$ of the $\alpha$ chain was linked through its carbonyl oxygen to thrombin Ser$^{195}$ Oy to form a reaction intermediate analogous to the Arg of PPACK in 1PPB (15). The conformation of fragment $\alpha$- (35–49) was templated on the protein fragments KILD-KVVERI KMDENVKL from Ia0e and AFWRLVECFQKISKDSDCRA from 1DCI. These fragments were selected from our data base of 48 million conformers constructed by fragmenting the entire protein data bank into peptides from 3 to 18 residues in length. The conformers were screened for optimal shape, length, and fit to the thrombin surface. The conformation of fragment $\alpha$- (46–57) was loosely threaded over the NGDFEEIPPEYLY hirugen peptide from the structures 1HAH (16) and 1INRS (17). The fibrinogen fragment $\beta$- (41–44) was threaded over the peptide LDPR from the structure 1INRS (17). Residue Arg$^{24}$ of the $\alpha$ chain was linked through its carbonyl oxygen to thrombin Ser$^{195}$.
The peptide is mainly in extended conformation with two short loops 28–33 (loop 1) and 39–48 (loop 2).

The Ao chain fragment explores different portions of the thrombin surface (Fig. 1). Residues 20–25 interact with exosite II. Residues 26–38 fit into the active site. Residues 39–51 bind to the groove connecting the active site with exosite I, and residues 52–62 dock onto exosite I. The change in exposed surface area upon formation of the complex between thrombin and Ao-(20–62) is 3501 Å² (25% of the thrombin surface), comparable to 3304 Å² in the thrombin-hirudin interface (24).

Half of the contact area maps outside the region from the aryl binding site to the S3′ site framing the portion of substrate interacting with the active site. The relevant contacts of the Ao-(20–62)-thrombin complex are reported in Fig. 2 and are compared with those of the templates.

There is a site of phosphorylation in the human fibrinogen Ao chain at Ao-Ser227 that results in an intermediate showing higher specificity for thrombin (25). Analysis of the 31P one-dimensional NMR spectral changes caused by the synthetic phosphorylated peptide Ao-(20–41) binding to thrombin suggests that this group contributed to binding (26). Transferred nuclear Overhauser effect experiments locate the contact of this group to the vicinity of Arg175 of thrombin, in exosite II. The phosphate seems to stabilize the otherwise disordered residues Ao-(22–26) in their interaction with thrombin (26). The backbone structure of residues Ao-(22–35) of our model of the Ao-(20–62)-thrombin complex is superimposable to the bundle of NMR conformers proposed by Maurer et al. (26). Our three-dimensional model suggests the interaction of the phosphate group within the pocket formed by the basic side chains of Arg165, Lys169, and Arg172. Ao-Asp21 interacts with Lys169, and Ao-Asp26 interacts with Arg97 to form ion pairs. The naturally occurring mutant fibrinogen Lille I (27) carries the substitution Ao-D26N and shows reduced clotting activity. The mutation should lead to breakage of this ion pair.

Ao-Phe27 is framed by Trp215, Ile74, and Leu99 forming the aryl binding site (15) and accounts for a major fraction of the binding specificity of fibrinogen toward thrombin. The Ao-F27Y mutant abrogates cleavage by thrombin in model peptides (28). A three-dimensional model of thrombin complexed with the Ao chain carrying the F27Y mutation shows that the hydroxyl group of Ao-Tyr27 H-bonds to the nitrogen atom of Ao-Val34 fixing the aromatic ring of Ao-Tyr27 into a distorted position that pulls thrombin Trp215 1.4 Å higher and out of the aryl binding site (Fig. 3). This perturbation increases the solvent exposure of the hydrophobic surface of the entire aryl binding site. Similar perturbations of Trp215 have been shown to produce drastic (up to 500-fold) reductions in clotting activity (29). Furthermore, the steric hindrance introduced in the thrombin mutant L99Y skews the Ao-Phe27 side chain away from the Trp215 ring, explaining the 260-fold decrease in specificity for FpA release (3).

Ao-Val34 is in close contact with thrombin Y60a and W60d in the S2/S3 sites. Ao-Glu10 is ion-paired with Arg173, and the naturally occurring fibrinogen Mitaka II (30) shows reduced clotting activity, presumably because of the loss of this ion pair.

Ao-Arg25 is the P1 residue of fibrinogen and is engaged in the expected ion pair with Asp189 in the S1 pocket via its side chain and an H-bond interaction with the carbonyl oxygen of Ser214 through its nitrogen atom (8,9). Mutation of this Arg to His (as in fibrinogen Petoskey I (31)) or Cys (as in fibrinogen Metz I (32)) results in drastic or complete loss of clotting activity.

Residues 31GGC35 are important for positioning Ao-Phe27 and Ao-Arg25 in their respective pockets. The mutation Ao-G31V in fibrinogen Rouen (33) causes reduced clotting activity. The intervening Gly in this sequence makes a contact with the
side chain of Glu$^{217}$ through its carbonyl oxygen atom. Removal of this contact in the E217A mutant causes a significant loss of fibrinogen clotting (34). Removal of both this contact and the strong coupling with Trp$^{215}$ in the aryl binding site in the double mutant W215A/E217A generates a thrombin mutant that has lost fibrinogen clotting, but retains activity toward the anticoagulant protein C (35). The nitrogen atom of A$^\alpha$/H$^{9251}$-Gly$^{33}$ H-bonds to the carbonyl oxygen of Gly$^{216}$. Residues A$^\alpha$/H$^{9251}$-Leu$^{28}$ and A$^\alpha$/H$^{9251}$-Pro$^{37}$ frame W60d. The A$^\alpha$/H$^{9251}$-P37L substitution in fibrinogen Kyoto II (36) has no effect on clotting. The model suggests that the Leu substitution can be accommodated well in the hydrophobic environment between Leu$^{41}$ and W60d of thrombin. On the other hand, the W60dS mutant of thrombin shows a significant perturbation of fibrinogen clotting (3, 37), consistent with a drastic disruption of the favorable hydrophobic interactions with A$^\alpha$/H$^{9251}$-Leu$^{28}$ and A$^\alpha$/H$^{9251}$-Pro$^{37}$ caused by the replacement of W60d with a polar side chain.

Residue A$^\alpha$/H$^{9251}$-Arg$^{38}$ makes a solvent-accessible ion pair with Glu$^{39}$. The energetic contribution of this ion pair is modest because the E39A mutant of thrombin has clotting activity comparable to wild type (3), and the fibrinogen mutants A$^\alpha$/H$^{9251}$-R38S Detroit (38), A$^\alpha$/H$^{9251}$-R38G Aarhus (39), and A$^\alpha$/H$^{9251}$-R38N Munich (40) have normal or slightly reduced clotting activity. The A$^\alpha$ and B$^\beta$ chains have a loop segment between the anchor positioned into the S1 pocket and the region binding to exosite I. Within this loop, A$^\alpha$/H$^{9251}$-Cys$^{47}$ is exposed to solvent in the A$^\alpha$-(20–62)-thrombin complex and is available for a disulfide bridge with A$^\alpha$/H$^{9251}$-Cys$^{47}$ from the second A$^\alpha$ chain of the fibrinogen dimer. A$^\alpha$/H$^{9251}$-Asp$^{51}$ is ion-paired with Arg$^{67}$ in exosite I. The mutation R67A equally affects FpA and FpB release (3), although there is no residue in the B$^\beta$ chain available for an ion pair with

Fig. 1. Three-dimensional models of thrombin bound to fragments of the fibrinogen A$^\alpha$ (A and B) and B$^\beta$ (C and D) chains. Thrombin is displayed as its solvent-accessible surface based on the coordinates 1PPB (15). The fibrinogen fragments A$^\alpha$-(20–62) and B$^\beta$-(31–72) are rendered as solid sticks in purple. The orientation is with the thrombin active site at the center (A and C), or exosite I at the center (B and D). The thrombin surface is color coded to reflect the extent of perturbation induced by the mutation of specific residues (labeled in the top panels) on the value of the specificity constant $k_{cat}/K_m$ for the release of FpA or FpB (3). The following scale is used: red (-3.5 to -2.5 log units), orange (-2.5 to -1.5 log units), green (-1.5 to -0.5 log units), cyan (-0.5 to 0.5 log units), and blue (0.5 to 1.5 log units). Residue Lys$^{70}$ is buried under Tyr$^{76}$ in exosite I and is not visible.
Arg^67. That suggests that the loss of thrombin activity toward the Aα and Bβ chains is due to perturbation of the buried ion-pair quartet involving Arg^67, Lys^70, Glu^80, and Glu^83 which holds the 70-loop and the all important residue Tyr^76 in their correct conformation (15). Indeed, the mutants R67A, K70A, E77A, and E80A alter the kinetics of FpA and FpB release drastically and to the same extent (3), even though Lys^70 and Glu^80 do not contact the Aα or Bβ chains. Furthermore, the effect of these mutations is practically identical to that caused by the Y76A mutation (3), suggesting that the role of the buried quartet is to orient Tyr^76 on the surface of the 70-loop for optimal interaction with fibrinogen.

The sequence WPF^54 of the Aα chain frames residue Tyr^76 making a hydrophobic cluster with Leu^65 and Ile^62. Aα-Pro^63 kinks the Aα chain into exosite I and enables optimal clipping of Tyr^76 by the flanking aromatic residues.

Other contacts made by residues of the Aα chain in this region are energetically dispensable. The sequence DED^59 of the Aα chain, also present in PAR1 as SDEE^60 (3), makes ion pairs with Lys^51 and Lys^54 through Aα-Asp^77, and with R77a through Aα-Glu^86. However, the K81A, K110A, and R77aA mutations have little effect on the release of fibrinopeptides (3).

Finally, the side chains of Aα-Cys^55 and Aα-Cys^66 are accessible to solvent and available for disulfide bridging.

**Interaction with the Bβ Chain**—The peptide Bβ-(31–148) produced by cyanogen bromide hydrolysis is not as good a substrate as the entire Bβ chain (41). We templated the conformation of FSAR^44 of the Bβ chain by homology with the peptide LDPR complexed with thrombin in 1NRS (17). The Bβ chain also shows a highly conserved proline-rich region in the range of contact with thrombin. The structures of LDPR-thrombin 1NRS (17), hirugen-thrombin 1HAH (16), and PAR1 σ3R1 (18) were used to build the core FSARGHRPLDKKREEAPSLRPAPPP bound to the SH3 domain 1ABO (18) were used to build the core FSARGHRPLDKKREEAPSLRPAPPP bound to the SH3 domain 1ABO (18). The root mean square deviations between the backbones of fragments Aα and Bβ relative to hirugen are 1.44 and 0.41 Å, respectively. Contacts with thrombin residues of less than 6 Å are listed in E for Aα-(20–57), Bβ-(31–65), and the templates LDPR and hirugen. Sequences in E are aligned according to the three-dimensional models of complexes. The positions of phosphate groups and disulfide bridges are indicated.

**Fig. 2.** Bound fragments Aα-(20–57) (blue in A and B) and Bβ-(31–68) (magenta in C and D) displayed over the thrombin surface of the active site (A and C) and exosite I (B and D) and superimposed to the following peptides used as templates: DFLAEGGGVR from 1BBR (green in A), hirugen NGDFEEPEYTL from 1HAH (green in B and D), and LDPR from 1NRS (green in C). The root mean square deviations between the backbones of fragments Aα and Bβ relative to hirugen are 1.44 and 0.41 Å, respectively. Contacts with thrombin residues of less than 6 Å are listed in E for Aα-(20–57), Bβ-(31–65), and the templates LDPR and hirugen. Sequences in E are aligned according to the three-dimensional models of complexes. The positions of phosphate groups and disulfide bridges are indicated.
47–55 dock into the groove connecting the active site to exosite I, and residues 56–72 interact with exosite I. The change in exposed surface area when fragment Bβ-(31–72) binds to thrombin is 3901 Å² (28% of the thrombin surface), a value that exceeds that of the Aα-(20–62)-thrombin complex by 11%. As much as 60% of the contact surface is located outside of the subsites from the aryl binding site to S3. The relevant contacts for the Bβ-(31–72)-thrombin complex are reported in Fig. 2 and compared with those of the templates.

The terminal amine and Bβ-Gly70 amide hydrogen interact with the carboxyl oxygen atoms of Asp77. The carbonyl oxygen atoms of Bβ-Oln31 interact with the guanidinium group of Arg173. Bβ-Asp35 interacts with Lys81, Bβ-Glu77 superimposes with Aα-Glu70 and makes a water-accessible ion pair with Arg173, whereas Bβ-Glu77 forms a solvent-accessible ion pair with Arg77. As for the Aα chain, these interactions involve solvent-exposed residues and are expected to be energetically dispensable.

In the active site region, Bβ-Phe41 superimposes well with Aα-Phe77 and fits in the aryl binding site formed by Trp215, Ile274, and Leu299. Mutations of Trp215 or Leu299 have a drastic impact on the release of FpB (3), underscoring the importance of this favorable hydrophobic interaction.

The side chain of Bβ-Phe41 superimposes with Aα-Leu28 and is in close contact with Y60a and W60d in the hydrophobic cluster acting as a lid over the S1 pocket. Mutation of W60d greatly affects the release of FpB (3, 37). Bβ-Arg44 is embedded into the S1 pocket with its side chain ion-paired with Asp189 and its nitrogen atom H-bonded to the carboxyl oxygen atom of Ser214. Mutation of this Arg to Cys, as in fibrinogen Ijmuiden (42), abrogates FpB release as expected.

The Gly residue in P1’ is conserved in all Aα and Bβ chains and comes into contact with Gly193. The P1’ Gly is replaced by Cys in fibrinogen Ise (36) leading to a reduction in clotting activity. The Bβ-His66 heterocycle at P2’ is staked against W60d as are residues Phe42 in PAR1, Phe45 in PAR3, and Tyr49 in PAR4 (3). Residue Bβ-Arg44 at the P3’ position makes a solvent-exposed ion pair with Glu80, whose energetic contribution to recognition is dispensable in view of the properties of the E93A mutant (3).

Unlike the Aα chain, the Bβ chain does not possess an exposed Cys available for disulfide bridging in the sequence upstream to the fragment that makes contact with exosite I. The carbonyl oxygen atom of Bβ-Pro60 interacts weakly with the nitrogen atom of Arg57 in exosite I. As for the Aα chain, the loss of the buried quartet involving Arg97, Lys100, Glu107, and Gly108 in PAR1, Phe110 in PAR3, and Tyr113 in PAR4 (3). Residue Bβ-Arg44 at the P3’ position makes a solvent-exposed ion pair with Glu80, whose energetic contribution to recognition is dispensable in view of the properties of the E93A mutant (3).
pro-aromatic triplet is to cage Tyr76 for optimal bridge binding. 60)-thrombin model indicates that the role of this aromatic-pro chain at position 27 with the standard chymotrypsin numbering. Primed residues pointing in the same direction, and caging another aromatic-Pro residue fills systematically the aryl binding site of all sequences in the S1 family of serine proteases shows the presence of a Pro residue located 13 residues downstream from the activation cleavage site in 83% of the cases (the Pro is often located downstream of the site of cleavage in a position to contact exosite I and appears to be highly conserved among all thrombin substrates, with the exception of factor V, factor XIII, and TAFI). The role of this Pro (residue 53 in the case of the Aα chain) is to kink the substrate backbone into exosite I at the thrombin surface. Hydrophobic/aromatic residues often flank this Pro (e.g. Aα chain, PAR3, and factor XI), and the Aα-(20–60)-thrombin model indicates that the role of this aromatic-Pro-aromatic triplet is to cage Tyr76 for optimal bridge binding of exosite I and the active site.

There are 838 examples of aromatic-Pro-aromatic triplets in the protein data bank. 20% of the examples show the aromatic residues pointing in the same direction, and caging another aromatic side chain in 5% of the cases. Interestingly, a screening of all sequences in the S1 family of serine proteases shows the presence of a Pro residue located 13 residues downstream from the activation cleavage site in 83% of the cases (the Pro is at position 27 with the standard chymotrypsin numbering). The origin of this conservation is unclear, although the analogy with thrombin substrates discussed above should draw attention to a possible “universal” role of this residue in optimizing the alignment of unprimed residues of substrate for cleavage by the target protease. Site-directed mutagenesis of this conserved Pro in different systems will be necessary to test this hypothesis.

REFERENCES
