Crystal Structure of Thrombin Bound to the Uncleaved Extracellular Fragment of PAR1*

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Abundant structural information exists on how thrombin recognizes ligands at the active site or at exosites separate from the active site region, but remarkably little is known about how thrombin recognizes substrates that bridge both the active site and exosite I. The case of the protease-activated receptor PAR1 is particularly relevant in view of the plethora of biological effects associated with its activation by thrombin. Here, we present the 1.8 Å resolution structure of thrombin S195A in complex with a 30-residue long uncleaved extracellular fragment of PAR1 that documents for the first time a productive binding mode bridging the active site and exosite I. The structure reveals two unexpected features of the thrombin-PAR1 interaction. The acidic P3 residue of PAR1, Asp39, does not hinder binding to the active site and actually makes favorable interactions with Gly219 of thrombin. The tethered ligand domain shows a considerable degree of disorder even when bound to thrombin. The results fill a significant gap in our understanding of the molecular mechanisms of recognition by thrombin in ways that are relevant to other physiological substrates.

Thrombin is a trypsin-like protease endowed with important physiological functions that are mediated and regulated by interaction with numerous macromolecular substrates, receptors, and inhibitors (1). Fenton (2) was the first to recognize that thrombin selectivity toward macromolecular substrates depends on interactions with exosites that extend beyond the active site. Exosite I is positioned some 15 Å away from the active site (3) and occupies a domain analogous to the Ca2+-binding loop of trypsin and chymotrypsin (4). Recognition of macromolecular substrates or receptors responsible for the procoagulant, prothrombotic, signaling, and anticoagulant functions of thrombin depends on the structural integrity of this exosite (1). In general, exosite-dependent binding is kinetically limiting as a recognition strategy of macromolecular targets by enzymes involved in blood coagulation (5). Structural and site-directed mutagenesis data document the important role of exosite I in the interaction of thrombin with fibrinogen (6–9) and the protease-activated receptors (PARs)2 PAR1 (7, 10–13) and PAR3 (7, 14, 15). Likewise, abundant structural and functional data document how thrombin recognizes substrates at the active site (3, 16, 17), including fibrinogen (18), PAR4 (15, 19), and factor XIII (20, 21). On the other hand, structural elucidation of how substrates bridge the active site and exosite I in their binding to thrombin has been challenging.

PAR1 is a premiere prothrombotic and signaling factor (22), the most specific physiological substrate of thrombin in terms of $k_{cat}/K_m$ values (7), and a most relevant target for crystallization studies. PARs are members of the G-protein-coupled receptor superfamily and play important roles in blood coagulation, inflammation, cancer, and embryogenesis (23–28). Four PARs have been cloned, and they all share the same mechanism of activation (22, 26, 29): thrombin and other proteases derived from the circulation and inflammatory cells cleave at a specific site within the extracellular N terminus to expose a new N-terminal tethered ligand domain, which binds to and activates the cleaved receptor (30). Thrombin activates PAR1 (10), PAR3 (14, 24), and PAR4 (31–33) in this manner but has no specificity toward PAR2, which is the target of other proteases. Cleaved PAR1 also acts as a cofactor for PAR4 activation on human platelets (34).

Major progress has been made recently in our structural understanding of how thrombin recognizes the extracellular domain of PAR4 and how cleaved PAR3 acts as a cofactor for PAR4 cleavage on murine platelets (15). On the other hand, our structural information on the binding mode of PAR1 remains confined to the acidic hirugen-like domain, which recognizes exosite I (12, 13) as predicted by mutagenesis data (7, 10). In a previous structure of thrombin bound to an extracellular fragment of PAR1, the cleavage site at Arg41 was directed toward the active site of a second thrombin molecule in the crystal lattice in a nonproductive binding mode (12). No details could be gleaned on the contacts made by the P1–P4 residues (35) of PAR1 with the active site of thrombin or on the precise conformation of the tethered ligand domain bound to the enzyme. That information is presented here for the first time.

MATERIALS AND METHODS

The human thrombin mutant S195A was constructed, expressed, and purified to homogeneity as described previously (36). A soluble extracellular fragment of human PAR1, S314-977-1183; E-mail: enrico@slu.edu.

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The atomic coordinates and structure factors (code 3LU9) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://.rcsb.org/).

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2 The abbreviations used are: PAR, protease-activated receptor; w, water molecule.
PAR1 fragment, also in the same buffer, was added to maintain the molar ratio at 1:11. Initial crystal screening was done using the PEGs Suite (Qiagen, Valencia, CA) containing 0.2 M Na+/K+ tartrate. Vapor diffusion with hanging drops was used to generate crystals. For each of the 96 screen conditions, a hanging drop was prepared by mixing 1 µl of thrombin-PAR1 complex and 1 µl of reservoir solution, and the drop was allowed to equilibrate with 500 µl of crystallization buffer at 22 °C. Diffraction quality crystals were obtained in 2 weeks. The crystals were triclinic, with space group P1 and unit cell parameters a = 46.3 Å, b = 50.2 Å, and c = 85.5 Å and α = 76.4°, β = 83.9°, and γ = 73.7°, with two molecules in the asymmetric unit. Crystals were cryoprotected with the appropriate buffer and 15% glycerol prior to flash-freezing. X-ray data were collected to 1.8 Å resolution at 100 K on an ADSC Quantum-315 CCD detector at beamline 14-BM-C of the Advanced Photon Source at Argonne National Laboratory (Argonne, IL). Data processing including indexing, integration, and scaling was performed using the HKL2000 software package (37). The crystal structure of thrombin S195A bound to the extracellular fragment of PAR1 was solved by molecular replacement using the coordinates of thrombin S195A bound to the extracellular fragment of PAR1 using the HKL2000 software package (37). The crystal structure including indexing, integration, and scaling was performed from the CCP4 Suite (38), and 5% of the reflections were selected randomly as a test for cross-validation. Model building was performed with COOT (39). In the final refinement stage, TLS (translation/libration/screw) tensors modeling rigid body anisotropic temperature factors were calculated and applied to the model. Statistics for data collection and refinement are summarized in Table 1. Atomic coordinates and structure factors have been deposited in the Protein Data Bank (code 3LU9).

RESULTS

It has long been recognized that the extracellular fragment of PAR1 carries all determinants for thrombin recognition (7, 10) and that these are the site of cleavage around Arg41, 38LDPR41, contacting the active site and the acidic hrugin-like motif 31KYEFP25 engaging exosite I. It was therefore expected that PAR1 would interact with thrombin by bridging the active site and exosite I (10, 40), as first observed for the potent natural inhibitor hrudin (41). Results from structural investigation of the thrombin–PAR1 interaction have documented the expected binding mode to exosite I (12, 13) but not the way PAR1 contacts the active site. Specifically, a previous low (3.1 Å) resolution structure of thrombin bound to a 23-residue long fragment of PAR1 encompassing the cleavage site revealed an artifactual conformation of the tethered ligand downstream of Arg41 resulting into nonproductive contacts with a second thrombin molecule in the crystal lattice (12). The artifactual conformation documented for PAR1 bound to thrombin in this previous structure (12) is not surprising because thrombin was used in its active wild-type form. Productive binding of the PAR1 fragment to the active site would have resulted in cleavage and either failure to crystallize or crystallization of a complex with the cleaved peptide bound. The structure presented here was solved using thrombin inactivated with the S195A mutation and reveals a binding mode of PAR1 to the E.Na+ (fast) form of thrombin that is consistent with all functional and mutagenesis data (7, 10).

The relatively high (1.8 Å) resolution of the present structure yields good electron density for the entire complex (Fig. 1). The extracellular fragment of PAR1 is bound to a single thrombin molecule in the crystal, and the surface of recognition bridges the active site and exosite I, as expected (7, 10, 40). Interestingly, the tethered ligand connecting the domains of PAR1 interacting with the active site and exosite I is for the most part disordered. Electron density is strong for Arg41 and Ser42 at the cleavage site, vouching for an intact PAR1 peptide, but becomes weaker for sequence 48LLRNP48 immediately downstream (Fig. 2). A solution structure of the cleaved fragment of human PAR1 captured the entire tethered ligand, 42SFLLRNPNP50, in a stable S-like conformation predicted to fold away from the thrombin surface (42). Recent crystallographic data were unable to detect strong electron density for fragments 38SFNGGP33 and 42SFLLRP54, corresponding to the tethered ligands of murine PAR3 and human PAR1 (13, 15). These findings, along with the current structure of the thrombin–PAR1 complex, suggest that the tethered ligand may be “under tension” in the uncleaved form of PAR1, ready to snap back onto the receptor surface and away from the enzyme as soon as cleavage at Arg41 takes place.

Intramolecular interactions stabilize the architecture of the extracellular fragment of PAR1 bound to thrombin. In the N-terminal portion upstream of the cleavage site, OH of Thr44

| TABLE 1 Crystallographic data for the thrombin-PAR1 complex |
|-------------------------------------------------------------|
| **Buffer/salt** | 0.2 M potassium/sodium tartrate |
| **PEG** | 20%, 3350 |
| **PDB code** | 3LU9 |

| Data collection | |
|-----------------|------------------------------------------------|
| **Beamline** | APS 14-BM-C |
| **Wavelength (Å)** | 0.9 |
| **Space group** | P1 |
| **Unit cell dimensions** | a = 46.3 Å, b = 50.2 Å, c = 85.5 Å; α = 76.4°, β = 83.9°, γ = 73.7° |
| **Molecules/asymmetric unit** | 2 |
| **Resolution range (Å)** | 40–1.8 |
| **Observations** | 196,399 |
| **Unique observations** | 63,011 |
| **Completeness (%)** | 96.0 (93.4) |
| **Rmerge (%)** | 5.8 (28.0) |
| **I/σ(I)** | 17.6 (3.8) |

| Refinement | |
|-------------|------------------------------------------------|
| **Resolution (Å)** | 40–1.8 |
| **| |
| **| |
| **Reflections (working/test)** | 57,541/3162 |
| **Protein atoms** | 5242 |
| **Solvent molecules** | 698 |
| **Na+/glycerol** | 2/1 |
| **r.m.s.d. bond length (Å)** | 0.031 |
| **r.m.s.d. angles (°)** | 1.5 |
| **r.m.s.d. Δφ (°; mm/ms/ss)** | 3.182/0.9/3.40 |
| **(B) protein (Å)** | 35.7 |
| **(B) solvent (Å)** | 24.4 |
| **(B) Na+/glycerol** | 26.9/35.3 |

| Ramachandran plot | |
|-------------------|------------------------------------------------|
| **Most favored (%)** | 98.8 |
| **Generously allowed (%)** | 0.7 |
| **Disallowed (%)** | 0.5 |

* r.m.s.d. from ideal bond lengths and angles and r.m.s.d. in B-factors of bonded atoms.
H-bonds to the backbone N of Ala\textsuperscript{36}. The water molecule at position 166 (w166) mediates an intramolecular interaction for the backbone Os of Ala\textsuperscript{36} and Leu\textsuperscript{48} with O\textsubscript{61} of Asp\textsuperscript{39}. A second water molecule, w167, links the backbone O of Thr\textsuperscript{37} to the backbone N and OH of Thr\textsuperscript{37}. The backbone O of Asn\textsuperscript{35} engages the backbone N of Leu\textsuperscript{38}. These intramolecular interactions result in a single 3\textsuperscript{10}-helix turn reminiscent of the architecture seen in fragments of fibrinogen and factor XIII bound to thrombin (18, 20). The C-terminal portion of the cleavage site presents the tethered ligand within 4 Å from the thrombin surface. Pro\textsuperscript{49} constrains Asn\textsuperscript{49} and Asp\textsuperscript{50} to fold toward the thrombin surface. An H-bond between the backbone O of Arg\textsuperscript{46} and the backbone Ns of Asn\textsuperscript{49} and Asp\textsuperscript{50} stabilizes the architecture of PAR1 in this region, along with H-bonds between OH of Tyr\textsuperscript{52} and the backbone O of Leu\textsuperscript{45} and between the backbone O of Asp\textsuperscript{50} and the side chain of Arg\textsuperscript{46}. The H-bonding network likely provides a spring-loaded mechanism to trigger the conformational transition of the tethered ligand following cleavage at Arg\textsuperscript{41} required to engage the receptor for activation and signaling.

The extracellular fragment of PAR1 engages the active site and exosite I of thrombin with a number of polar and hydrophobic interactions (Fig. 1) that are summarized in Table 2. The N terminus of the peptide is solvent-exposed. The backbone N of Thr\textsuperscript{34} interacts with the backbone O of Arg\textsuperscript{173}, and the side chain of Thr\textsuperscript{37} contacts Glu\textsuperscript{217}. The aryl-binding site of thrombin (3) formed by Trp\textsuperscript{215}, Ile\textsuperscript{174}, and Leu\textsuperscript{99} cages Leu\textsuperscript{38} at the P4 position of PAR1 (Fig. 3). The backbone N and O of the P3 residue Asp\textsuperscript{39} engage the backbone O and N of Gly\textsuperscript{216} in the canonical antiparallel β-strand conformation expected for a bound substrate (43–45). The side chain of Asp\textsuperscript{39} points away from the thrombin surface, but O\textsubscript{62} forms an H-bond with the backbone N of Gly\textsuperscript{219} (Fig. 3). w271 strategically bridges the backbone O of Thr\textsuperscript{37}, O\textsubscript{62} of Asp\textsuperscript{39}, and the backbone N of Gly\textsuperscript{216} and acts in concert with w166 to stabilize the side chain of Asp\textsuperscript{39}. The P2 residue of PAR1, Pro\textsuperscript{40}, fits snugly into a hydrophobic pocket formed by the benzyl ring of Tyr\textsuperscript{60}, the indole of Trp\textsuperscript{60d}, the imidazole of catalytic His\textsuperscript{57}, and the side chain of Leu\textsuperscript{99} (Fig. 3). These interactions are analogous to those observed for Pro residues at the P2 positions of PAR4 (15), factor XIII (20), and \(H\textsuperscript{-}D\textsuperscript{-}Phe\textsuperscript{-}Pro\text{-}Arg\text{-}CH\textsubscript{2}Cl\) (3, 17). The P1 residue of PAR1, Arg\textsuperscript{41}, is involved in extensive H-bonding interactions that are observed in practically all trypsin-like proteases bound to substrate (43–45). Two water molecules contribute to the network. w40 links NH1 of Arg\textsuperscript{41} to Phe\textsuperscript{227}, and w59 connects the backbone O of Asp\textsuperscript{39} and Ne and NH2 of Arg\textsuperscript{41} to O\textsubscript{2} of Glu\textsuperscript{192} and the backbone O of Gly\textsuperscript{219}. The guanidinium group of Arg\textsuperscript{41} engages the side chain of Asp\textsuperscript{189} in a strong bidentate H-bonding interaction and also the backbone Os of Ala\textsuperscript{190} and Gly\textsuperscript{219}. The backbone N of Arg\textsuperscript{41} inter-

![Image of X-ray crystal structure of thrombin in complex with the uncleaved extracellular fragment of PAR1.](image)

**FIGURE 1.** X-ray crystal structure of thrombin in complex with the uncleaved extracellular fragment of PAR1. Thrombin is shown in the standard Bode orientation (3), with the active site in the center and exosite I on the right. Thrombin is rendered in surface representation (wheat), and PAR1 is rendered in stick representation (yellow). The 2Fo – Fc electron density map (light green mesh) contoured at 0.9σ. Residues of thrombin interacting with PAR1 through molecular contacts within 4 Å are colored in orange (hydrophobic contacts) and marine (polar contacts). The uncleaved extracellular fragment of PAR1 engages both the active site and exosite I in productive binding modes.

**FIGURE 2.** Details of the cleavage site and tethered ligand of PAR1. Fragment \(\text{w}40\text{PRSFLL}\text{w}45\), spanning the P2–P4 positions, is shown with its 2Fo – Fc electron density map (light green mesh) contoured at 0.9σ. The peptide bond between Arg\textsuperscript{41} and Ser\textsuperscript{42} can be assigned with confidence, vouching for an intact PAR1 fragment. Downstream of Phe\textsuperscript{43}, the electron density becomes weaker, underscoring disorder in most of the tethered ligand.

### Table 2: H-bonding interactions between PAR1 and thrombin

| PAR1 | Thrombin | Distance | Molecule 1 | Molecule 2 |
|------|----------|----------|------------|------------|
| Thr\textsuperscript{35} N | Arg\textsuperscript{173} O | 3.5 | 4.7 |
| Thr\textsuperscript{37} Oy1 | Gly\textsuperscript{193} O2 | 2.6 | 2.6 |
| Asp\textsuperscript{39} O\textsubscript{62} | Gly\textsuperscript{219} N | 2.8 | 3 |
| Asp\textsuperscript{40} N | Gly\textsuperscript{216} O | 2.9 | 2.9 |
| Asp\textsuperscript{40} O | Gly\textsuperscript{216} N | 3.1 | 3.1 |
| Arg\textsuperscript{41} NH\textsubscript{1}, NH\textsubscript{2} | Asp\textsuperscript{189} O\textsubscript{61}, O\textsubscript{62} | 2.9, 2.9 | 2.8, 2.8 |
| Arg\textsuperscript{41} NH2 | Ala\textsuperscript{190} O | 3.4 | 3.5 |
| Arg\textsuperscript{41} NH2 | Gly\textsuperscript{193} O | 3 | 3.1 |
| Arg\textsuperscript{41} N | Ser\textsuperscript{214} C | 2.9 | 2.9 |
| Arg\textsuperscript{41} O | Ala\textsuperscript{195} N | 3 | 2.9 |
| Arg\textsuperscript{41} O | Asp\textsuperscript{194} N | 3.4 | 3.5 |
| Arg\textsuperscript{41} O | Gly\textsuperscript{193} N | 2.7 | 2.9 |
| Ser\textsuperscript{42} OH | Leu\textsuperscript{41} O | 2.3 | 2.8 |
| Arg\textsuperscript{45} Ne, NH\textsubscript{2} | Glu\textsuperscript{216} O | 3.1, 4.4 | 2.6, 3.1 |
| Asn\textsuperscript{36} N\textsubscript{62} | Lys\textsuperscript{149\textepsilon} O | 3.2 | NA |
| Asn\textsuperscript{36} O\textsubscript{61} | Lys\textsuperscript{149\textepsilon} Ne | 2.9 | NA |
| Tyr\textsuperscript{52} OH | Arg\textsuperscript{28} NH\textsubscript{1} | 3.2 | 2.5 |
| Tyr\textsuperscript{52} O | Glu\textsuperscript{216} Ne2 | 3.0 | 7.2 |
| Glu\textsuperscript{39} O\textsubscript{1e}, O\textsubscript{2e} | Arg\textsuperscript{53} NH\textsubscript{1} | 3.3, 3.2 | 7.8, 7.1 |
| Glu\textsuperscript{39} O1e | Tyr\textsuperscript{70} N | 3.1 | 3.2 |
| Glu\textsuperscript{39} N | Thr\textsuperscript{45} O | 2.9 | 3.0 |
| Phe\textsuperscript{43} benzene ring | Arg\textsuperscript{47} NH\textsubscript{1}, NH\textsubscript{2}, Ne | Cation–π | Cation–π |

NA, not applicable.
acts with the backbone O of Ser²¹⁴, whereas the backbone O of Arg⁴¹ occupies the oxyanion hole by engaging the backbone Ns of the mutated Ala¹⁹⁵ and Gly¹⁹³.

Downstream of the scissile bond, Leu⁴¹ of thrombin engages the side chain of Ser⁴² at the P¹ position of PAR1. Lys⁶⁰⁸ in the 60-loop of the enzyme bridges the backbone O and OH of Ser⁴² via w306 and w573. At the P² position, the benzylic ring of Phe⁵³ is in van der Waals interaction with Cβ and C of Gly¹⁹². Leu⁴⁴ at P³ stacks against Leu⁴¹. Ne of Arg⁴⁶ at P⁵ interacts with the backbone O of Gln³⁸ in the 30-loop of thrombin, and Asn¹⁷ at P⁶‘ makes a polar contact with Lys¹⁴⁹ε in the autolysis loop of the enzyme. Pro⁴⁸ induces a β-turn in the extracellular fragment of PAR1 that exposes the entire segment ⁴⁸-PNDK⁶⁰ to solvent. The rest of the interactions are identical to those documented in previous structures (12, 13). The OH and backbone O of Tyr⁵² interact with NH1 of Arg⁷³ and Ne2 of Gln⁹⁶, respectively. The side chain of Glu⁵³ is engaged by the side chain of Arg⁷⁵ and the backbone N of Tyr⁷⁶ in exosite I. The backbone N of Glu⁵³ interacts with the backbone O of Thr⁷⁴. w217 links the backbone N and O of Glu⁵³ with NH1 and NH2 of Arg⁶⁷ and the backbone O of Thr⁷⁴. Finally, Phe⁶⁵ is involved in a weakcation–π interaction with the side chain of Arg⁶⁷. Trp⁶⁶ and Gln⁶⁷ are solvent-exposed, and no electron density is detected for the rest of PAR1 fragment ⁵⁸-DEEKN⁶², in agreement with a previous structure (13) and mutagenesis data (10).

**DISCUSSION**

Information on how thrombin recognizes substrate at the active site has come from the structure bound to the irreversible active site inhibitor H-d-Phe-Pro-Arg-CH₂Cl (3). Arg at P¹ ion pairs to Asp¹⁸⁹ in the primary specificity pocket; Pro at P² fits snugly against Pro⁶⁰⁸, Pro⁶⁰⁷, and Trp⁶⁰³ in the 60-loop; and Phe at P₃, in the d-enantiomer, makes an edge-to-face interaction with Trp²¹⁵ in the aryl-binding site. This interaction, although energetically very strong (46, 47), is present because of the d-enantiomeric form of the P₃ residue. A residue at this position in the natural l-enantiomeric form would be directed to the solvent and away from the aryl-binding site. Indeed, the catalytic activity of thrombin depends little on the nature of the P₃ residue (48). Nonetheless, the H-d-Phe-Pro-Arg-CH₂Cl-inhibited structure reveals interactions that are relevant to recognition of natural substrates like fibrinogen (18), PAR4 (15, 19), and factor XIII (20, 21). The structure of thrombin in complex with the potent natural inhibitor hirudin has revealed how thrombin recognizes ligands that bridge the active site and exosite I (41). Hirudin blocks access to the active site of thrombin using its compact N-terminal domain and binds to exosite I via its extended acidic C-terminal domain. The mode of interaction of the C-terminal domain of hirudin was later documented in the structures of thrombin bound to hirugen (49), fibrinogen (8, 9, 50), PAR1 (12, 13), PAR3 (15), and thrombomodulin (51). The structure presented here in the complex with the extracellular fragment of PAR1 expands our knowledge of how thrombin recognizes macromolecular substrates at both the active site and exosite I.

The P₁–P₄ sequence ³⁸-LDPR⁴¹ of PAR1 is similar to the P₁–P₄ sequence ¹⁶⁶-VDP1⁶⁹ of the anticoagulant substrate protein C. Because no structure of the thrombin-protein C complex is currently available, the conformation of Asp³⁹ of PAR1 becomes relevant to recognition of protein C. It was originally believed that the presence of an acidic residue at the P₃ position of protein C would account for the poor specificity of thrombin toward this anticoagulant substrate in the absence of the cofactor thrombomodulin (52). It was argued that Asp at this position would clash with the side chain of Glu¹⁹² lining the thrombin active site (3). Indeed, thrombin mutants E192Q and E192A show slightly enhanced activity toward protein C (52, 53), and the protein C mutant in which the P₃ residue Asp¹⁶⁷ is mutated to Phe is activated more rapidly by thrombin (54). These observations support the idea that Asp at the P₃ position of protein C hinders binding to the enzyme by electrostatically clashing with the side chain of Glu¹⁹². Whether thrombomodulin moves Glu¹⁹² of thrombin or Asp¹⁶⁷ of protein C to produce a 1000-fold enhancement of protein C activation has long been the subject of debate (51–53, 55–60) and will likely remain so until a structure of thrombin bound to protein C is solved in the presence and absence of thrombomodulin. PAR1 carries an Asp residue at the P₃ position but, unlike protein C, is the physiological substrate cleaved by thrombin with the highest $k_{cat}/K_m$ value (7). Therefore, the presence of Asp³⁹ in PAR1 is not expected to provide much hindrance to binding to the active site of thrombin. Indeed, mutation of Asp³⁹ to Ala compromises PAR1 cleavage by thrombin to an extent comparable with that seen with the L38A and P40A mutations (61), supporting the conclusion that the side chain of Asp³⁹ interacts with the thrombin active site in a way that is energetically significant. Furthermore, the thrombin mutant E192Q has normal activity toward PAR1 (7).

The structure presented here shows that the side chain of Asp³⁹ makes direct and water-mediated H-bonds with the
backbone N of Gly219 of thrombin. This is an important observation for two reasons. First, it demonstrates that an acidic residue at the P3 position of substrate can be accommodated favorably within the active site of the enzyme, thereby explaining the very high specificity of thrombin toward PAR1. Second, it draws renewed attention to the way thrombin recognizes protein C and proves that Asp167 at the P3 position of protein C cannot contact thrombin as seen for Asp39 of PAR1. Mutagenesis data have shown that the recognition surface between thrombin and protein C changes upon thrombomodulin binding (53) and is reduced to only a few residues within the active site in the presence of cofactor. It is quite likely then that protein C approaches the active site of thrombin in a conformation that does not resemble the extended architecture seen for PAR1. The structure of thrombin bound to PAR4 has revealed how substrate can fold in a way to redirect itself away from exosite I to enable cofactor binding to that domain (15). That strategy may be used by protein C to leave exosite I free for thrombomodulin binding.

Gly219 assumes a potentially important role as a protein engineering target to construct thrombin variants with selective specificity toward macromolecular substrates. The interaction between O62 of Asp39 and the backbone N of Gly219 is unique to PAR1. In murine PAR4, the P3 residue is Asn instead of Asp, the O2 atom is N instead of O, and the side chain rotates by 39° to point away from the backbone N of Gly219 (15). Removing the H-bonding interaction between O62 of Asp39 and the backbone N of Gly219 with the G219P mutation, as done recently for the N143P mutant of thrombin (62), would be of significant interest. The G219P mutant may feature selective loss of PAR1 recognition and could afford a unique reagent void of prothrombotic and signaling functions.

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