Thrombin Functions through Its RGD Sequence in a Non-canonical Conformation*

Received for publication, June 10, 2005, and in revised form, June 30, 2005
Published, JBC Papers in Press, July 5, 2005
DOI 10.1074/jbc.C500248200

Matthew E. Papaconstantinou‡§,
Christopher J. Carrell‡, Agustin O. Pineda‡,
Kevin M. Bobofchak‡, F. Scott Mathews‡,
Christodoulos S. Flordellis¶,
Michael E. Maragoudakis§,
Nikos E. Tsopanoglou§, and Enrico Di Cera‡‡

From the 2Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine,
St. Louis, Missouri 63110 and the 3Department of Pharmacology, Medical School, University of
Patras, 26500 Patras, Greece

Previous studies have suggested that thrombin interacts with integrins in endothelial cells through its RGD (Arg-187, Gly-188, Asp-189) sequence. All existing crystal structures of thrombin show that most of this sequence is buried under the 220-loop and therefore interaction via RGD implies either partial unfolding of the enzyme or its proteolytic digestion. Here, we demonstrate that surface-absorbed thrombin promotes attachment and migration of endothelial cells through interaction with \( \alpha_\beta_3 \) and \( \alpha_\beta_1 \) integrins. Using site-directed mutants of thrombin we prove that this effect is mediated by the RGD sequence and does not require catalytic activity. The effect is abrogated when residues of the RGD sequence are mutated to Ala and is not observed with proteases like trypsin and tissue-type plasminogen activator, unless the RGD sequence is introduced at position 187–189. The potent inhibitor hirudin does not abrogate the effect, suggesting that thrombin functions through its RGD sequence in a non-canonical conformation. A 1.9-Å resolution crystal structure of free thrombin grown in the presence of high salt (400 mM KCl) shows two molecules in the asymmetric unit, one of which assumes an unprecedented conformation with the RGD sequence exposed to the solvent.

* This work was supported in part by National Institutes of Health Research Grants HL49413, HL58141, and HL73813 (to E. D. C.) and European Social Fund, Operational Program for Educational and Vocational Training II (EPEAEK II), Program Pythagoras II (to N. E. T.).

The atomic coordinates and structure factors (code 2A0Q) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

To whom correspondence may be addressed. Tel.: 30-2610-996171; Fax: 30-2610-994720; E-mail: ntsopan@med.upatras.gr.

To whom correspondence may be addressed. Tel.: 314-362-4185; Fax: 314-747-5354; E-mail: enrico@wustl.edu.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The possible functional role of the RGD sequence in thrombin is called into question by the crystal structure where Arg-187 is exposed to the solvent, but Gly-188 and Asp-189 are almost completely buried under the 220-loop (14). As a possible solution to this conundrum, Bar-Shavit et al. (7, 8) have suggested that thrombin functions through its RGD sequence only after denaturation or proteolytic cleavage, either by thrombin itself (7) or plasmin (8). However, recent studies have shown that surface-absorbed thrombin, or its active site inhibited form, promote attachment, migration, and survival of endothelial cells via the \( \alpha_\beta_3 \) integrin (15, 16). Other studies have provided evidence that thrombin in solution can interact with \( \alpha_\beta_1 \) integrin in a way that is inhibited by RGD mimetics (17, 18). Hence, the enzyme may expose the RGD sequence independent of denaturation or proteolysis, as reported here.

MATERIALS AND METHODS

Site-directed Mutagenesis—Site-directed mutants of human thrombin, tissue-type plasminogen activator (tPA), and rat trypsin were expressed, purified, and tested for activity as described (19–22). The thrombin mutant G188A (chymotrypsinogen numbering) was prepared in this study, and its k\(_{cat}/K_m\) values toward physiologic and synthetic substrates were found to be compromised up to 100-fold relative to wild type. All other thrombin mutants were characterized previously (11, 13). The trypsin mutant trypsin-RGD was made by replacing the 184a–188 sequence FLEGGK with the thrombin 184a–188 sequence YKPDEGKRG. The tPA mutant tPA-RGD was made by replacing the 184a–188 sequence DTRSGGPQANLH with the thrombin 186a–188 sequence DEGKR.

Cell Attachment Assay—Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Applications, Inc. (San Diego, CA).
CA. Forty-eight-well plates were coated overnight with 1 μg/well wild-type or mutant thrombins, tPA, or trypsins. Coating was confirmed and quantified by protein determination (Bradford assay) after solubilization in NaOH. In control experiments with mutants defective for attachment, the amount of protein was increased up to 30 μg/well. Bovine serum albumin (BSA) was used as a reference. The wells were then blocked for 60 min with 3% BSA in phosphate-buffered saline at 37 °C. HUVECs were detached with 0.526 mM EDTA/phosphate-buffered saline, suspended in serum-free medium containing 0.3% BSA (SFM-0.3% BSA) and incubated in the presence or absence of the GRGDSP peptide (Bachem, King of Prussia, PA) or mouse monoclonal antibody against human αβ3 integrin (clone LM609; Chemicon International Inc., Temecula, CA). The GRGDSP peptide is used for the affinity purification of fibronectin receptor (αβ3 integrin), as it contains the RGD integrin recognition site of the fibronectin cell binding domain. It is also a potent inhibitor of cell attachment to fibronectin through interaction with β3 integrin. Cell suspensions (10⁵/well) were then added to the wells, and the plates were incubated at 37 °C for 60 min. The non-adherent cells were aspirated and the number of the adhered cells was measured in triplicate wells by means of the endogenous enzyme hexosaminidase. In representative samples, endothelial cells attached on surface-absorbed proteins were fixed, stained, and photographed using an inverted microscope (Olympus IX70) equipped with photometric CoolSNAP HQ camera in ×10 objective lens magnification.

**Cell Migration**—Cell migration was studied using the modified Boyden chamber assay (Chemicon International Inc. kit: QCM™ 24-well colorimetric cell migration assay) where the upper and lower chambers are separated by 8-μm pore polycarbonate filters. In haptotactic cell motility assay, the undersurface of the membrane filter was precoated with 1 μg/well wild-type or mutant thrombins, tPA, or trypsin. In control experiments with mutants defective for migration, the amount of protein was increased up to 30 μg/well. BSA was used as a reference. To modulate the migration toward immobilized thrombin, lower chambers were filled with SFM-0.3% BSA containing the GRGDSP peptide or mouse monoclonal antibody against human αβ3 integrin (clone LM609). Endothelial cells were added to the upper compartment of the chamber at a density of 10⁵/μl in SFM-0.3% BSA and were incubated for 6 h at 37 °C allowing migration in the lower chamber. Cells on the filters that did not migrate (cells on upper surface) were removed by wiping with cotton swabs. Migrated cells were assessed according to the manufacturer's protocol. All experiments were carried out in triplicate.

**Crystalization Studies**—The R77aA mutant of thrombin, devoid of its site of autoproteolytic digestion (11), was crystallized to resolve the structure of thrombin bound to K⁺ and free of any inhibitors. The protein was concentrated to 5.6 mg/ml in 50 mM choline chloride, 20 mM Tris, pH 7.4. Crystallization was achieved at 25 °C by vapor diffusion in SFM-0.3% BSA and were incubated for 6 h at 37 °C allowing migration in the lower chamber. Cells on the filters that did not migrate (cells on upper surface) were removed by wiping with cotton swabs. Migrated cells were assessed according to the manufacturer's protocol. All experiments were carried out in triplicate.

**RESULTS AND DISCUSSION**

Recent studies have documented that surface-absorbed thrombin promotes endothelial cell attachment and migration via interaction with αβ3 integrin (15). The effect appears to be mediated by the RGD sequence, but no direct demonstration could be provided in that or any previous studies (6–8, 16). Fig. 1 documents the ability of immobilized wild-type thrombin to mediate the attachment of HUVECs. Inactivation of thrombin with the S195A mutation, which replaces the active site Ser with Ala, did not abrogate this property. This is in agreement with previous studies of thrombin inactivated at the active site with diisopropylphosphorylfluoridate (15). The ability to promote endothelial cell attachment was also retained by mutants significantly compro-
In cell attachment experiments, HUVECs were plated on wells coated with 1 of integrin antagonists was studied by addition of LM609 (10 g/ml) or GRGDSP (100 mM) with the cells to wells coated with wild-type thrombin (+Hirudin). The effect of integrin antagonists was studied by addition of LM609 (10 g/ml) or GRGDSP (100 mM) with the cells to wells coated with wild-type thrombin (+LM609, +GRGDSP) or S195A mutant (S195A +LM609, S915A +GRGDSP) thrombin. The same reagents at the same concentrations were used to coat the undersurface of membrane filters in migration experiments, and cells were added to the upper compartment of the Boyden chamber. Values obtained in the presence of BSA were used as controls in both attachment and migration experiments to calculate the fold increase shown in the plot. In migration assays, cells on the lower surface were also counted manually under the microscope in six predetermined high magnification microscopic fields. The number per field ranged from 89 ± 14 for wild-type thrombin to 14 ± 7 for mutants of the RGD sequence, with 4 ± 3 measured in the control with BSA.

to induce attachment and migration of HUVECs (Fig. 2). However, when residues 187 and 188 were replaced with Arg-Gly as found in thrombin (residue 189 is Asp in both trypsin and tPA), the mutants acquired the ability to induce attachment and migration of HUVECs as wild-type thrombin (Fig. 2). Need of the RGD sequence embedded in a proper protein scaffold is also demonstrated by the lack of activity of the thrombin peptide KRGDAC (Fig. 2), as opposed to the activity shown by the longer 23-amino acid thrombin peptide TP508 encompassing the RGD sequence (16).

Endothelial cell attachment and migration toward immobilized wild-type or S195A mutant thrombin was found to depend on $\alpha_v\beta_3$ and $\alpha_v\beta_1$ integrins. When endothelial cells were pre-treated with 10 $\mu$g/ml anti-$\alpha_v\beta_3$ antibody (LM609) or 100 $\mu$M GRGDSP peptide, which blocks the fibronectin receptor $\alpha_v\beta_1$, attachment and migration of cells were significantly inhibited (Fig. 2). The effect was observed for both wild-type thrombin and the S195A mutant.

To explore further the nature of thrombin conformation under the conditions employed in our cell attachment and migration assays, thrombin was challenged with the potent and highly selective inhibitor hirudin. Hirudin binds to thrombin in the femtomolar range (25) and is an exceptionally good probe of the conformational state of the enzyme (26) because it covers a serendipitous observation garnered from the crystal structure of the enzyme solved under experimental conditions never before explored. In an effort to understand the molecular basis of thrombin preference for Na$^+$ versus K$^+$ (28), the enzyme was crystallized free of any inhibitors in the presence of 400 mM KCl. The high concentration of K$^+$ was made necessary by the significantly weaker affinity of this cation compared with Na$^+$ (29). The two molecules in the asymmetric unit are related by a rotation of 173° (Fig. 3). Molecule-1 has K$^+$ bound to the Na$^+$ site and assumes a conformation similar to the Na$^+$-bound F form (11). The second molecule in the asymmetric unit, molecule-2, has no K$^+$ bound to the Na$^+$ site and assumes a conformation that has no counterpart in over 180 structures of thrombin currently deposited in the Protein Data Bank.

Packing of the two molecules in the asymmetric unit is quite different from that of the Na$^+$-bound F form of thrombin and includes a second K$^+$ bound at the interface. The intermolecular contacts are more extensive in the presence of KCl, with molecule-1 almost crushing molecule-2 in the contact region and squeezing the autolysis loop away from its canonical position (Fig. 3, blue). The entire segment from Asn-143 to Lys-149e comprising the autolysis loop (residues 144–149e) of molecule-1 is disordered and its electron density could not be traced, as typically found in thrombin structures (11). On the other hand, this region could be traced for molecule-2 up to Thr-147 and from Glu-151 on (Fig. 3, blue). Remarkably, the proximal segment of the autolysis loop of molecule-2 overlaps that of the Na$^+$-bound F structure of thrombin up to the Ca atom of Gly-140 and then repositions itself toward exosite I by making a sharp turn that dislodges the sequence around Glu-146 more than 20 Å away from its canonical position. The two backbone traces overlap again at the level of the backbone oxygen atom of Ser-153. One consequence of this sharp rearrangement of the autolysis loop of molecule-2 is the breaking of a key salt bridge between the side chains of Glu-146 and Arg-221a. At the other end of the interface, the entire 186-loop of molecule-2 is pulled and Pro-186 moves upward toward the 220-loop causing a total disorder in the region from Asp-221 to Gly-223 that cannot be traced in the electron density. These destabilizing interactions in the 186- and 220-loops are not seen in molecule-1, because the K$^+$ bound in the cation site...
stabilizes the architecture of the region. The massive disruption of the cation binding site in molecule-2 is completed by a collapse of the RGD sequence (in Corey-Pauling-Koltun model) in molecule-1, resulting in the exposure of the RGD sequence to the solvent (Fig. 4).

The drastic changes in molecule-2 reported here are not triggered by the R77aA mutation that resides far from the packing interface. The changes are likely the result of the crystallization conditions, namely the absence of any inhibitor and the presence of high (400 mM) KCl. Thrombin can therefore assume the non-canonical conformation where the 220-loop is disordered and away from its canonical position close to the 220-loop (11, 14). The site and the second at the interface. Note how the 218–225 region is stabilized the architecture of the region. The massive disruption of the cation binding site in molecule-2 is completed by a collapse of the β-strand from Gly-216 to Cys-220. The unprec.

The drastic changes in molecule-2 reported here are not triggered by the R77aA mutation that resides far from the packing interface. The changes are likely the result of the crystallization conditions, namely the absence of any inhibitor and the presence of high (400 mM) KCl. Thrombin can therefore assume the non-canonical conformation where the 220-loop is disordered and away from its canonical position close to the 220-loop (11, 14). The site and the second at the interface. Note how the 218–225 region is stabilized the architecture of the region. The massive disruption of the cation binding site in molecule-2 is completed by a collapse of the β-strand from Gly-216 to Cys-220. The unprec.

The drastic changes in molecule-2 reported here are not triggered by the R77aA mutation that resides far from the packing interface. The changes are likely the result of the crystallization conditions, namely the absence of any inhibitor and the presence of high (400 mM) KCl. Thrombin can therefore assume the non-canonical conformation where the 220-loop is disordered and away from its canonical position close to the 220-loop (11, 14). The site and the second at the interface. Note how the 218–225 region is stabilized the architecture of the region. The massive disruption of the cation binding site in molecule-2 is completed by a collapse of the β-strand from Gly-216 to Cys-220. The unprec.

The drastic changes in molecule-2 reported here are not triggered by the R77aA mutation that resides far from the packing interface. The changes are likely the result of the crystallization conditions, namely the absence of any inhibitor and the presence of high (400 mM) KCl. Thrombin can therefore assume the non-canonical conformation where the 220-loop is disordered and away from its canonical position close to the 220-loop (11, 14). The site and the second at the interface. Note how the 218–225 region is stabilized the architecture of the region. The massive disruption of the cation binding site in molecule-2 is completed by a collapse of the β-strand from Gly-216 to Cys-220. The unprec.

The drastic changes in molecule-2 reported here are not triggered by the R77aA mutation that resides far from the packing interface. The changes are likely the result of the crystallization conditions, namely the absence of any inhibitor and the presence of high (400 mM) KCl. Thrombin can therefore assume the non-canonical conformation where the 220-loop is disordered and away from its canonical position close to the 220-loop (11, 14). The site and the second at the interface. Note how the 218–225 region is stabilized the architecture of the region. The massive disruption of the cation binding site in molecule-2 is completed by a collapse of the β-strand from Gly-216 to Cys-220. The unprec.

The drastic changes in molecule-2 reported here are not triggered by the R77aA mutation that resides far from the packing interface. The changes are likely the result of the crystallization conditions, namely the absence of any inhibitor and the presence of high (400 mM) KCl. Thrombin can therefore assume the non-canonical conformation where the 220-loop is disordered and away from its canonical position close to the 220-loop (11, 14). The site and the second at the interface. Note how the 218–225 region is stabilized the architecture of the region. The massive disruption of the cation binding site in molecule-2 is completed by a collapse of the β-strand from Gly-216 to Cys-220. The unprec.

The drastic changes in molecule-2 reported here are not triggered by the R77aA mutation that resides far from the packing interface. The changes are likely the result of the crystallization conditions, namely the absence of any inhibitor and the presence of high (400 mM) KCl. Thrombin can therefore assume the non-canonical conformation where the 220-loop is disordered and away from its canonical position close to the 220-loop (11, 14). The site and the second at the interface. Note how the 218–225 region is stabilized the architecture of the region. The massive disruption of the cation binding site in molecule-2 is completed by a collapse of the β-strand from Gly-216 to Cys-220. The unprec.