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Thrombomodulin binding selects the catalytically active form of thrombin

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Abstract

Human α -thrombin is a serine protease with dual functions. Thrombin acts as a procoagulant, cleaving fibrinogen to make the fibrin clot, but when bound to thrombomodulin (TM), it acts as an anticoagulant, cleaving protein C. A minimal TM fragment consisting of the 4th, 5th, and most of the 6th EGF-like domain (TM456m) has been prepared that has much improved solubility, thrombin-binding capacity, and anticoagulant activity over previous TM456 constructs. In the present work we compare backbone amide exchange of human α -thrombin in three states: apo, PPACK-bound, and TM456m-bound. Beyond causing decreased amide exchange at their binding sites, TM and PPACK both cause decreased amide exchange in regions more distant from the active site, within the γ -loop and the adjacent N-terminus of the heavy chain. The decreased amide exchange in the N-terminus of the heavy chain is consistent with the historic model of activation of serine proteases, which involves insertion of this region into the β -barrel promoting the correct conformation of the catalytic residues. Contrary to crystal structures of thrombin, HDXMS results suggest that the conformation of apo-thrombin does not yet have the N-terminus of the heavy chain properly inserted for optimal catalytic activity, and that binding of TM allosterically promotes the catalytically active conformation.

Many coagulation proteases are activated by binding to cofactors, and a growing body of evidence supports the concept that these cofactors induce allosteric changes in their cognate enzymes (1). Whereas most coagulation proteases exist only in an inactive or active state, thrombin is a dual-action protease, with its cofactor, TM, modulating between two of its active states. In the absence of TM, thrombin acts as a procoagulant, cleaving fibrinogen to fibrin. However, when in complex with TM, thrombin acts as an anticoagulant by cleaving and thereby activating protein C (2, 3). For both procoagulant and anticoagulant activity,

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The PDB structures of thrombin are all numbered according to the chymotrypsin numbering scheme however HDXMS data is sequentially numbered. Therefore, throughout the manuscript residue numbers are given in both the sequential numbering and the chymotrypsin numbering (in parentheses with subscript CT). Please note that the chymotrypsin numbering uses the convention of adding letters to insertions so the numbering doesn't necessarily correspond. For example, residues 223-238 correspond to 182-192_{CT} because this region includes 184A and 186A-D. The loops are designated in the chymotrypsin numbering as these refer to the PDB file numbering.

thrombin must first be converted from its zymogen, prothrombin, to the active protease α -thrombin. As for all serine proteases, prothrombin is converted to α -thrombin by proteolysis of an internal peptide bond, exposing a new N-terminal Ile-NH₃. Crystal structures of thrombin and many other serine proteases has shown that the newly generated N-terminus is inserted in a pocket, known as the “Ile cleft” (4), where the new N-terminal amino group forms a salt bridge with a highly conserved aspartic residue (5). This salt bridge is thought to promote the formation of a correctly assembled S1 pocket of the active site and thereby full proteolytic activity. Using accelerated molecular dynamics (aMD) simulations, we recently showed that the N-terminus of trypsin inserts from a trypsinogen-like state into this pocket and remains in the pocket once inserted, indicating that the N-terminus is more stable inside the pocket than out (6). All crystal structures of α -thrombin show the N-terminus in this inserted conformation ([rcsb.org](https://www.rcsb.org)).

TM contains six EGF-like domains; the 5th domain interacts directly with thrombin at the fibrinogen binding site, anion binding exosite 1 (ABE1). It has been shown that a TM fragment consisting of only the 5th and 6th EGF-like domains (TM56) is sufficient to bind thrombin and inhibit fibrinogen cleavage, but the additional presence of the 4th EGF-like domain of TM (TM456) is critical to induce the anticoagulant activity of thrombin (7, 8). TM456 binding to thrombin significantly increases the association rate, k_a , of a variety of active site-directed inhibitors of thrombin (9-12), and the k_a for protein C binding is 1000-fold higher for the thrombin:TM456 complex compared to thrombin alone (13). TM binding dramatically improves the k_{cat} for protein C activation while only modestly affecting the K_M (8).

The mechanism by which TM456 enhances protein C cleavage is not yet fully understood and is particularly intriguing. The TM binding site in ABE1 is distal to the active site and the essential TM4 domain makes no direct contact with thrombin whatsoever (14). The simplest explanation for the dramatically increased association rates is that TM456 allosterically alters the conformation of the loops that surround the active site. However, a comparative analysis of the X-ray crystal structures of thrombin (1PPB) (15) and the thrombin-TM456 complex (1DX5) (14) revealed no significant structural differences in the thrombin active site loops. In addition, the N-terminus of the heavy chain is inserted in the Ile cleft in both structures. TM4 also forms an extended binding surface for protein C, providing optimal alignment for insertion into the active site and subsequent cleavage (14). Critical electrostatic interactions between protein C and residues in TM4, including Glu382 in the β 4- β 5 loop, Asp398, and Glu357, as well as hydrophobic interactions with the aromatic residues Tyr358 and Phe376, were identified. The importance of these residues for the activation of protein C has been empirically validated by alanine-scanning mutagenesis (16). However, the proposed ‘docking and optimal alignment’ mechanism is insufficient to explain the 1000-fold increase in observed association rates, particularly for smaller substrates and other inhibitors, which may enter the active site of thrombin without directly interacting with the TM4 domain.

Several studies including fluorescence (17), MALDI-TOF amide hydrogen/deuterium exchange mass spectrometry (HDXMS) (18), and isothermal titration calorimetry (19) have identified changes in the thrombin active site region that occur upon binding to different

constructs of TM in the absence of protein C. These studies suggest that the presence of TM4 may affect the dynamic properties of both the active site and the loops surrounding it by dynamic allostery (20-22). A very recent NMR/aMD study on thrombin bound to the inhibitor D-Phe-Pro-Arg-chloromethylketone (PPACK) identified significant dynamic motions in the active site loops across timescales ranging from picoseconds to tens of microseconds even with inhibitor bound (23).

The previous MALDI-TOF HDXMS studies were carried out using a small fragment of TM, TM45 (residues 346-426), which could be expressed and purified in large quantities. However, lacking EGF6, this fragment did not have full thrombin-binding capabilities. In addition, only 50% of the thrombin sequence could be covered using the MALDI-TOF instrumentation. In the present work we report HDXMS experiments using a newly engineered TM construct, TM456m (residues 346-449), which has full thrombin-binding capability. In addition, the experiments were carried out using a Waters Synapt G2Si instrument, yielding nearly 90% sequence coverage of thrombin. The results provide exciting new evidence of how TM activates thrombin for protein C cleavage.

Experimental Procedures

Design and production of TM456m

TM456m was subcloned from the TM456 gene, which had been previously synthesized using *E. coli*-optimized codons (8). PCR mutagenesis was used to introduce a stop codon to terminate TM456m at G449 and to change Cys448 to Ser. Details of the expression vectors, transformation, and transformant selection of *Pichia pastoris* clones were all carried out as previously described (8). In brief, freshly purified TM456m pPic9K expression plasmid was linearized using BglII, and introduced into spheroplasts of *P. pastoris* protease resistant strain, SMD1168, to achieve multicopy insertion of the expression plasmid into the chromosomal DNA resulting in stable transformants. The multicopy transformants were selected by replica plating on G418, a kanamycin analog that penetrates *P. pastoris*. The SMD1168 transformants that produced the highest levels of TM expression were stored as glycerol freezes at -80°C .

Preparation of TM456m

Fermentations of *P. pastoris* strains containing multiple copies of the TM expression vectors were carried out in a Bioflow 3000 fermentor (New Brunswick Scientific, N.J.) equipped with a 3L bioreactor. Both TM constructs (TM45 and TM456m) were expressed and purified from *P. pastoris* fermentations, as described previously (8). The TM proteins are secreted into the media, and initially captured by anion-exchange chromatography using a QAE Sephadex A-50 column. The protein is then further purified on a HiLoad 26/10 Q Sepharose High Performance column followed by reverse-phase C-18 HPLC using an acetonitrile gradient from 10 – 50% in 20mM NH_4OAc pH 5.65. The protein was lyophilized before being reconstituted in MilliQ H_2O and purified by Superdex 75 size-exclusion chromatography in 50 mM Tris 150 mM NaCl pH 7.4. Protein C activation assays (described below) were used at each step in the purification strategy to determine the highest activity fractions to be taken to the next step.

Preparation of Thrombin

All human α -thrombin was expressed and refolded from *E. coli* inclusion bodies, as previously described (23). Briefly, after purification of the refolded “pre-thrombin-2” by ion exchange chromatography, activation to α -thrombin was achieved by incubation of the “prethrombin-2” diluted to 50 mL with 50 mM Tris/HCl pH 7.4, 1 mL 1 M CaCl_2 , 1 mg/ml PEG8000, 5% (v/v) glycerol with 250 mg AEBSF pretreated *E. carinatus* venom (Sigma-Aldrich Cat. # V8250) at room temperature. Optimal activation times varied from 2-10 hr, as predetermined by a time dependent thrombin activation test. At the end of the activation period, the protein solution was diluted 1:1 with 50 mM Tris/HCl pH 7.4, 1 mg/ml PEG 8000 and loaded onto a MonoS cation exchange column (GE Healthcare Life Sciences). Separation of active α -thrombin from preactive (prethrombin-2, meizothrombin) and auto-proteolyzed forms (particularly γ -thrombin) is efficiently achieved using MonoS chromatography with a gradient of 100 mM to 500 mM NaCl in 25 mM phosphate pH 6.5. Fractions containing only active, α -thrombin with specific activity >3500 U/mg were pooled and stored at -80°C for no longer than 1 month before use. This method of thrombin purification, which avoids dialysis, has been shown previously to result in $>95\%$ active α -thrombin (24). In addition, previous NMR analyses of isotopically labeled thrombin prepared in the same way, demonstrated that only a single species, active α -thrombin was present (23). PPACK-thrombin was prepared by incubating α -thrombin with a 10X-molar excess of PPACK (Haematologic Technologies, Inc.) for 30 minutes at room temperature. The protein was then purified by Superdex 75 size-exclusion chromatography in 25 mM phosphate 100 mM NaCl pH 6.5. Aliquots of PPACK-thrombin were stored at -80°C for no longer than 1 month before use.

Protein C Activation Assays

Protein C activation assays were performed as previously described (8). Briefly, a discontinuous assay for TM activation of thrombin towards protein C cleavage was performed by first incubating TM with human α -thrombin (Hematologic Technologies, Essex Junction, VT) before adding human protein C (Hematologic Technologies, Essex Junction, VT). Following a 20 min incubation with protein C, the thrombin was inactivated by addition of heparin/antithrombin-III, and the activated protein C was assayed by addition of the aPC-specific chromogenic substrate, S-2366 (Diapharma, West Chester, OH). The cleavage of the chromogenic substrate results in a linear increase in absorbance at 405 nm over time, which is proportional to the amount of aPC. TM is an essential activator of thrombin. Classical kinetic descriptions of such systems include the Michaelis constant for the essential activator (TM), $K_{M, TM}$, a second Michaelis constant for the substrate (PC), $K_{M, PC}$, and the catalytic constant, k_{cat} (25). These kinetic constants (k_{cat} , $K_{M, TM}$, and $K_{M, PC}$) were determined for each TM construct as discussed previously (8). $K_{M, TM}$ was measured by varying the TM concentration at constant protein C. $K_{M, PC}$ was measured by varying the amount of protein C at constant TM concentration. The V_{max} determined from both experiments was then used to determine the k_{cat} . All protein C assays used to calculate kinetic constants were repeated in triplicate; values agreed within 15%.

Hydrogen-Deuterium Exchange Mass Spectrometry (HDXMS)

PPACK-thrombin and α -thrombin samples were prepared for HDXMS from frozen aliquots (~7.4 μ M) using pre-rinsed 0.5 mL 3K MWCO Amicon concentrators, spinning in a microcentrifuge at 14,000 g in 5 minute intervals at room temperature. The appropriate amount of each protein was concentrated to make 400 μ L with a final concentration of 10 μ M in 25 mM phosphate 100 mM NaCl pH 6.5. A portion, 50 μ L, of each protein was removed for peptide identification analysis, and the remaining 350 μ L was diluted to 5 μ M for the deuterium exchange experiments. The TM456m: α -thrombin complex was prepared for HDXMS by preparing a 40 μ M TM456m sample that was buffer-exchanged into 25 mM phosphate 100 mM NaCl pH 6.5 using a pre-rinsed 15 mL 3K MWCO Amicon concentrator. The appropriate amount of α -thrombin and TM456m were mixed and concentrated (as above) in a pre-rinsed 0.5 mL 3K MWCO Amicon to make 5 μ M α -thrombin:150 μ M TM456m in a final volume of 100 μ L. This ratio was determined using the $K_{M, TM}$ as an upper bound of the K_D and computing the percent bound using the quadratic binding equation as follows: In the HDXMS experiment, this sample is diluted 12-fold (see below) resulting in final concentrations of 420 nM α -thrombin and 12.5 μ M TM456m, which would result in a thrombin sample that is >99 % bound with TM456m.

HDXMS was performed using a Waters Synapt G2Si system with HDX technology (Waters Corporation). Deuterium exchange reactions were prepared using a LEAP HDX PAL autosampler (Leap Technologies, Carrboro, NC). D₂O buffer was prepared by lyophilizing 10mL of 25 mM phosphate 100 mM NaCl pH 6.5 and resuspending in 99.96% D₂O immediately before use. Each deuterium exchange time point (0 min, 30 sec, 1 min, 2 min, 5 min, 10 min) was measured in triplicate. For each deuteration time point, 5 μ L of protein (or complex) was mixed with 55 μ L of D₂O buffer at 25°C and allowed to incubate for 0-10 min. The deuterium exchange was quenched for 1 min at 1°C by combining 50 μ L of the deuteration reaction with 50 μ L of 3M Guanidine HCl, 0.1% Formic Acid, 250 mM TCEP pH 2.66. The quenched sample was then injected into the 50 μ L sample loop, followed by digestion on an in-line pepsin column (immobilized pepsin, Pierce, Inc.) at 15°C. The resulting peptides were captured on a BEH C18 Vanguard pre-column, separated by analytical chromatography (Acquity UPLC BEH C18, 1.7 μ M, 1.0 $\times$ 50 MM, Waters Corporation) using a 7-85% acetonitrile in 0.1% formic acid over 7.5 min, and electrosprayed into the Waters SYNAPT G2Si quadrupole time-of-flight mass spectrometer. The mass spectrometer was set to collect data in the Mobility, ESI+ mode; mass acquisition range of 200–2,000 (m/z); scan time 0.4 s. Continuous lock mass correction was accomplished with infusion of leu-enkephalin (m/z = 556.277) every 30 s (mass accuracy of 1 ppm for calibration standard). For peptide identification, the mass spectrometer was set to collect data in MS^E, ESI+ mode instead. The peptides were identified from triplicate analyses of 10 μ M α -thrombin, and data were analyzed using PLGS 2.5 (Waters Corporation). Peptides masses were identified using a minimum number of 250 ion counts for low energy peptides and 50 ion counts for their fragment ions; the peptides also had to be larger than 1500 Da. The following cut-offs were used to filter peptide sequence matches: minimum products per amino acid of 0.2, minimum score of 8, maximum MH⁺ error of 3 ppm, a retention time RSD of 5%, and the peptides had to be present in all three ID runs collected. The peptides identified in PLGS were then analyzed in DynamX 3.0 (Waters

Corporation). The relative deuterium uptake for each peptide was calculated by comparing the centroids of the mass envelopes of the deuterated samples with the undeuterated controls following previously published methods (26).

Results

Properties of TM456m

Unlike full-length TM456 (residues 345-462), TM456m (residues 346-449) is soluble, stable, and can be concentrated without aggregating. Long-term measurement of specific activity of a purified and highly concentrated (1.4 mM) sample of TM456m stored at 4°C showed that TM456m retained full activity and remained soluble over several months. Compared to the previously studied TM45 (8), TM456m includes an additional 23 residues of EGF6, terminating at G449. The measured specific activity of TM456m was 3 times higher than TM45 and the measured $K_{M,TM}$ was 5 times lower (tighter) than the $K_{M,TM}$ of TM45 (Table S1). The measured $K_{M,PC}$ and k_{cat} for TM456m were the same as for TM45 and TM456 (Table S1) (8). The measured $K_{M,TM}$ of TM456m was 30 ± 5 nM as compared to 170 ± 30 nM for TM45. Previous work from our lab has shown that the $K_{M,TM}$ of TM45 is 10-fold weaker than the K_d (as measured by SPR) so the K_d for TM456m can be estimated at 3 nM, close to what we measured for full-length TM456 (8, 24). TM456m thrombin binding affinity was substantially improved as compared to TM45 and the stability of purified TM456m was equivalent to that of TM45.

HDXMS Coverage

Stringent filtering of the peptides identified by PLGS resulted in 46 peptides that covered 87.8% of the α -thrombin sequence with a redundancy of 2.47 (Figure 1). Despite inclusion of high concentrations of TCEP in the quench solution, three of the regions missing in the coverage map, residues 64-67 (42-45_{CT}), 77-89 (55-60G_{CT}), and 223-238 (182-192_{CT}) correspond to regions in and around disulfide bonds. Another region (residues 136-138) is surrounded by a large number of peptic cleavage sites, likely resulting in peptides that were too short to be unambiguously identified.

Decreased solvent accessibility upon PPACK binding to α -thrombin

Several peptides that showed significantly decreased deuterium incorporation upon PPACK binding were classified as directly binding because they contained residues with amides within 5 Å of the PPACK molecule itself (as determined from the 1PPB crystal structure of PPACK-bound thrombin). This was a conservative determination of the amides that are physically protected by the PPACK molecule, and therefore are likely to show decreased solvent accessibility. Three peptides with decreased deuterium incorporation covered residues Asn98-Leu99_{CT}, which are within 5 Å of PPACK: residues 117-132 (residues 85-99_{CT}, MH+ 2144.140), residues 117-135 (residues 85-102_{CT}, MH+ 2530.295), and residues 118-132 (residues 86-99_{CT}, MH+ 2031.056). The deuterium uptake plot for the peptide corresponding to residues 117-132 is shown in Figure 2A, and the rest of the uptake plots are shown in SI Figure S1. Also within 5 Å of PPACK are residues Asp194-Ser195_{CT}, which were covered by one peptide with decreased deuterium incorporation: residues 239-255 (residues 193-207_{CT}, MH+ 1895.886). It should be noted that this peptide should

not be observed if the PPACK were attached to serine 195, but it is known that chloromethylketones attach sometimes to the serine but mostly to His57 (27).

In addition, significant decreases in deuterium incorporation were seen in the 170s loop upon PPACK binding. This region was covered by three peptides: residues 197-221 (residues 156-180_{CT}, MH+ 2896.550), residues 202-221 (residues 161-180_{CT}, MH+ 2343.227), and residues 202-222 (residues 161-181_{CT}, MH+ 2490.296). The associated deuterium uptake plots can be found in SI Figure S1, and a representative plot of the data for residues 202-221 is shown in Figure 2B. Residues 214-215 (Arg173_{CT}-Ile174_{CT}) are considered substrate binding residues although they are not quite within our stringent 5 Å distance from the PPACK molecule. The 170s loop may thus be expected to be influenced by PPACK binding. All residues with amides near PPACK are highlighted in yellow in Figure 3A.

One region further than 5 Å from the PPACK molecule were also observed to have decreased deuterium incorporation when PPACK was bound; residues 117-128 (residues 85-96_{CT}, MH+ 1631.869).

Decreased amide exchange in the sodium-binding loop upon PPACK binding

Residues Trp215-Glu217_{CT}, Cys220-Asp221_{CT}, and Gly226-Phe227_{CT}, all found within the Na⁺-binding loop, have amides within 5 Å of PPACK. Three peptides covering this region all showed lower deuterium incorporation in PPACK-bound thrombin: residues 256-275 (residues 208-227_{CT}, MH+ 2267.985), residues 259-275 (residues 211-227_{CT}, MH+ 1845.822), and residues 260-275 (residues 212-227_{CT}, MH+ 1788.801). The deuterium uptake plot for the peptide corresponding to residues 259-275 is shown in Figure 2C and the rest of the uptake plots are shown in SI Figure S1.

Decreased amide exchange in the N-terminus of the thrombin heavy chain upon PPACK binding

Several important regions of thrombin were not covered in the previous HDXMS studies. These included the N-terminus of the thrombin heavy chain for which a dramatic decrease in amide exchange was observed in PPACK-bound thrombin as compared to apo-thrombin. The decreased exchange was observed in two different N-terminal peptides, one of which covered residues 37-44 (residues 16-23_{CT}, MH+ 819.373) and the other covered residues 37-54 (residues 16-33_{CT}, MH+ 1961.54). The plot showing deuterium uptake in residues 37-44 in apo-thrombin versus PPACK-thrombin is shown in Figure 2D and the uptake plot for residues 37-54 is shown in SI Figure S1.

Decreased amide exchange in the γ-loop upon PPACK binding

Three peptides corresponding to the γ-loop residues were analyzed. The peptide covering residues 166-177 (residues 130-141_{CT}, MH+ 1335.717) (Figure 5A) and the peptide covering residues 181-196 (residues 145-155_{CT}, MH+ 1714.912) (Figure 5C) did not show differences between α-thrombin and PPACK-thrombin. However, the peptide corresponding to residues 167-180 (residues 131-144_{CT}, 1506.781 Da) (Figure 5B) showed lower amide exchange in PPACK-bound thrombin. This trend was also observed in the peptide

corresponding to residues 166-180 (residues 130-144_{CT}, MH+ 1619.865), shown in SI Figure S1.

Decreased solvent accessibility of the TM456m binding site on α -thrombin

Similar to what was observed in previous work with TM45 (28), TM456m binding was observed to protect amides in ABE1, the TM binding site, which includes the 30s loop and the 70s loop (14). Three peptides covered the 30s loop: residues 54-61 (residues 33-39_{CT}, MH+ 1004.552), residues 55-61 (residues 34-39_{CT}, MH+ 891.468), and residues 55-63 (residues 33-41_{CT}, MH+ 1117.636). The 70s loop was also covered by three peptides, covering residues 96-110 (residues 65-78_{CT}, MH+ 1885.063), residues 96-112 (residues 65-80_{CT}, MH+ 2127.189), and residues 97-116 (residues 66-84_{CT}, MH+ 2473.357). All peptides within ABE1 showed significantly lower deuterium incorporation when TM456m was bound; the deuterium uptake plot for the peptide corresponding to residues 54-61 is shown in Figure 4A and the deuterium uptake plot for the peptide corresponding to residues 96-112 is shown in Figure 4B. These peptides are highlighted in yellow in Figure 3B.

Differences between HDXMS results in studies of TM45 and TM456m

We previously reported slight decreases in amide exchange of the 90s loop and the C-terminal helix of thrombin upon TM45 binding (18, 28) which were attributed to allosteric changes in the thrombin active site upon TM45 binding. These decreases in amide exchange were not observed when thrombin was bound to the new, higher affinity TM456m, even though the same peptides that were observed before were also observed in the current studies. The 90s loop and the C-terminal helix form part of ABE2, which, like ABE1, is a positively charged patch on the surface of thrombin. The experiments presented here were performed at much lower protein concentrations (5 μ M) than the previous experiments (100 μ M). It is possible that the fifth EGF-like domain, which is highly negatively charged, and was fully exposed in TM45, may have been interacting non-specifically with ABE2. We suspect that the addition of most of the sixth EGF-like domain prevents this non-specific interaction.

Decreased amide exchange in the sodium-binding loop upon TM456m-binding

Deuterium uptake into the Na⁺-binding loop was lower in TM456m-bound thrombin than in apo-thrombin. Although the differences weren't as large as in the PPACK-thrombin versus apo-thrombin experiment, the difference was observed in three different peptides, all of which covered this loop. The uptake plot for the peptide covering residues 259-275 (residues 211-227_{CT}, MH+ 1845.822) is shown in Figure 4C and the uptake plot for the peptides covering residues 256-275 (residues 208-227_{CT}, MH+ 2267.985) and residues 260-275 (residues 212-227_{CT}, MH+ mass of 1788.801) are shown in SI Figure S2. It is important to note that the PPACK moiety is within 5 Å of several residues within this loop, so some of the decreased exchange observed for PPACK-thrombin is likely due to direct alterations of solvent accessibility due to PPACK occlusion of this region. This region is very far from TM456 in the crystal structure, suggesting that TM affects the Na⁺-binding loop by an allosteric mechanism.

Decreased amide exchange in the N-terminus of the thrombin heavy chain upon TM456m binding

Similar to PPACK binding, the N-terminus of the thrombin heavy chain showed significantly decreased deuterium uptake in TM456m-bound thrombin as compared to apo-thrombin. The same two N-terminal peptides in which decreased amide exchange was observed upon PPACK-binding also showed decreases in amide exchange upon TM456m-binding. The uptake plot for the peptide corresponding to residues 37-44 (residues 16-23_{CT}, MH+ 819.373) is shown in Figure 4D and the uptake plot for residues 37-54 (residues 16-33_{CT}, MH+ 1961.54) is shown in SI Figure S2. Again, whereas PPACK occupies the active site and may be expected to promote burial of the N-terminus of the heavy chain, the TM456 binding region is far from the active site. Thus, the decreased amide exchange in the N-terminus of the heavy chain must be caused allosterically.

Decreased amide exchange in the γ -loop upon TM456m-binding

TM456m binding caused similar decreases in deuterium uptake into the γ -loop as were observed for PPACK-binding. Comparative analysis of the deuterium uptake of several overlapping peptides (Figure 5D-F and SI Figure S2 for residues 166-180 (residues 130-144_{CT}, MH+ 1619.865)), showed that the same region affected by PPACK-binding was also most affected by TM456m-binding. Interestingly, the decreases in amide exchange upon TM456m-binding appeared to be sustained over much longer times as compared to the change caused by PPACK-binding (compare Figure 5B with Figure 5E).

Discussion

A newly designed TM456 analog, TM456m, which contains all of the residues of TM456 except residues P₄₅₀ through C₄₆₂ (the majority of the last loop of EGF6), was prepared. This analog has higher activity, better thrombin binding, and improved stability against aggregation compared to full-length TM456. Using this new analog, we have been able to perform amide HDXMS analysis of the effects of TM binding on thrombin at lower concentrations of the two proteins. As previously reported with TM45, TM456m causes reduced amide exchange throughout the thrombin molecule (18, 28). Direct binding of TM456m reduces exchange in the 30s and 70s loops of ABE1 in accord with the TM binding site observed in the crystal structure of the thrombin-TM456 complex (14). Although we previously reported decreased amide exchange in the 90s loop and C-terminal helix of ABE2, this was not observed with TM456m. These differences can be attributed to possible non-specific interactions of the exposed negative charges in EGF5 in the previously studied TM45 construct.

TM456m binding reduced exchange in the Na⁺-binding loop and in the 170s loop, both of which are known to contribute to substrate specificity. The Na⁺-binding loop contains Trp215, which, if mutated to Ala or Ile result in improved anticoagulant activity (29). The loop corresponding to 170s loop in factor VIIa is known to be important for allosteric regulation of the catalytic activity of this coagulation protease (30, 31).

Here we report, for the first time, that TM binding causes burial of the N-terminus of the heavy chain, a region of thrombin that was not covered in previous MALDI-TOF-based HDXMS studies (18, 28). The stabilization of the N-terminus of the heavy chain in the “Ile cleft” is observed in PPACK-bound thrombin, as well as in TM456m-bound thrombin. The first conclusion one can draw from these results is that in apo-thrombin, the N-terminus of the heavy chain is not properly positioned. This terminal segment may be entering and exiting the “Ile cleft” or it may be mostly exposed, as indicated by the surprisingly high level of deuterium incorporation observed for residues 37-44 (16-23_{CT}) of apo-thrombin (Figure 3D and 4D). In fact, no structures of thrombin, including those of apo-thrombin (PDB code 1JOU (32)) show the N-terminus exposed, probably because the protein would not crystallize in this form. The exchange kinetics also suggest that the burial of the N-terminus may be a slow event, consistent with our unsuccessful attempts to observe the N-terminus exiting the “Ile cleft” of thrombin in aMD simulations (Markwick, PRL, unpublished observations). Starting from a model of trypsin with the N-terminus artificially removed from the “Ile cleft”, we were able to observe this insertion event in long aMD simulations (6). As with thrombin, all crystallographically determined structures of trypsin have the N-terminus of the heavy chain buried (rcsb.org). It will be interesting to see whether solution analyses, perhaps by NMR, can reveal the dynamics of this critical region.

It can be expected that covalent substrate-analog binding (i.e. PPACK binding) would stabilize the N-terminus of the heavy chain in the “Ile cleft”, which is the catalytically active form of thrombin. However, it was wholly unexpected to observe such a phenomenon upon TM binding at ABE1, which is far from the active site and in no way connected to it. The HDXMS results provide an additional clue as to how TM may be altering the stability of the bound N-terminus of the heavy chain. Decreases in deuterium uptake in the γ -loop (also known as the autolysis loop) had been previously reported upon binding of various ligands to thrombin (28, 33, 34) and were also observed in our current studies upon both PPACK-binding and TM456m-binding. The improved HDXMS instrumentation used here provided data from several overlapping peptides allowing localization of the difference in uptake to three residues, Gly178-Leu180, which directly abut the N-terminus of the heavy chain (Figure 5G). The differences in deuterium uptake were only observed at measurement times under 5 min for PPACK-thrombin, but were sustained beyond 10 min for TM456m-bound thrombin. It is likely that PPACK binding organizes the active site effectively “pulling” the N-terminus of the heavy chain into its proper location. It may be that once the N-terminus is in the “Ile cleft”, the γ -loop residues Gly178-Leu180 adjacent to it are stabilized. We can hypothesize that in the case of TM binding, the opposite process occurs. We previously reported that TM binding increases correlated motions in the γ -loop as observed in aMD simulations (35). Thus, TM may stabilize the γ -loop, which, in turn may stabilize the N-terminus of the heavy chain. A conformational selection process is likely given the dynamic nature of apo-thrombin (23, 36).

The TM-induced allosteric stabilization of the N-terminus-in-conformation provides a mechanistic explanation for the dramatic increase in k_a for protein C binding and k_{cat} for protein C cleavage (8, 13). Decreases in exchange in the N-terminus of coagulation factor VIIa were also observed upon Tissue Factor (TF) binding at ABE2 (30, 31). Tissue factor

also caused decreased amide exchange throughout the factor VIIa molecule but the changes that could be correlated with the activity enhancement upon TF binding were the burial of the N-terminus of the heavy chain and stabilization of the 170s loop. It is interesting that TM binding to thrombin causes only very modest stabilization of the 170s loop whereas PPACK binding dramatically stabilizes the 170s loop (Figure 2B and SI Figures 1 and 2). Our results suggest that two separable conformationally selected stabilizations may occur for maximal coagulation protease activity; insertion of the N-terminus of the heavy chain and active site conformational selection specifically in the Na⁺-binding and 170s loops. Although many thrombin substrates do not require binding at an allosteric site, it is fascinating to surmise that fibrinogen, which binds to both ABE1 and the active site, can conformationally select the active form of thrombin since its interaction with ABE1 would stabilize the N-terminus-in-conformation and its interaction with the active site would stabilize the 170s loop. In contrast, since protein C only interacts with the active site, it requires TM to interact with ABE1 and select for the N-terminus-inconformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

TM	Thrombomodulin
EGF	Epidermal growth factor-like domain
ABE	Anion Binding Exosite
HDX	Hydrogen-deuterium exchange
MS	Mass spectrometry
PPACK	D-Phe-Pro-Arg-chloromethylketone
aMD	accelerated molecular dynamics
SI	supplementary information

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Figure 1.

(A) HDXMS coverage map for α-thrombin. Peptides are marked in grey bars underneath the amino acid sequence and sequential numbering of α-thrombin. Peptide masses are printed in white within each grey bar. The amino acid sequence is highlighted in different colors to mark important regions of α-thrombin: the light-chain is colored pink, the 30s loop is colored orange, the 60s loop is colored yellow, the 70s loop is colored blue, the 90s loop is colored red, the γ-loop is colored purple, the 170s loop is colored green, the 180s loop is colored light green, and the Na⁺-binding loop is colored brown. The chymotrypsin numbering is given for some important residues in bold over the amino acid sequence. (B) A crystal structure of PPACK-thrombin (1PPB) with loops colored, as identified in (A), and PPACK drawn in black sticks. A crystal structure of thrombin bound to TM456 (1DX5) was used to align TM456m with the PPACK-thrombin structure to indicate the TM binding site. TM456m is shown in cyan.

Figure 2.

Relative deuterium uptake plots for peptides in PPACK-thrombin in black versus unbound α -thrombin in blue. (A) The 90s loop, which directly contacts PPACK, residues 117-132 (residues 85-99_{CT}, MH+ mass of 2144.140), (B) the 170s loop, residues 202-221 (residues 161-180_{CT}, MH+ mass of 2343.277), (C) the Na⁺-binding loop, residues 259-275 (residues 211-227_{CT}, MH+ mass of 1845.822) which also directly contacts PPACK, and (D) the N-terminus of the heavy chain, residues 37-44 (residues 16-23_{CT}, MH+ mass of 819.373).

Figure 3.

Differences in deuterium exchange between (A) PPACK-thrombin versus α -thrombin and (B) TM456m-bound α -thrombin versus unbound α -thrombin displayed on the thrombin structure (1PPB). These differences in deuterium protection were calculated by comparing the deuterium uptake after 10 minute incubation with deuterium. Residues are colored with a gradient of white to red, with residues colored in red showing the highest degree of deuterium protection upon binding and residues colored in white showing no deuterium protection upon binding. Residues colored black were not covered in the experiment. In (A), PPACK is shown in blue sticks, and all residues, covered in the HDXMS experiment, which have amides within 5 Å of PPACK are colored in yellow. In (B), residues constituting the TM binding site are colored in yellow.

Figure 4.

Relative deuterium uptake plots for peptides in TM456m-bound α -thrombin in red versus unbound α -thrombin in blue. (A) The 30s loop, residues 54-61 (residues 33-39_{CT}, MH+ mass of 1004.552), (B) the 70s loop in ABE1, residues 96-112 (residues 65-80_{CT}, MH+ mass of 2127.189), (C) the Na⁺-binding site, residues 259-275 (211-227_{CT}, MH+ mass of 1845.822), and (D) the N-terminus of the heavy chain, residues 37-44 (residues 16-23_{CT}, MH+ mass of 819.373).

Figure 5.

Comparison of the deuterium uptake in three regions covering the γ -loop in α -thrombin versus PPACK-thrombin (A-C) and α -thrombin versus TM456m-thrombin (D-F): (A, D) residues 166-177 (residues 130-141_{CT}, MH+ mass of 1335.717), (B, E) residues 167-180 (residues 131-144_{CT}, MH+ mass of 1506.781), and (C, F) residues 181-196 (residues 145-155_{CT}, MH+ mass of 1714.912). (G) Structure of thrombin (1PPB) showing the interaction between the residues Gly178-Leu180 (residues 142-144_{CT}) of the γ -loop (purple spheres) with residues Ile37-Gly40 (residues 16-19_{CT}) of the N-terminus of the heavy chain (blue spheres). The same loop colorings from Figure 1B are used.