BIOORGANIC CHEMISTRY 23, 519–527 (1995)

Structure/Activity of the Region of Thrombomodulin That Binds Thrombin[1]

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Received June 21, 1995

Peptides corresponding to the thrombin-binding loop of the fifth EGF-like domain of thrombomodulin (TM) extending from C407 to E426 (CECPEGYILDDGFICTDIDE) have been synthesized and their analysis has afforded an understanding of the structure and function of this region of TM. The results have implications for the design of thrombin inhibitors and give insight into the energetic factors that drive protein–protein interactions. Previous work had shown that the peptide corresponding to C409–E426 but missing I420 binds to thrombin by an induced-fit mechanism and that the "tail" residues C-terminal to the last cysteine are critical for thrombin binding. In this study, we probe the requirements for the C409–C421 disulfide bond and for each of the tail amino acids. Thrombin binding was assayed as inhibition of fibrinogen clotting and as inhibition of the thrombin–TM interaction that results in activation of protein C. Peptides with C407–C421 disulfide bond and C409 changed to A were inhibitors of thrombin, but were weaker than peptides with the C409–C421 disulfide bond. Peptides from the des-I420 series showed an absolute requirement for I424 while the native sequence peptides did not, and the peptides from the des-I420 series that contain I424 were more potent thrombin inhibitors than the native sequence peptides. Analysis of the thrombin-bound structures suggests that deletion of I420 places I424 on the other side of the β-pleated sheet where it undergoes a favorable intramolecular hydrophobic interaction with I414 stabilizing the bound conformation.

Thrombomodulin (TM) is an endothelial cell surface glycoprotein that binds tightly to thrombin. TM has a direct anticoagulant effect by inhibiting the thrombin cleavage of fibrinogen to form a fibrin clot. TM is also the essential cofactor which promotes thrombin cleavage of protein C (*1a*, *1b*) causing inactivation of factors Va and VIIIa and thus inhibition of thrombin production. Although TM is a 70-kDa membrane-bound protein, the fragment of TM composed of only the fourth and fifth EGF-like domains has almost full TM anticoagulant activity (*2*, *3*). Experiments demonstrating that TM competes for

[1] This work was supported by NIH Grant HL47463, by the Rita Allen Foundation, and by the Searle Scholars Program.

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0045-2068/95 $12.00

thrombin binding with hirudin and fibrinogen show that TM binds to the anion-binding exosite of thrombin (*4*, *5*). The region of TM consisting of the fifth and sixth EGF-like domains inhibits fibrinogen cleavage and is a competitive inhibitor of protein C activation, but it possesses no cofactor activity (*6–8*). Within this fragment, it is the fifth domain which is the major site of thrombin binding (*2*, *9*, *10*).

Peptides corresponding to regions of the fifth EGF-like domain, especially the sequence from E408 to E426, have been shown to bind to thrombin (*2*, *8*). Alanine scanning mutagenesis has identified amino acids Y413–D417 within the C409–C421 loop and D423–E426 within the connecting region between the fifth and sixth domains as functionally important (*11*). Recent work on the peptide corresponding to the C409–E426 sequence has shown that a cyclic structure (i.e., a disulfide bond between C409 and C421) is essential for thrombin binding. Furthermore, the connecting region or "tail" amino acids must be attached to the cyclic loop (*12*). The loop–tail peptide appears to bind to thrombin by an induced-fit mechanism because it is not structured when free in solution, but adopts a tri-stranded β-sheet structure in the presence of thrombin (*13*). It is not known whether the peptide containing a disulfide bond between C407 and C421 can adopt the same thrombin-bound structure, and which amino acids within the tail are essential for thrombin binding.

MATERIALS AND METHODS

Peptide synthesis. Peptides were synthesized by standard solid-phase Fmoc chemistry on a Milligen 9050 peptide synthesizer employing the pentafluorophenyl esters of the amino acids with further activation by hydroxybenzotriazole. The peptides were cleaved from the resin using trifluoroacetic acid contianing 5% thioanisole, 2.5% ethanedithiol, and 1.25% anisole. The cleaved, deprotected peptides were isolated by ether precipitation overnight at −20°C and the precipitated peptides were collected on a sintered glass funnel and lyophilized. Typical yields were 80–90% of the theoretical yield or between 200 and 500 mg depending on the molecular weight of the peptide.

The crude cysteine-containing peptides were reduced at a concentration of 10 mg/ml in 20 mM dithiothreitol, 100 mM potassium phosphate buffer, 1 mM EDTA, pH 8.2, for 30 min at 37°C under nitrogen. The peptide solution was diluted to 0.1 mg/ml in MilliQ H_2O, the pH was adjusted to 8.1, and the peptide was air-oxidized by stirring for 2–4 days at room temperature to afford the intramolecular disulfide-bonded product. The peptides were purified using standard reverse-phase HPLC on a Waters DeltaPak 300 Å semiprep column with a 0 to 50% 0.1% TFA–CH_3CN gradient over 1 h at a flow rate of 10 ml/min. Since the oxidized peptide was in a large volume (approximately 1–2 liters), the entire solution was pumped onto the HPLC through the solvent pump at a flow rate of 10 ml/min. Typical yields of the purified peptides were approximately 30–50 mg. The identity of each peptide was confirmed by mass spectrometry, amino acid analysis, and N-terminal sequencing.

The molecular weights were within 1 mass unit of the calculated molecular weight. Ellman's Reagent was used to confirm the absence of free thiols in the oxidized peptides (*14*).

The peptides were dissolved in MilliQ H_2O at a nominal concentration of 3.6 mg/ml and stored in small portions at −20°C. The concentration of each peptide in these stock solutions was determined by amino acid analysis using a known amount of norleucine as a standard. Before each assay, portions of the peptide solution were thawed and an appropriate amount of a 10× solution of Tris-buffered saline (TBS) was added so that the final concentration of Tris–HCl and NaCl was the same as that of the TBS (20 mM Tris–HCl, 100 mM NaCl, pH 7.4) used in the assays described below. The pH of the resulting peptide solution was adjusted when necessary by adding a few microliters of 0.1 N NaOH and matching the pH of the peptide solution to that of TBS as assessed by pH paper.

Clotting assays. The direct inhibition of thrombin-induced clot formation by TM has been previously demonstrated by measuring the time it takes for thrombin to induce clot formation at different concentrations of TM in a solution containing fibrinogen (*15*). The TM peptides were used in place of TM in this assay at concentrations up to 1 mM but for ease of comparison of the data, the results are tabulated as the peptide concentration required to double the clotting time. The assays were carried out by incubating several different concentrations of each peptide with 6.6 U of human thrombin (a generous gift of Dr. John Fenton) in 200 μl Tris-buffered saline (TBS) containing 5 mM $CaCl_2$ and 1.76 mg/ml PEG 8000 for easier clot visibility. After 10 min, 200 μl of a solution of 2 mg/ml purified human fibrinogen (Calbiochem) in TBS was then added and the time for the clot to form was measured as described previously (*12*). The amount of peptide required to double the clotting time was calculated from the equation of each line, and the errors were determined from the standard error of the slope of the line generated by least-squares analysis of the data.

Assay for inhibition of protein C activation. Inhibition of protein C activation was measured by incubating various concentrations of each peptide with human thrombin (0.4 μg/ml, 0.875 U/ml, 11 nM) and rabbit TM (20 ng/ml, 0.29 nM) in TBS containing BSA (1 mg/ml) and $CaCl_2$ (5 mM). After 10 min, protein C (7.25 μg/ml, 120 nM, Hematologic Technologies) was added and the mixture was incubated for 20 min. The thrombin activity was quenched with heparin–antithrombin III (80 and 220 ng/ml, respectively) and assayed as described previously (*12*). Correlation coefficients for each line were at least 0.95 and experiments were repeated at least twice. The K_i for each peptide was determined by performing the protein C assay described above at several different TM concentrations (0.14, 0.21, and 0.29 nM) and the data were plotted as $1/v$ vs the concentration of peptide in a Dixon plot. Occasionally, the K_i was determined from the IC_{50} as described previously (*12*). The errors were determined in the same manner as for the clotting assays.

Transferred NOE measurements. Structures of the thrombin-bound peptides were determined from transferred NOE experiments in the presence of bovine thrombin as described previously (*13*). The detailed structural analyses will be published elsewhere.

TABLE 1
Sequences and Molecular Weights of the TM Peptides[a]

Peptide	Sequence	Molecular weight[b]		
		Expected	M + H$^+$	M + Na$^+$
TM52	CPEGYILDDGFIC	1442.7	1443	
TM52+1	CPEGYILDDGFICT	1543.8		1566
TM52+2	CPEGYILDDGFICTD	1658.8	1658	1681
TM52+3	CPEGYILDDGFICTDI	1772.0	1771	
TM52+4	CPEGYILDDGFICTDID	1887.1	1886	1907
TM52+5	CPEGYILDDGFICTDIDE	2016.0	2016	
TM52,4-6	CEAPEGYILDDGFICTDIDE	2214.4	2215	
TM52-1	CPEGYILDDGFC	1329.5	1330	1352
TM52-1+1	CPEGYILDDGFCT	1430.6		1453
TM52-1+2	CPEGYILDDGFCTD	1545.7	1546	1568
TM52-1+3	CPEGYILDDGFCTDI	1658.6		1681
TM52-1+4	CPEGYILDDGFCTDID	1773.9	1774	1796
TM52-1+5	CPEGYILDDGFCTDIDE	1902.8	1903	1925
TM52-1,4-6	CEAPEGYILDDGFCTDIDE	2102.2	2102	

[a] The numerical notation for the peptides is as follows: TM52 denotes the peptide derives from the last loop of the fifth EGF-like domain of TM. The "-1" denotes des-Ile-420; the "+1, +2, +3," denotes addition of the "tail" residues TDIDE C-terminal to the last cysteine of the fifth domain; the "4-6" denotes a disulfide bond between the fourth and sixth cysteine instead of between the fifth and sixth cysteine.

[b] The molecular weight is that determined by mass spectrometry. The expected molecular weight for each peptide was determined from the calculated exact mass less 2 mass units for the two hydrogen atoms that are missing due to the disulfide bond. In some cases only the protonated form or the sodium adduct was observed in the FAB mass spectrum.

RESULTS

The sequences of the peptides prepared for this study along with the mass spectral data confirming the identity of each peptide are presented in Table 1. Each peptide contained a single disulfide bond between the two cysteine residues. Peptides denoted "−1" are missing Ile-420. The experiments to determine the specificity for disulfide bond as well as the experiments to determine the role of each "tail" amino acid were carried out in the context of both the native sequence as well as the des-I420 sequence.

The disulfide bonding pattern of EGF and of the three EGF-like domains for which the disulfide bonding pattern has been determined is (1-3,2-4,5-6). This result suggests that all EGF-like domains will have the same disulfide bonding pattern; however, recent work has shown that the major product upon refolding of a synthetic fifth EGF-like domain from TM has the (1-3,2-5,4-6) disulfide bonding pattern and that this domain also binds to thrombin (*10*). In EGF-like domains, the fourth and fifth cysteines are always separated by a single amino acid, so it is possible that the C-terminal loop structure could form correctly with a disulfide bond either between