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Thermodynamics and the role of allostery in the thrombin-thrombomodulin interaction

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Thermodynamics and the Role of Allostery in the Thrombin-Thrombomodulin Interaction

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Chemistry by Muneera Aina Beach

Committee in charge:
Professor Elizabeth A. Komives, Chair
Professor Kenneth Kaushansky
Professor Andrew McCammon
Professor Susan Taylor
Professor Robert Tukey

2008
The dissertation of Muneera Aina Beach is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2008
For my husband, Daymon, who is my best friend; you are as necessary as the air I breathe, and to all of my ancestors whose shoulders I stand upon.
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<tbody>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>ABEI</td>
<td>Anion Binding Exosite 1</td>
</tr>
<tr>
<td>ABEII</td>
<td>Anion Binding Exosite 2</td>
</tr>
<tr>
<td>AUC</td>
<td>Analytical Ultra Centrifugation</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism Spectroscopy</td>
</tr>
<tr>
<td>D</td>
<td>Dalton</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EGF-like</td>
<td>Epidermal Growth Factor-like</td>
</tr>
<tr>
<td>EGRCK</td>
<td>D-Glu-Gly-Arg chloromethylketone</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
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<td>FEGRCK</td>
<td>Fluorescein- D-Glu-Gly-Arg chloromethylketone</td>
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<td>FFPRCK</td>
<td>Fluorescein-D-Phe-Pro-Arg chloromethylketone</td>
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<tr>
<td>glcNAC</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
</tr>
<tr>
<td>$k_{ex}$</td>
<td>Rate constant for exchange</td>
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<tr>
<td>$k_a$</td>
<td>Association rate constant</td>
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<tr>
<td>$K_a$</td>
<td>Association equilibrium constant</td>
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<tr>
<td>$k_d$</td>
<td>Dissociation rate constant</td>
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<tr>
<td>$K_d$</td>
<td>Dissociation equilibrium constant</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix-Assisted Laser Desorption Ionization with Time of Flight Detection</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
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<td>PPACK</td>
<td>D-Phe-Pro-Arg chloromethylketone</td>
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<tr>
<td>PSD</td>
<td>Post Source Decay</td>
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<tr>
<td>Q-TOF MS</td>
<td>Quadrupole Time-of-Flight Mass Spectrometer</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris-carboxyethylphosphine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
</tr>
<tr>
<td>Th</td>
<td>Thrombin</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
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<tr>
<td>TMEGF45</td>
<td>The fourth and fifth EGF-like domains of thrombomodulin</td>
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<td>TMEGF456mini</td>
<td>Truncated version of the fourth, fifth, and sixth EGF-like domains of thrombomodulin</td>
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<td>Surface Plasmon Resonance</td>
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in physical chemistry. I still have my “Honk if you passed P-Chem” bumper sticker. Dr. Linda Roberts gave me an opportunity to do research in her lab and served as my mentor in Sacramento. She helped me pick what graduate programs to apply to and how to fashion my essays. I am eternally grateful for all of her help. I would also like to thank the Science Educational Equity office team of Pam King and Dr. Juanita Barrera. The SEE program served as my family away from home and nurtured my confidence so that I could be successful in graduate school.

My graduate school career began by doing a rotation in Dr. Dan Donoghue’s lab. In the past years he has become my career and scientific mentor. I have learned a great deal from him over the past years about science and integrity. My graduate advisor has been Elizabeth Komives. I have loved my project and thank her for allowing me to explore many avenues that uncovered findings which will be very important to the scientific community. Simon Bergqvist taught me about isothermal titration calorimetry and Biacore. I would also like to acknowledge the entire Komives lab for their support and intellectual conversations; Carla Cervantes, Amy Davenport, Ingrid DeVries, Diego Ferreiro, Brian Fuglestad, Mike Guttman, Mela Mulvihill, Nick Treuheit, Stephanie Truhlar, and past group members Gina Betts, Carrie Hughes Croy, Johnny Croy, Julia Koepppe, Mathew Mitchell, and Helena Prieto.
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PUBLICATIONS


Beach, M.A., Treuheit, N., Bergqvist, S., Komives, E.A. Allostery between anion binding exosite I and the active site of thrombin studied by ITC and fluorescence spectroscopy. (Manuscript in preparation)

FIELDS OF STUDY

Major Field: Biochemistry
Studies in Biochemistry and Biophysics
Professor Elizabeth A. Komives
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<tr>
<td>2003 – 2006</td>
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<tr>
<td>2003</td>
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<td>2002</td>
<td>Commencement Speaker, College of Natural Sciences and Mathematics, CSU Sacramento</td>
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<td>2002</td>
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<tr>
<td>2001 – 2002</td>
<td>Sally Casanova Pre-Doctoral Scholar, California</td>
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<tr>
<td>2000 – 2002</td>
<td>McNair Scholar, California State University, Sacramento</td>
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Thrombin is a serine protease that has both procoagulant and anticoagulant functions in the blood clotting cascade. The blood contains a high concentration of prothrombin, which must be proteolytically cleaved at two sites to generate active α-thrombin. Active thrombin cleaves fibrinogen to create fibrin which polymerizes into clots. Very little α-thrombin is ever generated, and this is rapidly captured by either thrombomodulin (TM) and/or antithrombin III. Regulation of thrombin is imperative to maintaining normal hemostasis and to restrict clot extension. TM binds at anion binding exosite I (ABE I) of thrombin, which is the recognition site for fibrinogen and hirudin. TM acts as a molecular switch, changing the specificity of thrombin from procoagulant activity, defined as cleavage of fibrinogen, to anticoagulant activity,
defined as cleavage of protein C. This work investigates the allosteric influence TM has on the active site of thrombin by fluorescence spectroscopy and isothermal titration calorimetry (ITC).

Chapter II describes the expression, purification, and inhibition activity of TMEGF56 which binds ABE I of thrombin but lacks EGF4 and therefore does not have cofactor activity toward protein C. We show that we have produced large quantities (20 mg/fermentation) of protein from Pichia pastoris that has the same binding affinity as TMEGF56 made from Chinese hamster ovary cells. In Chapter III several mutations in TMEGF45 and TMEGF456 were studied that affect protein C activation but do not contact thrombin according to the crystal structure of the thrombin-TMEGF456 complex. We measure changes in fluorescence of a covalently bound active site fluorophore when thrombin is titrated with TM. TM variants with the most cofactor activity exhibit the largest affect on fluorescence of the active site fluorophores. The results show that mutations in TM that do not contact thrombin directly decrease cofactor activity and decrease the way TM alters the active site of thrombin. Chapter IV presents a study of the thermodynamics of thrombin in the presence of its cofactor, TM, by ITC. Here we measured the binding thermodynamics of a non-covalent thrombin active site ligand. When various molecules were bound at ABE I of thrombin, an active TM cofactor (TMEGF45), inactive TM cofactor (TMEGF56), or a DNA aptamer, the thermodynamic profile observed for the active site ligand changed. We suggest that TM is more than a docking site for PC. It allosterically influences the active site of thrombin, without an overall change in $\Delta G$. This enthalpy-entropy compensation may be a hallmark of dynamic allostery.
Chapter I

Introduction
A. The function of thrombin in the clotting cascade

Thrombin has both procoagulant and anticoagulant activity. Many coagulation factors, including thrombin, exist in their zymogen form. Upon clotting cascade activation, coagulation factor Xa cleaves prothrombin at two sites, producing active $\alpha$-thrombin (Figure 1.1). Thrombin converts soluble fibrinogen into fibrin by cleaving the amino-terminal ends of the fibrinogen $\alpha$A and $\beta$B chains. The resulting cleaved pieces can then polymerize and ultimately lead to the formation of a fibrin clot. Thrombin can also upregulate the clotting cascade by catalyzing activation of blood factors XI, VIII, and V (Esmon 2000). Thrombomodulin (TM) is a key molecular switch that diverts thrombin from its procoagulant activities to anticoagulant function and is one of the major mechanisms for cessation of blood clotting (Esmon 2000). The thrombin-TM complex cleaves protein C to generate activated protein C much more efficiently than thrombin alone. Activated protein C then cleaves and inactivates two limiting and essential cofactors, Va and VIIIa, required in the activation of zymogens in the cascade (Figure 1.2) (Walker, Sexton et al. 1979; Suzuki, Stenflo et al. 1983; Fulcher, Gardiner et al. 1984; Srinivasan, Hu et al. 1994). Thrombin is also regulated by inhibition via antithrombin III (Rosenberg and Lam 1979; Fenton 1986).

Uncontrolled clotting, called thrombosis, blocks the flow of blood through the circulatory system. Thrombosis is the primary cause of heart attacks, strokes, pulmonary emboli, and venous thrombosis and is therefore the leading cause of mortality in the US (National Center for Health Statistics, US department of health
Figure 1.1. Thrombin is a dual action protease in the clotting cascade. Thrombin (Factor IIa) is highlighted in pink box. The procoagulant action of thrombin in this cascade is to cleave fibrinogen and factor XIII in order to form a stable fibrin clot. Thrombin’s anticoagulant activity involves formation of the thrombin-TM complex, which leads to cleavage of PC and ultimately cleavage of cofactors VIIIa and Va. Factor X can no longer be activated, thus haulting the clotting cascade. This diagram is from Trends Cardiovasc Med 1998; 8: 340–350.
Figure 1.2. Thrombin’s role as an anticoagulant in the clotting cascade. Thrombin is bound to TM at the cell surface and this complex cleaves protein C much more efficiently (3 orders of magnitude) than thrombin alone. Activated protein C then inactivates essential cofactors V and VIII, which are required to activate factor X. Factor X can no longer cleave thrombin, thereby shutting down the clotting cascade.
and human services, CDC, data up to 2005 available in PDF at www.cdc.gov/nchs/FASTATS/death.htm). Mice models which have knocked out TM only in the endothelial cells die a short time after birth due to massive spontaneous thrombosis in veins and arteries (Isermann, Hendrickson et al. 2001). Persons who are heterozygous for protein C deficiency have severe recurrent thrombotic episodes and homozygous protein C deficiency is usually lethal early in life (Griffin, Ecatt et al. 1981). A mutation that causes Factor V not to be cleaved by protein C, called the Factor V Lieden mutation, results in protein C-resistant Factor V (Zoller, Svensson et al. 1994). Persons who are homozygous for this mutation have a significantly increased risk of thrombosis (Zoller, Svensson et al. 1994). Thus by studying the thrombin-thrombomodulin interaction we hope to gain a mechanistic understanding of the first step in the anticoagulant pathway, the interaction of thrombin with TM and their activation of protein C.

B. The Thrombin-TM interaction

TM is a multifunctional glycosylated ~78 kD transmembrane protein that resides primarily on the endothelial cell surface (Figure 1.3). The short cytoplasmic tail (residues 522-557) was recently found to activate endothelial nitric oxide synthase 3 and play a role in G protein-coupled signaling (David-Dufilho, Millanvoye-Van Brussel et al. 2005). The lectin-like domain (amino acids 1-154) is important for receptor endocytosis, maintaining the integrity of cell-cell interactions, and may have roles in tumor growth and inflammation (Conway, Pollefeyt et al. 1997; Conway, Van
An EGF-like domain consisting of six repeats (residues 223-462) followed by a

**Figure 1.3.** Schematic drawing of the domains of thrombomodulin with an expanded view of the cofactor active fragment detailing the role of each EGF-like domain.
Ser/Thr rich region (residues 463-497) constitutes the rest of the extracellular domain. EGF1-6 are thought to promote fibroblast stimulation, 3-6 are required for TAFI activation, and thrombin binds and activates protein C via epidermal growth factor like domains (EGFs) 4, 5, and 6 (Esmon and Owen 1981; Nesheim, Wang et al. 1997). The Ser/Thr-rich domain enhances protein C activation (Tsiang, Lentz et al. 1992). It has been shown that the minimal fragment that binds to thrombin but still retains cofactor activity is TMEGF 45 (White, Hunter et al. 1995). TMEGF456 binds thrombin 10-fold more tightly than TMEGF45 (White, Hunter et al. 1995).

Alanine scanning has revealed amino acids essential in the activation of protein C of both TM and thrombin (Nagashima, Lundh et al. 1993; Hall, Nagashima et al. 1999). A crystal structure of the thrombin-TM complex has helped identify contact residues in the thrombin-TM interaction (Figure 1.4) (Fuentes-Prior, Iwanaga et al. 2000). When the collective knowledge of both studies is evaluated, it becomes clear that some residues within the fourth domain of TM that do not contact thrombin are critical for cofactor activity. Residues in the fifth domain of TM are also critical, and only some of these are in contact with thrombin. Domain deletion experiments showed that the fourth domain contains the residues essential for cofactor activity, but it can not function without the fifth domain, which is required for binding. The fifth domain binds thrombin in the absence of the fourth domain, but binding affinity increases 20-fold when the fourth domain is present (Stearns, Kurosawa et al. 1989; Zushi, Gomi et al. 1989; Hayashi, Zushi et al. 1990; Tsiang, Lentz et al. 1992; White, Hunter et al. 1995). Three residues within the sixth domain were also shown to be
important, although deletion of the sixth domain only increases thrombin binding by 10-fold and there is no difference in the $k_{cat}$ for protein C activation between

**Figure 1.4.** A) Schematic drawing of TMEGF45 showing the residues identified as important for the cofactor activity of TM. Residues are colored according to location: fourth domain in green, linker region in purple, Met388 in cyan, and fifth domain in red. B) Crystal structure of the thrombin-TMEGF456 complex. The important TM residues are shown as sticks and colored as in A. ABE1 of thrombin is blue and shown in sticks. The active site catalytic triad of thrombin is shown as green sticks.
TMEGF45 and TMEGF456 (White, Hunter et al. 1995). Structural studies performed by Wood et al and Prieto et al. have indicated that Phe376 is important for the structure of TM. It shows nuclear Overhauser effects (NOEs) with M388 and this may be how the fourth domain communicates with the fifth domain to elicit anticoagulant cofactor activity (Wood, Becvar et al. 2003; Prieto, Sampoli Benitez et al. 2005; Wood, Helena Prieto et al. 2005). Backbone dynamics experiments have also shown that the C-loop of EGF5 has higher hNOE values in TMEGF45 than in EGF5 alone, indicating that presence of the fourth domain orders key thrombin binding residues and thus may be responsible for the modulation of dynamics in the fifth domain (Prieto, Sampoli Benitez et al. 2005).

Amide H/D exchange experiments via mass spectrometry have enabled us to follow subtle conformational changes in the active site upon TM binding to thrombin, revealing a pathway from the TM-binding site (ABE I) to the active site. Only TM fragments containing the fourth domain were able to decrease amide exchange in the 90sC loop near the active site (Koeppe and Komives 2006). This is strong evidence that TM does, in fact, alter the active site of thrombin directly; TM is doing more than providing a docking site for protein C. Others have seen the allosteric effects that TM has on the active site of thrombin as well. Arg35 is a residue that makes up the basic core of ABE I, and its guanidinium group points near the active site Ser195. Mutation of this residue to Glu diminishes calcium dependence and TM dependence of protein C activation (Bode, Turk et al. 1992; Rezaie and Yang 2003). Two Asp residues
located at P3 and P3’ positions in PC are known to have inhibitory effects on PC activation in the absence of TM, but not in the presence of TM. When Glu-192 and Glu-39, residues that are known to confer P3 and P3’ specificity, are mutated to Gln or Lys, TM requirement is nullified (Le Bonnec, MacGillivray et al. 1991). Changing either PC Asp to Gly relieves inhibitory repulsion. These examples lend credence to the hypothesis that TM may change the orientation of active site residues to alleviate steric hindrance and/or electronic repulsion.

Although the results of all of these experiments can really only be explained if TM causes allosteric changes in thrombin, the crystal structure of thrombin bound to TMEGF456 showed no differences in thrombin compared with PPACK-inactivated thrombin (Bode, Turk et al. 1992; Fuentes-Prior, Iwanaga et al. 2000). The research presented here will investigate the proposed allostery in the thrombin-TM interaction. First, mutants of TMEGF45 will be presented that have different effects on the fluorescent properties of a covalently bound active site inhibitor. Isothermal titration calorimetry experiments probing the cooperatively between the exosite 1 and the active site have uncovered an unusual thermodynamic coupling in which binding at one site changed the balance of ΔH and -TΔS but not the overall ΔG. We propose that enthalpy-entropy compensation may be a hallmark of the new type of allostery, termed dynamic allostery, in which no large conformational changes are elicited by affector binding; instead a transmission of dynamic changes is observed. In this thesis functional assays have been combined with conservative mutagenesis and binding studies to ascertain how TM allosterically regulates thrombin towards activation of protein C.
Chapter II

Characterization of TMEGF56
A. Introduction

TM binds to thrombin via epidermal growth factor like domains (EGFs) 4, 5, and 6 (Figure 2.1). It has been shown that the minimal fragment that binds to thrombin but still retains cofactor activity is TMEGF45 (White, Hunter et al. 1995). The binding affinity of TMEGF456 is 10 fold tighter. Structural studies of these domains have revealed much about the thrombin-TM interaction. The 5th EGF-like domain of TM contains a novel uncrossed disulfide bonding pattern of 1-2, 3-4, 5-6 rather that the typical EGF-like pattern of 1-3, 2-4, 5-6 (White, Hunter et al. 1996). This pattern makes TMEGF5 more dynamic as compared to TMEGF4, and presumably TMEGF6, which have the normal EGF-like bonding arrangement (Prieto, Sampoli Benitez et al. 2005). TMEGF5 also contains most of the residues found to be important for binding to thrombin (Nagashima, Lundh et al. 1993). This presents the possibility that thrombomodulin may bind to thrombin by an induced fit mechanism. Therefore, the flexibility of the 5th domain may be important to thrombin binding.

Much is still unknown about the relationship between the 5th and 6th domains of TM. The crystal structure solved by Fuentes-Prior et al. shows that there is a calcium binding site located between the 5th and 6th domain that may add stability to the thrombin binding loops and order them into a conformation to fit anion binding exosite I of thrombin (Fuentes-Prior, Iwanaga et al. 2000). There is no solution or crystal structure of TMEGF456 alone. A solution structure of TMEGF456 in complex with thrombin is also not available. It remains unclear if the 6th domain orders the 5th domain before thrombin binding or if the ordering happens as TM bind to thrombin.
Figure 2.1. Schematic drawing of the domains of thrombomodulin with an expanded view of the cofactor active fragment detailing the role of each EGF-like domain.
TMEGF456 aggregates at concentrations required for solution studies (Komives lab, unpublished). To investigate the relationship between the 5th and 6th domains, therefore, TMEGF56 was expressed and characterized. It was our hope that the solution structure and dynamics of TMEGF56 would be solved alone and bound to thrombin. The results of these experiments would reveal the effect that the 6th domain of TM has on the 5th domain and what is the structure of the 5th domain before, and upon binding to thrombin.

The traditional method of making disulfide bonded and glycosylated proteins is to make them in mammalian cells. It is not possible, however, to isotopically label proteins expressed in mammalian cells. Previous lab members have tried expressing TM constructs in E. coli and subsequently refolding the protein. This led to decreased yields as there were improperly folded constructs, which had no activity. Even in E. coli systems that secrete protein into the periplasmic space there are issues of glycosylation and inefficient disulfide bond formation, leading to decreased levels of correctly folded protein (Lueking, Holz et al. 2000). E. coli expression systems are not generally suitable for expressing protein with a high level of disulfide connectivity and do not have the means by which to handle posttranslational modifications such as glycosylation, which alters the solubility of TMEGF like domains. For the reasons enumerated above, our lab has invested its energy in making our protein in Pichia.
B. Materials and Methods

1. Expression of TMEGF56

TMEGF56 was cloned from the TMEGF456 gene which was synthesized using optimized E. coli codons made by Chris White and expressed in *Pichia pastoris* similar to that which was described by White *et al.* (White, Hunter et al. 1995). DNA was amplified by PCR and then cloned into the pPIC9 vector. This step was necessary because the expression vector pPIC9K contains a second XhoI cleavage site in the kanamycin resistance gene. Retention of this XhoI at the KEX2 cleavage site is required for post-translational processing of the α-factor leader sequence used to direct secretion of the protein. The protein sequence begins at Gln387 and continues to the end of the 6th domain Lys 465 using XhoI and EcoRI as restriction sites. The SacI–SalI fragment of pPIC9 containing TMEGF56 gene was subcloned into pPIC9K to produce the final *P. pastoris* expression vector. The TMEGF56 protein produced by this vector contains two additional amino acids at the 5’ end; histidine and methionine, due to an NdeI site necessary for subcloning into a short shuttle vector pBKsh2. Plasmid DNA was transformed into *P. pastoris* KM71 and SMD1168 cells, the latter of which had previously been proven to yield large quantities of TMEGF45 (White, Hunter et al. 1995). The SMD1168 strain is defective in the vacuole peptidase A (pep4). Since this enzyme is responsible for activating carboxypeptidase Y and protease B1, SMD1168 cells also lack these proteases. The KM71 strain, however, contains only an alternate gene called AOX2 instead of AOX1 to produce alcohol oxidase enzyme (Daly and Hearn 2005). The AOX2 enzyme has the same specific
activity as AOX1 but has a much lower expression level due to a weaker promoter and can only consume methanol slowly, giving rise to the phenotype termed ‘methanol utilization slow’ (Mut'). It was thought that this slower expression may give the protein an increased opportunity to have the correct glycosylation and folding pattern. Expression was monitored as described previously (White, Hunter et al. 1995).

For purification from shake flasks, *P. pastoris* supernatant was first purified by anion-exchange chromatography (HiLoad 26/10 Q Sepharose) equilibrated in 50 mM MES buffer, pH 6.5. A step gradient of 0, 15 and 30% buffer B (50 mM MES, pH 6.5, 1 M NaCl) was used, and the TM fragments eluted in the 30% fraction. This step was followed by reverse-phase HPLC as described (Wood and Komives 1999). Finally, TMEGF56 was purified using size-exclusion chromatography (HiLoad 16/60 Superdex 75, Amersham/GE Healthcare) equilibrated in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. For purification of TMEGF56 from the fermentor, an initial purification step on QAE sephadex column was added using the same buffer as the HiLoad Q step.

2. **Protein C activation inhibition assay**

TMEGF56 binds to thrombin but is not able to cause thrombin to activate protein C. In order to measure the TMEGF56 thrombin-binding activity, a competition assay was set-up in which TMEGF56 was added at different concentrations to an assay in which TMEGF456 activation of thrombin cleavage of protein C was measured. In this way, the thrombin-binding was assayed as the ability to compete for TMEGF456 activity. To measure the thrombin-TM activation of protein C, 15 μL of active thrombin (0.004 mg/mL in TBS) was mixed with 10 μL of TMEGF456 (0.001 mg/mL in TBS) in 80 μL of TBS 0.1% BSA, 5 mM CaCl₂ in a 96-
well plate for 10 min, allowing the thrombin-TM complex to form. For the TMEGF56 competition, 10 µL TMEGF56 (typically 0.02 mg/ml) was added during this incubation period. After 10 min, 20 µL of 0.06 mg/mL human protein C (Haematologic Technologies, Essex Junction, VT) was added. After 20 min the reaction was quenched by addition of 40 µL of a solution of heparin (0.075 mg/mL, Calbiochem, San Diego, CA) and antithrombin III (0.070 mg/mL, Haematologic Technologies, Essex Junction, VT). The amount of activated protein C was determined using a chromogenic substrate, S2366 (Diapharma, West Chester, OH). An enzymatic unit is 1 nmol of activated protein C produced per min and was determined from a standard curve generated from activated protein C added to S2366 under the same conditions as the assay (White, Hunter et al. 1995). To determine the potency of TMEGF56 made from Pichia, we used human TMEGF56 made in mammalian Chinese Hamster Ovary (CHO) cells as a standard, a gift from Tim Mather at the Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma. This TMEGF56 standard was used to measure the percent inhibition caused by a known amount of TMEGF56. Percent activity was then calculated using the Units of inhibition activity min⁻¹ mg⁻¹. This calculation was used to measure the purity and activity at each purification step.

3. Clotting inhibition Assay

The clotting inhibition reaction was carried out by adding 10 µL of 10–50 µg/ml TMEGF56 fractions to 10 µL 0.03 mg/ml thrombin in the presence of a 5mM Ca²⁺, 0.88mg/ml PEG solution and 80 µL Tris-HCL, 150 mM NaCl, pH 7.4. The
solution was allowed to incubate for 2 min. Fibrinogen (200 µL of a 4 mg/ml solution in TBS) was then added. Normal clotting time for bovine thrombin is under 20 sec. The concentration of standard TMEGF56 (Mather) needed to double clotting time was \( \sim 10 \mu g/ml \).

4. Mass Spectrometry

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Voyager DE-STR instrument (Applied Biosystems) as previously described (Wood and Komives 1999). The matrix used was 10 mg/ml sinapinic acid (Sigma-Aldrich) dissolved in a solution containing 700 µL 2% TFA and 300 µL acetonitrile.

5. Analytical Ultracentrifugation

Sedimentation velocity experiments were conducted with an Optima XL1 analytical ultracentrifuge (Beckman Coulter). Since TMEGF45 is known to self associate with any buffer present (Prieto, Sampoli Benitez et al. 2005), we decided to test different concentrations of TMEGF56 to see how it would behave under NMR concentration conditions. TMEGF56 was washed into H₂O and the pH was adjusted using 100mM NaOH or 100mM HCl, then concentrated to 0.2mM, 0.5mM, or 0.8 mM. Protein concentrations were determined by BCA assay (Pierce). All experiments were performed at 20°C. Six-channel cells were used in the sedimentation equilibrium experiments containing 110 µL of sample and 125 µL of reference buffer solution. Six rotor speeds were selected, 18000 rpm, 22000 rpm, 26000 rpm, 30000 rpm, 34000 rpm, and 38000 rpm. Data collection was started initially after 20 hours
of equilibration and replicate scans were collected every two hours. Subsequent spins were allowed to equilibrate for 12 hours after which replicate scans were performed.

All data analysis was carried out using the Beckman Origin software package. Buffer density ($\rho$) and partial specific volume ($\upsilon$) calculations have been carried out using SEDNTERP. Partial specific volume was calculated to be 0.69 ml/g for TMEGF56 according to the method described by S. Shire for glycoproteins with different percentages of carbohydrates (S.J. Shire, Beckman Resource Center, 1998).

6. Solution NMR

TMEGF56 was uniformly labeled with $^{15}$N during fermentation as described (Wood and Komives 1999). Samples were 0.2 mM with a final volume of 0.45 mL in 90% H$_2$O/10% $^2$H$_2$O with 0.02% NaN$_3$, pH 6.5. The pH was adjusted by adding aliquots of 100 mM NaOH. $^1$H-$^{15}$N HSQC spectra were collected at 298 K on a Bruker DRX 600MHz with a 5mm triple-resonance indirect Cryoprobe, or a Bruker Avance 800MHz equipped with a 5mm 1H ($^{13}$C/$^{15}$N) triple-resonance indirect XYZ gradient probe as previously described (Wood, Sampoli Benitez et al. 2000; Wood, Becvar et al. 2003).

C. RESULTS

1. TMEGF56 can be fermented from Pichia and purified effectively

Traditionally TMEGF45 constructs had been expressed in SMD1168 cells mainly because the lack of pep4 did not lead to cleavage of important residues at the end of the 5th domain necessary for thrombin binding as GS115 cells had been found
to in past studies. TMEGF456 however, could not be concentrated to above 0.2 mM and did not give good yields when expressed in the SMD1168 strain. We therefore tried the approach of expressing TMEGF56 in both KM71 and SMD1168 P. pastoris cells.

Fermentation of TMEGF56 was similar to method described by Wood et al. (Wood and Komives 1999). For KM71 cells, methanol induction was started slow and stayed at 50% methanol instead of being switched to 100% methanol at the latter stages of fermentation. The SDS-Page gel in Figure 2.2 shows the induction of KM71 cells over the course of 33 hours. It was found that 30 hours was a sufficient induction time and longer inductions did not increase protein production in either KM71 or SMD1168 strains.

Purification of TMEGF domain proteins usually involves anion exchange chromatography, size exclusion chromatography and then a final purification step. Final purification of TMEGF45 is carried out by analytical reversed phase HPLC. Since TMEGF456 does not retain its known activity of $1 \times 10^6$ U ml$^{-1}$ min$^{-1}$ after the harsh conditions of HPLC, it is usually further purified on MonoQ chromatography. Purification of TMEGF56 followed the procedures for TMEGF456. TMEGF56 elutes in the 30% B fraction on the HLQ, similar to TMEGF456 (Fig 2.2). Further purification led to two products that exhibited clotting inhibition in the size exclusion step of purification, one 22 kD and 11 kD. Both fractions were further purified by mono Q chromatography separately using a gradient of 100 mM NaCl to 500 mM NaCl. In the last mono Q step, the protein was divided into multiple peaks, most with the same activity. We concluded that some of these different peaks may be
glycoforms of the protein, but this purification step was not beneficial because the process gave a protein yield of less than 1mg of protein. It is interesting that the same phenomenon was observed for TMEGF456 (A. Baerga unpublished results). Gel electrophoresis and MALDI mass spectrometry both revealed a single species present. An HPLC step was incorporated between the HLQ and SEC in our purification scheme to discern if the specific activity could be increased.

**Figure 2.2.** Purification of TMEGF56 after fermentation. Gel fractions are A) Pre Induction, B) 9 hrs post induction, C) 14 hrs post induction, D) 33hrs post induction, E) Molecular Weight Marker, F) TMEGF56 standard. HLQ) TMEGF56 elutes at 30%B in the gradient. RP-HPLC) The rise of the labeled peak in the most active fraction. SEC) Two peaks can be purified from the HPLC step, one eluting at 20 kD and another at 12 kD.
There was a notable difference in the specific activity with HPLC and without as is demonstrated in table 2.1. It is clear that the HPLC step does add some level of purification not previously attained by just HLQ and SEC. It is also clear that the 20 kD protein fraction is more active than the 11 kD protein. Deglycosylation with Endo H, which cleaves between the two GlcNAc residues N-linked to Asn391 indicated that the size difference between the 20 kD and the 12 kD proteins was the amount of mannose residues attached. Fig 2.3 shows the mass spectra of the 20 kD protein glycosylated and deglycosylated. In the glycosylated spectra, all of the different glycoforms create a small hill of mass at around 20 kD. The counts are very low, around 150 counts, even though the protein concentration is 1 mg/ml. This phenomenon is due to the fact that different glycoforms divide the protein concentration into so many different species. After deglycosylation, however, the same protein at 1 mg/ml produced a peak of the correct mass of 8902 Da (protein plus one GlcNAc). The counts were 8000 instead of 150, indicative of the fact that it is now a single species.
Figure 2.3. MALDI mass spectra of high molecular weight TMEGF56 A. glycosylated and B. deglycosylated. Sequence of TMEGF56 and expected molecular weight of deglycosylated sample.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Expected MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMQMFCNQTACPADCDPNTQASCECPEGYILD DGFICTDIDECENGGFCSGVCHNLPGTFECICGP DSALARHIGTDCDSGK</td>
<td>8902</td>
</tr>
</tbody>
</table>
2. TMEGF56 binds at ABEI of thrombin with the same affinity as TMEGF456, and inhibits protein C activity and clotting activity.

The ability of TMEGF56 (produced by limited proteolysis of rabbit TM) to inhibit clotting formation and protein C activation has been well documented by Esmon and others (Esmon and Owen 1981). We tested our expressed TMEGF56 produced in both KM71 cells and SMD1168 cells. It was thought that growing TMEGF456 in KM71 cells, which have AOX2 site so that cell growth in methanol is slowed, may produce more correctly folded and glycosylated protein than SMD1168 cells.

Protein C inhibition assays were performed to monitor the purification process and the activity of each TMEGF56 construct. TMEGF456, TMEGF56 and thrombin were added together and allowed to incubate for 10 min to allow TMEGF56 to compete for TMEGF456 in binding to thrombin. Protein C was then added and the amount of activated protein C that was generated was quantified by the cleavage of chromogenic substrate S-2366 monitored at A405 as depicted in the following rate equation.

\[
\begin{align*}
T + TM & \rightarrow T(TM + PC) \\
& \rightarrow T(TM(PC)) \\
& \rightarrow aPC \\
& \rightarrow A_{405}
\end{align*}
\]

Since TMEGF56 does not promote protein C activation, it is expected to reduce the amount of protein C activation by TMEGF456 in the assay. Table 2.1 shows the specific activity of TMEGF56 throughout the purification of the protein.
Different SMD1168 and KM71 clones were compared to determine which strain expressed the best protein. It can be seen that protein produced in each Pichia strain had similar specific activities. The purification was similar as well. The SMD1168 strain was more predictable in the fermentation process, and for this reason we used this strain in subsequent experiments. The most active fraction of the SEC samples is the large molecular weight fraction, even when deglycosylated. Table 2.1 also illustrates the difference in activity between glycosylated and deglycosylated protein forms. Deglycosylation caused a loss of specific activity. These data are consistent with the fact that deglycosylation causes TMEGF456 to aggregate and lose activity.

Thrombin affinity toward TMEGF domains have been measured by Ye et al. and the $K_d$ were determined to be 7.4 nM for TMEGF1-6 and 29 nM for TMEGF56 (Ye, Liu et al. 1992). Abel Baerga-Ortiz in our lab obtained similar results for TMEGF456 produced from Pichia in protein C activity assays and in SPR experiments (White, Hunter et al. 1995; Baerga-Ortiz, Rezaie et al. 2000). To further characterize the binding affinity of TMEGF56 to thrombin, we conducted surface plasmon resonance experiments. Figure 2.4 shows kinetic constants for PPACK-thrombin binding to TMEGF fragments are very close to the reported values by Ye et al.
Table 2.1. Specific activity of TMEGF56. Sample 1 is human TMEGF56 made from CHO cells and was used as the standard for the basis of 100% activity. Samples 4-7 were purified by HLQ and SEC. Samples 8 and 9 were purified by HLQ, HPLC, and SEC.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>aPC slope</th>
<th>Inhibition activity</th>
<th>TMEGF56 concentration (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 TMEGF456 std</td>
<td>89.64</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 TMEGF456 + TMEGF56 std</td>
<td>12.35</td>
<td>86.22</td>
<td>0.2</td>
<td>431</td>
<td>100</td>
</tr>
<tr>
<td>3 TMEGF456 + TMEGF56std Degly.</td>
<td>22.07</td>
<td>75.38</td>
<td>0.2</td>
<td>377</td>
<td>87</td>
</tr>
<tr>
<td>4 TMEGF456 + 20 kD TMEGF56</td>
<td>30.75</td>
<td>65.70</td>
<td>0.2</td>
<td>328</td>
<td>76</td>
</tr>
<tr>
<td>5 TMEGF456 + 20 kD Degly.</td>
<td>34.97</td>
<td>60.99</td>
<td>0.2</td>
<td>305</td>
<td>71</td>
</tr>
<tr>
<td>6 TMEGF456 + 12 kD TMEGF56</td>
<td>41.95</td>
<td>53.20</td>
<td>0.2</td>
<td>266</td>
<td>62</td>
</tr>
<tr>
<td>7 TMEGF456 + 12 kD Degly.</td>
<td>49.3</td>
<td>45.00</td>
<td>0.2</td>
<td>225</td>
<td>52</td>
</tr>
<tr>
<td>8 TMEGF456 + 20 kD TMEGF56</td>
<td>13.56</td>
<td>84.87</td>
<td>0.2</td>
<td>424</td>
<td>98</td>
</tr>
<tr>
<td>9 TMEGF456 + 12 kD TMEGF56</td>
<td>21.76</td>
<td>75.73</td>
<td>0.2</td>
<td>379</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 2.2. Specific activity of TM proteins expressed in different Pichia strains.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>aPC</th>
<th>Inhibition activity</th>
<th>TMEGF56 concentration (mg/ml)</th>
<th>Specific activity (U/mg)</th>
<th>% activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEGF456 std</td>
<td>67.42</td>
<td>0.00</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMEGF456 + TMEGF56 std</td>
<td>11.39</td>
<td>83.11</td>
<td>0.2</td>
<td>415.53</td>
<td>100</td>
</tr>
<tr>
<td>TMEGF56 SMD13</td>
<td>19.16</td>
<td>71.58</td>
<td>0.2</td>
<td>357.91</td>
<td>86</td>
</tr>
<tr>
<td>TMEGF56 KM12</td>
<td>22.78</td>
<td>66.21</td>
<td>0.2</td>
<td>331.06</td>
<td>79</td>
</tr>
<tr>
<td>TMEGF56 KM7</td>
<td>25.66</td>
<td>61.94</td>
<td>0.2</td>
<td>309.70</td>
<td>74</td>
</tr>
</tbody>
</table>
Figure 2.4. A) BIAcore sensorgrams of human PPACK-thrombin binding to TMEGF56. B) BIAcore sensorgrams of human PPACK-thrombin binding to TMEGF456. For both experiments thrombin concentrations were 7.42 (purple), 4.95 (blue), 3.3 (cyan), 2.2 (green), 1.47 (red), 0.979 (magenta), and 0.653 (orange) nM.

Table 2.3. Kinetic constants for PPACK-thrombin binding to TMEGF fragments.

<table>
<thead>
<tr>
<th>protein</th>
<th>$K_a$ (1/Ms)</th>
<th>$k_d$ (1/s)</th>
<th>$R_{max}$ (RU)</th>
<th>$K_A$ (1/M)</th>
<th>$K_D$ (M)</th>
<th>Chi²</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM56</td>
<td>3.63E+06</td>
<td>0.0255</td>
<td>278</td>
<td>1.42E+08</td>
<td>7.03E-09</td>
<td>7.96</td>
</tr>
<tr>
<td>TM456</td>
<td>5.35E+06</td>
<td>0.0228</td>
<td>193</td>
<td>2.35E+08</td>
<td>4.25E-09</td>
<td>14.7</td>
</tr>
</tbody>
</table>
3. Solution NMR demonstrates that TMEGF56 may be less structured than TMEGF45 or TMEGF5

Sedimentation equilibrium experiments were performed under various concentrations and pH conditions. Previous research had established that TMEGF45 acts as a dimer in buffer as evidence by size exclusion and AUC studies but as a monomer in pure H2O solutions (Wood et al., 2000). TMEGF56 does not elute from the size exclusion column as a dimer, but is divided into a more active highly glycosylated form and a lower activity low molecular weight form. We did not know how TMEGF56 would behave at high concentrations necessary for NMR studies. Therefore the focus of AUC experiments was to find a suitable pH and concentration for NMR experiments.

Figure 2.5 shows sedimentation equilibrium results at several different speeds. Since the 18,000 rpm and 22,000 rpm spins did not exhibit exponential curvature, these data were excluded from the analysis. All data gave dispersed residuals between 0.015 and -0.015. Data for all of the runs gave very similar results, however the runs for 26,000 rpm 30,000, and 34,000 rpm gave the best residuals and were used in the molecular weight analysis. All sedimentation data was analyzed by direct nonlinear least squares fitting and standard deviation was determined for runs at three speeds.

Table 2.4 shows all results from sedimentation experiments conducted at 20°C. At 0.2 mM the average molecular weight is very close to the average molecular weight of 11 kD as determined by mass spectrometry. The molecular weight seems to be unaffected by a pH variation between 6.5 and 7.5. At higher concentrations
Figure 2.5. Characterization of TMEGF56 by AUC. Data represents 0.2 mM TMEGF56 at pH 7.5. Overlay of sedimentation experiments 18000 rpm red, 22000 rpm green, 26000 yellow, 30000 white, 34000 blue, 38000 gray. All experiments were performed at 293K.
Table 2.4. AUC data from TMEGF56 low molecular weight samples at various pH and concentration at 293K.

<table>
<thead>
<tr>
<th>Sample Concentration (mM)</th>
<th>pH</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>6.5</td>
<td>10686 ± 112</td>
</tr>
<tr>
<td>0.2</td>
<td>7.0</td>
<td>10121 ± 186</td>
</tr>
<tr>
<td>0.2</td>
<td>7.5</td>
<td>11146 ± 83</td>
</tr>
<tr>
<td>0.2</td>
<td>7.0</td>
<td>9016 ± 216</td>
</tr>
<tr>
<td>0.5</td>
<td>7.0</td>
<td>7053 ± 84</td>
</tr>
<tr>
<td>0.8</td>
<td>7.0</td>
<td>6081 ± 125</td>
</tr>
</tbody>
</table>
TMEGF56 has a molecular weight that is lower than the average calculated molecular mass. At 0.5 mM the molecular weight is 7053 g/mol and at 0.8 mM the molecular weight is 6081 g/mol. It is known that proteins in unbuffered solutions can exhibit non-ideal behavior, which can be seen during sedimentations experiments. The same behavior was noted by Sampoli-Benitez during her characterization of TMEGF45 where the molecular weight obtained in H₂O was 4,000 Da lower that the average molecular weight obtained by mass spectrometry (Sampoli-Benitez thesis). The data presented shows that TMEGF56 does behave as a monomer in H₂O, thus NMR data were first collected in H₂O at various concentrations and pHS.

Several solution structures of TM have been solved to date. The structures of TMEGF4 (Meininger, Hunter et al. 1995), TMEGF5 (Sampoli Benitez, Hunter et al. 1997), and TMEGF45 (Wood, Sampoli Benitez et al. 2000), along with relaxation studies of these domains have given some insight into how these domains function (Prieto, Sampoli Benitez et al. 2005). When compared to the 4th domain of TM (EGF4), the 5th domain has a low number of nuclear Overhauser effects (NOEs) alone and in TMEGF45, suggesting that this domain may be disordered (Prieto, Sampoli Benitez et al. 2005). The C-loop of the 5th domain, however, where some thrombin binding residues are located, seems to be the most ordered of the three loops (Fig. 2.6). Backbone dynamic studies have also found that the C-loop of the 5th domain may be more ordered in TMEGF45 than in TMEGF5 (Prieto, Sampoli Benitez et al. 2005). These data may indicate that the 4th domain of TM orders the 5th domain, and this ordering may allow tighter binding to thrombin. Indeed, TMEGF45 binds to thrombin 10 fold more tightly than TMEGF5 (White, Hunter et al. 1995).
Figure 2.6. Schematic of TMEGF456. M388 linker residue between EGF4 and EGF5 is colored brown. EGF5 residues important for thrombin binding are in purple. Residues that participate in the calcium binding site are yellow. Note that Residues in the linker region between EGF5 and EGF6 are important to binding thrombin and calcium binding.
The HSQC of TMEGF45 with resonance assignments as solved by Wood et al. is shown in Figure 2.7 (Wood, Sampoli Benitez et al. 2000). The 5th domain peaks (circled) reside primarily in the center of the spectrum indicating that 5th domain appears to be less uniquely structured than the 4th domain of TMEGF45. There are also some peaks in the 5th domain that have multiple resonances associated with them. These are indicated by an arrow and are D400, E426, L415, and N402. D400, N402, and E426 have been found to be important to thrombin binding and cofactor activity (Nagashima, Lundh et al. 1993) (Fuentes-Prior, Iwanaga et al. 2000). L415 makes hydrophobic contacts with I420 in the free solution structure, but is free in the thrombin bound structure and makes contact with thrombin (Fuentes-Prior, Iwanaga et al. 2000; Wood, Sampoli Benitez et al. 2000).

TMEGF56 was fermented and enriched with 15N ammonium sulfate (Cambridge Isotopes) as described previously (Wood and Komives 1999). The protein was purified and washed into water then concentrated. Isotopic enrichment was high as expected 99.9% as can be seen in Figure 2.8 for the 11 kD TMEGF56 and was the same for the 20 kD TM as well. We used the low molecular weight fragment for NMR studies primarily, and then looked at the high molecular weight TMEGF56 as well to see if we could get any better results. Presented here are that data for the low molecular weight TMEGF56, the high molecular weight TM did not produce better results.

Direct comparison of 15N-TMEGF56to 15N-TMEGF45 is made in Figure 2.9. Both proteins are at 0.8 mM in 90% H2O/10% D2O, 0.02% Azide, pH 6.5 at 298K.
The 5\textsuperscript{th} domain peaks are more dispersed in TMEGF45 than in TMEGF56. TMEGF56 has more resonances that are in fluctuation than in TMEGF45.

Figure 2.7. HSQC of 15N-TMEGF45 with 5th domain peaks circled. From Woods et al, 2000.
Figure 2.8. MALDI mass spectra of low molecular weight 15N-TMEGF56 glycosylated and deglycosylated at 1mg/ml protein concentration.
Figure 2.9. HSQC of A. 15N-TMEGF45 of 600 mHz 298K and B. 15N-TMEGF56 800 mHz Both samples are 0.8mM in 90% H2O, 10% D2O, at 298K.
possible 78 peaks are seen in $^{15}$N-HSQC of TMEGF56. Not including side chain peaks, 60 of 78 possible peaks can be seen.

Multiple attempts were made to improve the quality of HSQC spectra by using different buffer conditions. As mentioned each molecular weight construct behaved similarly under NMR conditions. There is a calcium site shared by residues in the 5th and 6th domain. Calcium was added to the NMR samples in concentrations up to 20 mM. Some improvement to the spectra was seen at 5 mM CaCl$_2$ as seemed to become more dispersed and five peaks appeared. No added improvement was noted with concentrations up to 20 mM CaCl$_2$. The spectra of TMEGF56 appeared to be aggregated with the addition of salt and buffer. Table 2.5 summarizes most NMR buffer conditions used. Since the spectra can be seen at 0.2 mM sample concentration, buffer conditions were worked out at this concentration to conserve protein. Several other conditions were attempted including the use of 5 mM CHAPS in case there were interactions of the mannose residues with hydrophobic patches of protein, but were unsuccessful. Conversely, 25 mM NaCl was added to slightly increase ionic strength in the hopes that charged residues would be more content. This strategy did not improve the spectra. Finally, after 5 mM CaCl$_2$ was determined to be the best buffer condition, the protein was concentrated and a HSQC was performed. Even though five new peaks appeared, the spectrum was still very crowded in the center and many peaks have non-uniform intensities.

The difference between TMEGF45 and TMEGF56 are accentuated in the overlay of the two $^{15}$N-spectra in Figure 2.10. It is clear that the 5th domain peaks collapse of $^{15}$N-TMEGF56 (red) in the center. Non-uniform peak intensities are
present in the entire spectra, including the new peaks that are present between 135 and 120 ppm in the $^{15}$N dimension, presumably from the 6th domain, indicating that the amides are undergoing chemical shift perturbations and moving at different rates.

**Table 2.5. HSQC sample conditions.** All Samples in 90% H2O/10% D2O and 0.02% Azide.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Buffer</th>
<th>Spectrometer</th>
<th>Temperature</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 mM</td>
<td>H$_2$O, pH 7.0</td>
<td>800</td>
<td>298K</td>
<td>Peaks in center</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>H$_2$O, pH 6.5</td>
<td>600</td>
<td>298K</td>
<td>same</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>H$_2$O, pH 7.5</td>
<td>600</td>
<td>298K</td>
<td>same</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>H$_2$O, pH 7.5, 7.0 7.5, 20 mM CaCl$_2$</td>
<td>800</td>
<td>298K</td>
<td>65 peaks seen</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>25 mM Kphos, pH 6.5</td>
<td>600</td>
<td>298K</td>
<td>Protein looks aggregated</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>25 mM Tris, 7.5</td>
<td>600</td>
<td>298K</td>
<td>Looks bad</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>H$_2$O, pH 7.5, 7.0, 6.5</td>
<td>600</td>
<td>310K</td>
<td>Protein still has many peaks in the center</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>H$_2$O, pH 5.0</td>
<td>600</td>
<td>298K</td>
<td>Only 43 peaks</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>H$_2$O, pH 7.5, 7.0, 6.5, 5mM CaCl$_2$</td>
<td>800</td>
<td>298K</td>
<td>Can see 65 peaks clearly</td>
</tr>
<tr>
<td>0.75 mM</td>
<td>H$_2$O, pH 6.5, 5mM CaCl$_2$</td>
<td>800</td>
<td>298K</td>
<td>65 peaks as well</td>
</tr>
</tbody>
</table>
Figure 2.10. Overlay of HSQC of 15N-TMEGF45 (black) and 15N-TMEGF56 (red). 70 of possible 78 peaks are seen in 15N-HSQC of TMEGF56. 15N-TMEGF56 has 5 mM CaCl2.
We also compared our spectra to $^{15}$N-TMEGF5 alone at the same conditions except the pH was 7.0 instead of 6.5. It is evident from the spectral overlay in Figure 2.11 that $^{15}$N-TMEGF56 (red) peaks are more populated at the center than $^{15}$N-TMEGF5 (blue). So it seems that TMEGF56 may have more random coil structure than even the 5th domain alone. The spectrum definitely has missing peaks and tremendous amount of overlapping peaks.

D. Discussion

We expressed TMEGF56 in KM71 cells because we hypothesized that folding and disulfide bond formation may be the rate limiting process for making active protein. Therefore, it may be more advantageous to use a Mut$^+$ strain for expression because the rate of induction is slower and thus might produce more of our active construct. In both KM71 and SMD1168 strains, the active protein was over glycosylated. Fortunately, the hyperglycosylation did not abrogate binding to ABEI of thrombin demonstrated by its ability to bind thrombin at the same affinity as TMEGF456, block protein C and fibrinogen clotting activity.

Attempts were made to shift glycosylation toward the less mannose substituted form by adjusting temperature and pH. When glycoform was shifted to mostly the low molecular weight product, the activity drops to below the normal activity of 12 kD TMEGF56 of 88% active to ~70% active determined by protein C inhibition assay. This may be an indication that the protein may not be folded correctly. Another
Figure 2.11. HSQC overlay of 15N-TMEGF5 (blue) and 15N-TMEGF56 (red). Data was acquired in 90% H2O/10% D2O, 0.02% azide, pH 7.0 at 298K. 15N-TMEGF56 has 5 mM CaCl2
hypothesis is that several glycoforms are made, elute at the same molecular weight, and are difficult to separate.

Data from the \((^{15}\text{N}, ^{1}\text{H})\) HSQC shows us that both molecular weight products are very similar, which is consistent with their very close inhibition activities. In both forms, it is clear that the 5\(^{\text{th}}\) domain appears to be unstructured, and the spectra resemble those of an aggregated protein. However, we are certain that the protein is not aggregated from protein C inhibition assay performed post sample preparation, from the size exclusion chromatography results, and from analytical ultracentrifugation. It is possible that TMEGF56 is in fact less structured than TMEGF45 or TMEGF5 alone. TMEGF56 share a calcium binding site between the linker region of the 5\(^{\text{th}}\) domain and the second disulfide bond region of the 6\(^{\text{th}}\) domain. Loss of the 6\(^{\text{th}}\) domain decreases cofactor activity 10 fold. It was believed that the calcium binding site would order residues necessary for thrombin binding. While it may be true that select residues are ordered by the calcium binding site, most of the domain could remain flexible. This would be congruent with an induced fit mechanism; flexibility may be more important the thrombin-thrombomodulin interaction than we realized.
Chapter III

Mutations in the fourth EGF-like domain affect thrombomodulin-induced changes in the active site of thrombin
A. Introduction

When thrombin binds thrombomodulin (TM), the substrate specificity of thrombin changes from cleavage of fibrinogen to cleavage of protein C. This also changes the role of thrombin in the blood clotting cascade from a procoagulant to an anticoagulant (Esmon 2000). TM contains several domains including six EGF-like domains of which the fourth, fifth and sixth are necessary and sufficient for thrombin binding and subsequent cleavage of protein C by the thrombin-TM complex (Stearns, Kurosawa et al. 1989; Hayashi, Zushi et al. 1990). The fourth EGF-like domain is necessary for protein C activation, while the fifth and sixth EGF-like domains are involved in binding thrombin (Kurosawa, 1988 #28; White, 1995 #64).

Alanine scanning mutagenesis of thrombin and TMEGF456 in two separate studies identified residues in both TM and thrombin that are important for protein C activation (Nagashima, Lundh et al. 1993; Hall, Nagashima et al. 1999). Alanine scanning of thrombin identified anion binding exosite 1 (ABE1), the site of fibrinogen binding, as the TM binding site and this was later confirmed by amide H/$^2$H exchange experiments and x-ray crystallography (Mandell, Falick et al. 1998; Fuentes-Prior, Iwanaga et al. 2000). Residues in the active site of thrombin were also identified as important for TM binding (Hall, Nagashima et al. 1999).

The alanine scan of TMEGF456 identified 22 residues that were critical for protein C activation. These residues were located throughout the fourth, fifth, and sixth EGF-like domains. All of the critical residues were highly conserved in TM
from several different species (Figure 3.1) (Nagashima, Lundh et al. 1993). Several
negatively

**Figure 3.1.** Sequence alignment of TMEGF456 from five different species. Several of the critical residues identified by alanine scanning are indicated with arrows above the sequence.
charged residues in TMEGF456 were critical, which was expected because ABE1 of thrombin is positively charged. A crystal structure of thrombin in complex with TMEGF456 clarified the importance of many of these critical residues from TMEGF456 but raised questions about others (Fuentes-Prior, Iwanaga et al. 2000). Figure 3.2 shows the important residues from the alanine scan of TM mapped onto the thrombin-TMEGF456 crystal structure. Several residues in the fifth and sixth EGF-like domains of TM are in direct contact with thrombin. Mutation of these residues to alanine resulted in weakened TM binding as expected (Nagashima, Lundh et al. 1993).

Surprisingly, the crystal structure showed that critical TM residues in the fourth EGF-like domain were far from the thrombin molecule (Fuentes-Prior, Iwanaga et al. 2000). Despite the lack of direct contact between the fourth EGF-like domain and thrombin in the crystal structure, studies have shown that the fourth EGF-like domain is required to induce changes in the active site of thrombin. Ye et al. performed fluorescence experiments to investigate the differences between two different TM fragments. When fluorescein-FPR or ANS-FPR was attached to the active site histidine, the FPR linkage positioned the fluorophore away from the active site serine. Interestingly, this fluorescent label responded specifically to TM(1-6) and not to TMEGF56 (Ye, Liu et al. 1992). Amide H/$^2$H exchange experiments further confirmed these findings and showed that the 90s loop in thrombin was altered when TM fragments containing the fourth EGF-like domain were bound (Mandell, Baerga-Ortiz et al. 2001; Koepp, Seitova et al. 2005). The amide H/$^2$H exchange results provide a putative "allosteric" mechanism for the alleviation of substrate repulsion.
suggested by the elegant studies of Rezaie's group (Rezaie and Yang 2003; Rezaie and Yang 2005).

Figure 3.2. A) Schematic drawing of TMEGF45 showing the residues identified as important for the cofactor activity of TM. Residues are colored according to location: fourth domain in green, linker region in purple, Met388 in cyan, and fifth domain in red. B) Crystal structure of the thrombin-TMEGF456 complex. The important TM residues are shown as sticks and colored as in A. ABE1 of thrombin is blue and shown in sticks. The active site catalytic triad of thrombin is shown as green sticks.
Thus, the fourth EGF-like domain of TM is required for alteration of the thrombin active site, but it does not directly contact thrombin according to the crystal structure. There are two possible explanations of this apparent discrepancy. Either the crystal structure has captured an inactive state and under solution conditions the fourth EGF-like domain does contact thrombin, or it may communicate with the thrombin active site through the fifth EGF-like domain. We favor the latter hypothesis. NMR studies of the minimally active fragment of TM, TMEGF45, showed communication between the fourth and fifth EGF-like domains by way of Met 388 in the linker between the two domains (Wood, Sampoli Benitez et al. 2000; Prieto, Sampoli Benitez et al. 2005). Our hypothesis is that the fourth EGF-like domain of TM is responsible for a number of functions. It interacts directly with protein C, and it alters the binding of the fifth EGF-like domain so that repulsion of protein C both at Arg 35 (Yang, Manithody et al. 2006) and at Asp 102 is alleviated (Rezaie and Yang 2005).

In order to further investigate the role of the critical amino acids in the fourth EGF-like domain, mutations were introduced in the minimally-active fragment, TMEGF45, and assayed for thrombin binding and ability to cause thrombin-mediated activation of protein C. For each mutant TMEGF45, the $K_M$ of TMEGF45-binding to thrombin, the $K_M$ of protein C binding to the thrombin-TMEGF45 complex and the $k_{cat}$ for protein C activation was measured. Mutations included both alanine substitutions and more conservative changes. Some mutations caused loss of TM binding despite the fact that they are not in the binding site. Others caused decreased protein C binding (as measured by the $K_M$ for protein C activation). Some mutants caused a loss
of \( k_{\text{cat}} \) for protein C activation but retained nearly full thrombin-binding affinity. These mutants were further analyzed for their ability to cause changes in the active site of thrombin using the fluorescent labels previously described by Ye et al. (Ye, Esmon et al. 1991). Taken together, the results show definitively that certain residues in TM that are far from thrombin in the crystal structure are important for TM-binding and TM-mediated changes in the active site of thrombin.

**B. Materials and Methods**

1. **Site-directed mutants of TMEGF45 and TMEGF456**

   Mutants of TMEGF45 and TMEGF456 were made using the QuikChange site directed mutagenesis kit from Stratagene (San Diego, CA). Double stranded pPIC9K vector with the synthetic TMEGF45 and TMEGF456 genes with \( E. \ coli \) optimized codons was used as a template {White, 1995 #64}. The sequence of each mutated gene was determined by DNA sequencing.

   Each TMEGF fragment was expressed in *Pichia pastoris* yeast as described by White et al. {White, 1995 #64}. The protein was first purified by anion-exchange chromatography (HiLoad 26/10 Q Sepharose) and then by reverse-phase HPLC as described (Wood and Komives 1999). Active HPLC fractions were lyophilized, reconstituted in 50 mM Tris, pH 7.4, and 150 mM NaCl and then further purified by HiLoad 16/60 Superdex 75 size-exclusion chromatography (Amersham/GE Healthcare).
2. Preparation of thrombin

Human thrombin was obtained from purified prothrombin (Haematologic Technologies) as described previously (Croy, Koepepe et al. 2004). Optimal yields were obtained when the prothrombin concentrate was dissolved in 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 1mg/mL PEG-8000 so that the final prothrombin concentration was 1.6 mg/mL. The prothrombin was then activated for two hours at 37°C with 5 mg/mL Echis carinatus venom, and purified by cation exchange chromatography on a Mono-S HR 10/10 column (Amersham/GE Healthcare) using a gradient of 100 - 500 mM NaCl in 25 mM KH₂PO₄, pH 6.5. Alpha-thrombin was identified by fibrinogen clotting assay, and protein concentration was determined by absorbance at 280 nm (ε=1.92 cm mL unit⁻¹ mg⁻¹).

3. Protein C activation assay

To measure the thrombin-TM activation of protein C, active thrombin (0.1nM in the assay) was mixed with TMEGF45 (10 – 500 nM in the assay) for 10 min at 25°C to allow the thrombin-TM complex to form. Human protein C (0.1 – 0.8 µM; Haematologic Technologies, Essex Junction, VT) was added and protein C activation was allowed to occur for 20 min in a microtiter plate, volume 150µL, in TBS with 0.1% BSA added. The reaction was quenched by addition of 40 µL of a solution of heparin (0.075 mg/mL, Calbiochem, San Diego, CA) and antithrombin III (0.070 mg/mL, Haematologic Technologies, Essex Junction, VT) for 10 min. The amount of activated protein C was determined using a chromogenic substrate, aPC (Diapharma, West Chester, OH). The amount of activated protein C was determined by
comparison to a standard curve prepared with activated protein C under the same conditions {White, 1995 #64}. A non-linear fit of the Michaelis-Menten plot was used to determine the concentration of TMEGF45 that gave $\frac{1}{2} V_{\text{max}}$ when the concentration of thrombin was 0.1 nM. This concentration of TMEGF45 was then used in a protein C activation assay in which varying concentrations of protein C (from 0.1 to 1µM) were used. The results of the two assays were combined and the values of $K_{M,\text{TM}}$, $K_{M,\text{PC}}$, and $k_{\text{cat}}$ were determined.

4. Biacore surface plasmon resonance

Surface plasmon resonance experiments were performed using a BIACORE 3000 surface plasmon resonance instrument (Biacore, Inc., Piscataway, NJ) as described in detail elsewhere (Baerga-Ortiz 2000). Biotin-labeled TMEGF456 (570 response units) was coupled to a SA (streptavidin) sensor chip on a BIACORE 3000 instrument. Sensorgrams were collected for PPACK-thrombin as the flowing analyte (0.78 nM, 1.56 nM, 3.125 nM, 6.25nM, 12.5 nM, 25 nM) in 10 mM Hepes buffer, 150 mM NaCl, 2.5 mM CaCl$_2$ (pH 7.4) at a flow rate of 80 µl/min and at a sampling rate of 5 Hz. No surface regeneration was required for the thrombin-TMEGF456 interaction. Rate constants for association ($k_a$) and dissociation ($k_d$) and the dissociation constant ($K_D$) were obtained by globally fitting the data from five injections of thrombin using the BIAevaluation software version 3.0 using the simple 1:1 Langmuir binding model. Statistical analysis of the curve fits for both dissociation and association phases of the sensorgrams show low $X^2$ values.
5. NMR spectroscopy

TMEGF45 Y358A was uniformly labeled with $^{15}$N during fermentation as described (Wood and Komives 1999). Samples were 0.25 mM with a final volume of 0.45 mL in 90% H$_2$O/10% $^2$H$_2$O with 2 mM NaN$_3$, pH 6.5. The pH was adjusted by adding aliquots of 100 mM NaOH. $^1$H-$^{15}$N HSQC were collected at 310 K on a Bruker DRX600 or a Bruker DMX500 as previously described (Wood, Sampoli Benitez et al. 2000; Wood, Becvar et al. 2003).

6. Fluorescence Spectroscopy

Fluorescently labeled thrombin samples were prepared by incubation of thrombin (1 mg/mL in 25 mM KH$_2$PO$_4$, 300 mM NaCl, pH 6.5) with a 20-fold excess of fluorescein EGR-chloromethylketone (FEGRCK; Haematalogic Technologies, Essex Junction, VT) for at least 3 hrs at 25°C. After the incubation, excess label was removed by size exclusion chromatography on a HiLoad 16/60 Superdex 75 column (Amersham/GE Healthcare). The chromatography also served as a buffer exchange step as the column was equilibrated in 50 mM Tris, 150 mM NaCl, 2.5 mM CaCl$_2$, pH 7.4. The stock thrombin samples were 2 µM and were stored in small aliquots at -80°C until use but not more than 2 weeks.

Samples of TMEGF45 mutants were prepared from lyophilized protein which was reconstituted in Tris buffer (50 mM Tris, 150 mM NaCl, 2.5 mM CaCl$_2$, pH 7.4) to make stock solutions of 200 µM concentration. These samples were stored at -80°C until use. Fluorescence spectroscopy was performed on a FluorMax-2 fluorimeter (Spex). Wavelengths from 500 nM to 600 nM were scanned in an increment of 1 nM.
For the fluorescein labeled samples, the excitation was 490 nM with emission at 520 nM. Samples of fluorescent thrombin (10 nM in 50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 7.4) were prepared from the stock solutions described above. TMEGF45 (22 µM in 50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 7.4) was titrated into the fluorescent thrombin, mixing thoroughly after each addition. The concentration of TMEGF45 in the cell increased from 0 nM to 500 nM during the course of the titration. This represents an increase from a 0-fold to a 50-fold molar excess of TMEGF45 to thrombin. The emission intensity was measured after each addition of titrant and corrected for dilution. All data were collected at 25 °C.

C. RESULTS

1. Protein C activation by TMEGF45 mutants.

A steady state kinetic assay was used to measure the individual binding and catalytic steps in the production of activated protein C {White, 1995 #64}.

As shown in Scheme 1, the $K_M$ for binding of each TM variant to thrombin ($K_{M,TM}$) is measured by varying the concentration of each TMEGF45 mutant protein keeping the thrombin and protein C concentrations constant. The $K_M$ for binding of protein C to the thrombin-TMEGF45 mutant complex ($K_{M,PC}$) was determined by varying the protein C concentration in a second experiment carried out at the measured $K_M$ for the
particular TM variant. The $k_{\text{cat}}$ was determined from the apparent $V_{\text{max}}$ measured in both experiments. This assay was used previously to demonstrate that TMEGF45 has the same $k_{\text{cat}}$ and $K_{\text{M,PC}}$ for activation of protein C as full-length TM, and has a 10-fold weaker $K_{\text{M,TM}}$ \textit{\{White, 1995 #64\}}. Table 3.1 summarizes the steady state kinetic data we collected for the TMEGF456 mutants. We surveyed both alanine mutants and conservative mutations at the same sites in order to differentiate between structural

**Table 3.1.** Steady state kinetic constants measured for TMEGF456 mutants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{\text{cat}}$ (s(^{-1})) TMEGF456</th>
<th>$K_{\text{M,TM}}$ (nM) TMEGF456</th>
<th>$K_{d}$ (nM) 456</th>
<th>$K_{\text{M,PC}}$ (µM) TMEGF456</th>
<th>Primary Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>4.6±0.1</td>
<td>12±0.6</td>
<td>ND</td>
<td>0.6±0.2</td>
<td>N/A</td>
</tr>
<tr>
<td>D349A</td>
<td>1.8±0.4</td>
<td>100±10</td>
<td>&gt;100</td>
<td>0.6</td>
<td>thrombin binding</td>
</tr>
<tr>
<td>D349N</td>
<td>4.2±0.8</td>
<td>21±2</td>
<td>4.2</td>
<td>1.7±0.2</td>
<td>$k_{\text{cat}}$</td>
</tr>
<tr>
<td>E357A</td>
<td>0.17±0.04</td>
<td>212</td>
<td>28</td>
<td>0.5</td>
<td>thrombin binding</td>
</tr>
<tr>
<td>E357Q</td>
<td>0.33±0.03</td>
<td>30±12</td>
<td>5.2</td>
<td>0.7±0.5</td>
<td>$k_{\text{cat}}$</td>
</tr>
<tr>
<td>E357D</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>$k_{\text{cat}}$</td>
</tr>
<tr>
<td>Y358A</td>
<td>&lt;0.1</td>
<td>ND</td>
<td>&gt;3</td>
<td></td>
<td>folding</td>
</tr>
<tr>
<td>Y358F</td>
<td>7.5±0.2</td>
<td>14.0±0.3</td>
<td>2.6</td>
<td>0.90±0.06</td>
<td>hyperactive</td>
</tr>
<tr>
<td>F376A</td>
<td>&lt;0.1</td>
<td>ND</td>
<td>20</td>
<td>ND</td>
<td>folding</td>
</tr>
<tr>
<td>F376W</td>
<td>3±0.2</td>
<td>127±1</td>
<td>ND</td>
<td>1.9±0.2</td>
<td>Hyperactive in 45</td>
</tr>
<tr>
<td>M388L</td>
<td>6.0±0.7</td>
<td>12±3</td>
<td>4.4</td>
<td>0.6±0.2</td>
<td>hyperactive</td>
</tr>
</tbody>
</table>
defects and the role of specific side chains for TM function. Mutations that increased \( K_{M,TM} \) were interpreted as being important for thrombin binding. Mutations that retained near wild type \( K_{M,TM} \) but were deficient in \( K_{M,PC} \) were interpreted as playing a role in protein C binding. Mutations that decreased \( k_{cat} \) with near wild type binding were taken on for further study to see if they were able to cause TM-mediated changes in the thrombin active site as assessed by fluorescence experiments that probe changes in the thrombin active site.

2. **Alanine substitutions often affected folding of the TM fragments**

Alanine substitution at either Tyr 358 or Phe 376 was highly deleterious, dramatically increasing the \( K_{M,TM} \) and dramatically decreasing the \( k_{cat} \). This was true in the context of TMEGF456 and TMEGF45. The heteronuclear single quantum coherence (HSQC) NMR spectrum of the Y358A mutant of TMEGF45 is shown in Figure 3.3. The missing fourth domain cross peaks is consistent with the idea that this mutant has a folding defect. This result led us to make more conservative changes at the critical residues. In the context of TMEGF45, mutation of Tyr 358 to His resulted in protein with measurable activity, and mutation to Phe or Trp improved activity. Also in the context of TMEGF45, several mutations at Phe 376 to other hydrophobic amino acids were made, including to Leu and His, but none of these produced protein with any measurable activity (Table 3.2). It is possible that because TM is a non-globular protein, it may be more sensitive to folding defects upon single site mutation than a globular protein (Blaber, Baase et al. 1995).
Figure 3.3. Overlay of the NMR HSQC spectra of TMEGF45 wild type with the mutant Y358A. The wild type spectrum is in blue, and the mutant spectrum is in red. Samples were 0.25 mM with a final volume of 0.45 mL in 90% H2O/10% 2H2O with 2 mM NaN3, pH 6.5. 1H-15N HSQC were collected at 310 K on a Bruker DRX600 (wild type) or a Bruker DMX500 (Y358A). Many of the cross peaks that are missing from the spectrum of the Y358A mutant can be assigned to the fourth EGF-like domain suggesting that mutation of Tyr 358 to Ala results in a defect in folding of this domain.
3. Mutations that improved the activity of TM fragments

As reported previously, mutation of Met 388 to Leu results in hyperactivity (Clarke, Light et al. 1993). This linker substitution improved $k_{cat}$ for protein C activation by approx. 1.5 fold but did not affect TM binding thrombin (Table 3.1). Several other substitutions also resulted in hyperactivity. Y358F in TMEGF456 had a 1.5 fold increase in $k_{cat}$ for protein C activation. Y358W in TMEGF45 bound to thrombin with a 2-fold improved affinity. F376W in TMEGF45 also resulted in hyperactivity with a 3-fold increase in $k_{cat}$. This mutation was actually deleterious in TMEGF456, which also contained the M388L mutation. When the F376W mutation was made in TMEGF45 also containing the M388L mutation, it was also deleterious. Thus, the hyperactivity associated with the F376W mutation requires Met at 388. These results show that there are many ways to improve the activity of TM. Interestingly, none of these residues are anywhere near thrombin in the crystal structure (Figure 3.2).

4. Mutations in the fourth domain of TM sometimes directly affect thrombin binding

Mutation of Asp 349, also distant from thrombin in the crystal structure had a dramatic affect on $K_{M,TM}$ with mutation to Ala resulting in a 5-fold weakening of thrombin binding affinity and mutation to the more conservative Asn resulting in a 3-fold weakening. Similarly, mutation of Glu 357 to either Ala or Asp weakened thrombin-binding although the most structurally conservative change to Gln retained
thrombin-binding affinity at wild-type levels. Again, these residues are not near thrombin in the crystal structure. Importantly, we were able to measure direct binding to thrombin by Biacore surface plasmon resonance and these results corroborate the binding affinities inferred from $K_{M,TM}$ measurements for TMEGF456 mutants (Table 3.1). Also, it is reassuring that the fold decrease in $K_{M,TM}$ was similar in the context of both TMEGF45 and TMEGF456 despite the fact that these proteins have a 10-fold difference in binding. Thus, the conclusion that mutations at these fourth domain residues directly affect thrombin binding affinity is a strong one.

Conservative mutations in the fourth domain of TM affect the way TM alters the active site of thrombin. Several mutations of key fourth domain residues were found to primarily affect $k_{cat}$ for protein C activation while retaining nearly wild type $K_{M}$'s. These mutants were: D349N, E357Q, Y358H, and D400E (bold in Table 3.2). These mutants were carried on for further investigation of the mechanism underlying their $k_{cat}$ defect.

The emission spectrum of FEGRCK-thrombin upon addition of TMEGF45 showed an overall loss of emission intensity upon TMEGF45 binding. This was seen for the wild type TMEGF45 protein as well as the mutants M388L and E357Q mutants. For the mutants D349N and D400E, however, this same loss in emission intensity was not observed (3.5).
Table 3.2  Steady state kinetic constants measured for TMEGF45 mutants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{cat}$ (s$^{-1}$) TMEGF45</th>
<th>$K_{M,TM}$ (nM) TMEGF45</th>
<th>$K_d$ (nM)$^1$ TMEGF45</th>
<th>$K_{MLPC}$ (µM) TMEGF45</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>5.0±0.1</td>
<td>140±12</td>
<td>68</td>
<td>0.32±0.1</td>
</tr>
<tr>
<td>D349N</td>
<td>0.69±0.01</td>
<td>440±38</td>
<td>43</td>
<td>0.77±0.2</td>
</tr>
<tr>
<td>E357Q</td>
<td>0.36±0.06</td>
<td>149±29</td>
<td>55</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>E357D</td>
<td>0.22±0.02</td>
<td>542±41</td>
<td>ND</td>
<td>0.62±0.26</td>
</tr>
<tr>
<td>Y358A</td>
<td>0.08±0.003</td>
<td>623±100</td>
<td>ND</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Y358H</td>
<td>0.2±0.02</td>
<td>163±16</td>
<td>ND</td>
<td>1.6±0.16</td>
</tr>
<tr>
<td>Y358W</td>
<td>5.6±1.6</td>
<td>61±9</td>
<td>ND</td>
<td>0.8±0.5</td>
</tr>
<tr>
<td>F376W</td>
<td>15±4</td>
<td>208±19</td>
<td>ND</td>
<td>0.9±0.14</td>
</tr>
<tr>
<td>M388L</td>
<td>7.8±0.5</td>
<td>140±40</td>
<td>57</td>
<td>0.38±0.1</td>
</tr>
<tr>
<td>D400N</td>
<td>0.04±0.001</td>
<td>616±131</td>
<td>ND</td>
<td>0.7±0.04</td>
</tr>
<tr>
<td>D400E</td>
<td>0.17±0.01</td>
<td>237±16</td>
<td>49</td>
<td>1.36±0.1</td>
</tr>
</tbody>
</table>

$^1$ $K_d$ was determined by Scatchard analysis of the fluorescein-EGR-chloromethylketone-labeled thrombin binding data.
Figure 3.4. A) Change in fluorescence emission at 520 nm for fluorescein-FPRchloromethylketone-labeled thrombin (25 nM) upon addition of either TMEGF45 (■) or TMEGF56 (●) (up to 20-fold molar excess). B) Change in fluorescence emission at 520 nm for fluorescein-EGRchloromethylketone-labeled thrombin (25 nM) upon addition of either TMEGF45 (■) or TMEGF56 (●) (up to 20-fold molar excess).
Figure 3.5. Change in fluorescence emission at 520 nm for fluorescein-EGRchloromethylketone-labeled thrombin (25 nM) upon addition of TMEGF45 wt (■), M388L (□), D349N (○), D400E (◊), E357Q (v), or TMEGF56 (●) (up to 20-fold molar excess).
D. Discussion
1. Discovery of other hyperactive TM mutants

The results presented here expand the survey of TM mutations beyond those previously reported. One surprise was the number of ways activity could be increased by mutation. Substitution of Tyr 358 with either Phe or Trp resulted in activity increases similar to those seen for the M388L mutant. We also observed improved $k_{cat}$ for F376Y mutation, but this was only observed in the context of the smallest (TMEGF45) form of TM. We have previously shown using NMR that the residue at position 388 is critical for communication between the fourth and fifth EGF-like domains of TM (Wood, Sampoli Benitez et al. 2000; Wood, Becvar et al. 2003; Prieto, Sampoli Benitez et al. 2005). It is interesting that Tyr 358 is one of the residues that is connected to the residue at position 388 through long-range Nuclear Overhauser effects (NOEs). It also showed significant changes in backbone dynamics when the linker Met 388 was oxidized (Prieto, Sampoli Benitez et al. 2005). More specifically, long range NOEs were observed between the Leu 388 and Tyr 358 even though these had not been observed for Met 388. We hypothesized at the time that the hyperactivity of the M388L may be linked to stronger connectivity through the fourth domain. It will be interesting to investigate whether the substitution of Y358 with F and/or W increases the NOE connectivities in the context of Met 388, providing a mechanism for the hyperactivity observed for these mutants as well.
2. Structural defects of alanine mutants

Alanine scanning mutagenesis experiments often rely on the implicit assumption that replacement of one residue, particularly a surface one, with alanine will not cause structural defects. Indeed, in globular proteins this is most often the case (Blaber, Baase et al. 1995). TM, however, is not a globular protein and to our surprise many of the alanine mutants either could not be expressed or showed large structural defects. Thus, for TM, many of the residues thought to be important for protein C activation are actually important for the structural integrity of TM itself. In order to discover functional rather than structural defects, we made conservative changes to TM at the same sites shown to be important by alanine scanning (Nagashima, Lundh et al. 1993). These experiments revealed that substitution of Tyr 358 and Phe 376 nearly always had large structural consequences.

Although this study avoided mutation of any TM residue in the thrombin binding site, a surprising number of the mutants including some substitutions at Asp 349, Glu 357, Tyr 358, and Asp 400 showed deficiencies in thrombin binding. Asp 400 is pointing away from thrombin in the crystal structure, but if the TM molecule retains some flexibility in the complex, it is possible that this residue could contact thrombin directly (Fuentes-Prior, Iwanaga et al. 2000). The rest of the TM sites are so far removed from thrombin in the crystal structure that another explanation must be invoked. For at least some of these residues, NOE connections were observed by NMR leading to the idea that they may play a role in dynamically connecting the fourth and fifth EGF-like domains for optimal TM activity (Wood, Sampoli Benitez et al. 2000; Wood, Becvar et al. 2003; Prieto, Sampoli Benitez et al. 2005). Our
hypothesis is that mutations at these residues have large effects on the thrombin binding ability of TM because they transmit changes in the conformation of the fifth domain.

3. Mechanistic analysis of $k_{\text{cat}}$ defective mutants

Many of the mutations had detrimental effects on the $k_{\text{cat}}$ for activation of protein C by thrombin yet retained near-wild type binding affinity towards thrombin and protein C. These included D349N, E357Q, Y358H, and D400E. Our studies suggest that at least D349 and D400 are altering the catalytic activity of thrombin by changing the conformation of the active site, which is sensed by the fluorescence of fluorescein-EGRCK. If the crystal structure is correct, we must conclude that the fourth domain alters the way the fifth domain interacts with thrombin. Although E357 also showed a $k_{\text{cat}}$ defect, it was able to cause most of the change in fluorescence of fluorescein-EGRCK. Taken together, these results suggest that E357 probably affects $k_{\text{cat}}$ for protein C activation by altering protein C towards thrombin cleavage, and it is likely that it is doing this by direct contact with protein C in the enzyme-substrate complex.

The results presented here solidify the idea that dynamics and communication between the fourth and fifth EGF-like domains is important for function, and that somehow the fourth domain communicates via the fifth domain to increase the catalytic activity of thrombin towards protein C. These results strongly support a dual role for the fourth EGF-like domain. It is acting as a docking site for protein C and functionally changing the active site of thrombin indirectly through the fifth EGF-like domain. Several different mutations in the fourth EGF-like domain of TM specifically
decreased $k_{\text{cat}}$ for protein C activation, and at least two of these mutants concomitantly lost their ability to cause conformational changes at the active site of thrombin. 

These results clearly demonstrate that residues in TM that are far from the thrombin binding site affect both TM binding to thrombin and TM-mediated changes in the active site of thrombin. Although it is unusual that non-contacting residues would cause such long-range (perhaps indirect) effects, the results can be explained by the fact that TM is a non-globular protein and NMR experiments have shown that the conformation of TM is highly plastic and sensitive to mutation (Wood, Wood, Prieto).
Chapter IV

Using Thermodynamic to Allosteric Changes in Thrombin upon Active Site Occupation
A. INTRODUCTION

1. The Function of Thrombin in the Blood Clotting Cascade

Thrombin is a multifunctional serine protease which plays a central role in regulating the blood clotting cascade by having both procoagulant and anticoagulant functions (Figure 4.1). Thrombin’s procoagulant function is to initiate the final step of the blood clotting cascade, cleavage of fibrinogen. Soluble fibrinogen protein is converted into an insoluble fibrin aggregate by cleaving the amino-terminal ends of the αA and βB chains of fibrinogen which can then undergo polymerization and culminate in fibrin clot formation. Thrombin further amplifies procoagulant activity by also catalyzing activation of blood factors V, VII, VIII, and IX (Esmon, 2000). The procoagulation activities of thrombin are quickly diminished either as a result of capture by the endothially-bound protein, thrombomodulin (TM), or as a result of inhibition by antithrombin III. TM binds at a site on the opposite side of thrombin from the active site. The TM binding site is also the binding site for fibrinogen, and this site is called anion binding exosite 1 or ABE I (Ayala et al 2001, Pineda et al. 2002, Esmon et al 2000). When TM binds to ABE1, the substrate specificity of thrombin is altered away from fibrinogen and towards protein C, initiating anticoagulant function. The mechanism of substrate specificity alteration is not just that TM competes for binding with fibrinogen. The rate of protein C activation by thrombin is enhanced 20,000 fold upon binding of calcium and TM (Esmon 2000). Protein C activation leads to the degradation of activated factors V and VIII, resulting in the shut down of the coagulation cascade (Musci, Berliner et al. 1988).
Figure 4.1. Thrombin’s dual role in the clotting cascade. The procoagulant function of thrombin is to cleave fibrinogen into fibrin. Fibrin peptides then self-assemble into insoluble fibrin clots. The anticoagulant function of thrombin is initiated when thrombin is captured by TM. Thrombin is bound to TM at the cell surface and this complex cleaves protein C much more efficiently (3 orders of magnitude) than thrombin alone. Activated protein C then inactivates essential cofactors V and VIII, which are required to activate factor X. Factor X can no longer cleave thrombin, thereby shutting down the clotting cascade.
The molecular switch TM causes in specificity of thrombin is an integral mechanism for natural hemostasis as unchecked procoagulation leads to clot extension and thrombi formation (Griffin, Ecatt et al. 1981).

Our studies are focused on the biochemical and biophysical characterization of the thrombin-TM interaction. Through H/D exchange mass spectrometry, we have observed that exchange at two active site loops, the autolysis loop and the 90s_CT loop slowed when TM was bound (Koepp, Seitova et al. 2005). To answer the question of whether these changes at the active site were linked to TM cofactor activity, the amide H/D exchange experiment was used to probe how both active (TMEGF45) and inactive (TMEGF56) TM fragments altered the exchange behavior of the active site loops of thrombin. Both TM fragments caused the autolysis loop to exchange less, but only the cofactor-active TMEGF45 caused the lower exchange in the 90s_CT loop (Koepp, Seitova et al. 2005). The major thrombin binding loop (the 70s_CT loop) is directly connected to the 90s_CT loop by a surface strand which we will call a "transmission line" which extends from the active site all the way to ABE I (Figure 4.2). We have also furthered the work of Ye et al. in showing that point mutations in the 4_{th} domain have a diminished effect on active site fluorescent labels as compared to fully active TMEGF45 (Beach et al, submitted), (Ye, Esmon et al. 1991; Ye, Liu et al. 1992).

In this chapter, I describe the use of isothermal titration calorimetry (ITC) to measure binding of substrate analogs in the presence and absence of both active (TMEGF45) and inactive (TMEGF56) fragments of TM and a single stranded DNA aptamer that also binds to ABE1 (Bock et al. 1992). These experiments will probe
how binding enthalpy and entropy are coupled when ligands bind at both the active site and at ABE1. We had previously shown that binding of TMEGF45 is not accompanied by an enthalpy change (Baerga-Ortiz, Bergqvist et al. 2004). Here, we are less interested in the thermodynamics of binding at exosite 1 as we are in how a bound ligand at exosite 1 affects the thermodynamics of binding at the active site. Therefore, the lack of an observed enthalpy change upon binding of the TM fragments to exosite 1 is inconsequential. To probe the thermodynamic coupling between the two binding sites, we will first measure binding of the active site ligand to bovine α-thrombin, and compare the results to binding of the active site ligand to the thrombin-TM complexes as well as the thrombin-aptamer complex.
Figure 4.2. Structure of thrombin showing the "transmission line connecting the 70sCT TM binding site loop with the 90sCT loop. The red color indicates where TMEGF45 completely blocks solvent accessibility, the pink is where TMEGF45 partially blocks solvent accessibility. The cyan is the 90sCT loop and the blue is the "transmission line". Data from H/D experiments in Croy et al. 2005.
B. MATERIALS AND METHODS

1. Proteins and DNA aptamer.

Thrombin from bovine blood was used as the main source of protein in this study because we could obtain larger quantities per preparation, and it was less prone to aggregate at higher concentrations than the human protein. Bovine thrombin was purified from a barium citrate eluate (prepared from bovine plasma) according to previously published methods (Ni, Konishi et al. 1990). The eluate powder was redissolved overnight in 200mL of 100mM EDTA, 150mM NaCl, 10mM NaCitrate, containing 11.2g ammonium sulfate and 0.03g of benzamidine. Following resuspension, the concentration of ammonium sulfate was increased from 10% to 35%. After centrifugation at 10000g, the supernatant was kept, brought to 70% ammonium sulfate and centrifuged. The pellet, containing prothrombin was dissolved in 10mL of 25mM Tris, pH 7.5, 100mM NaCl, loaded onto a G-25 sephadex gel filtration column (2.5 x 100 cm) to remove the ammonium sulfate, and the fraction containing protein was collected. The prothrombin was activated by incubating it with 2.0mg/mL *Echis carinatus* venom, 10mM CaCl$_2$, 1mg/mL PEG-8000 for 60 minutes at 37°C. The mixture was loaded onto a second G-25 sephadex column (2.5 x 100 cm) equilibrated in 25mM KH$_2$PO$_4$ pH 6.5, 100mM NaCl, and the protein fraction was collected. Finally, the G-25 fraction containing active thrombin was loaded onto a MonoS FPLC column 16/10 (Amersham-Pharmacia) equilibrated with buffer A (25mM KPhos pH 6.5, 100mM NaCl). The thrombin was eluted with a linear gradient of B (25mM KPhos pH 6.5, 500mM NaCl) over 1 hr. Alpha-thrombin was identified
by fibrinogen clotting assay, and protein concentration was determined by absorbance
at 280nm ($\varepsilon = 1.92$ cm mL unit$^{-1}$ mg$^{-1}$). The yield of bovine thrombin preparation is
usually from 6-10 mg of pure protein. For the ITC experiments, bovine thrombin was
stored in 2ml aliquots with 10 mM benzanidine added to prevent autoproteolysis and
kept at -80°C for no more than 3 weeks. Aliquots were then purified and buffer
exchanged by size exclusion into the given ITC buffer. Protein was then concentrated
to 6.5 µM using an Amicon Centriprep with a 10 kD molecular weight cut off
(Millipore) at 4°C. After concentration, the protein was immediately complexed with
an ABE I ligand or used in an experiment. Protein that was not for use immediately
was stored at -20°C until use and for no longer than one day. It is important not to
store repurified protein at -80°C as multiple freeze/thaw cycles diminishes its activity.
Thrombin cannot be left at 4°C for an extended period of time due to autoproteolysis.
Therefore, the protein was kept on ice until used in ITC experiments. Each ITC
experiment requires 2.4 mL of 6.5 µM thrombin. A typical bovine thrombin prep
yields a maximum of six ITC experiments. It should be noted that experiments
performed in this chapter were from multiple protein preparations.

A protease-inactive form of bovine thrombin was obtained by incubating α-
thrombin with a thirty-fold molar excess of the inhibitor, D-Phe-Pro-Arg
chloromethylketone (PPACK) (Bachem) or D-Glu-Gly-Arg chloromethylketone
(EGRCK) (Haematologic). After a two hour incubation at room temperature, the
PPACK-bound or EGRCK-bound thrombin was repurified on a S-75 size exclusion
column as above.
TMEGF45 has a $k_d$ of 0.37 sec$^{-1}$, and as a result, binds thrombin 10-fold less tightly than TMEGF456 (Baerga-Ortiz, Hughes et al. 2002). Because TMEGF456 aggregates, experiments such as ITC and mass spectrometry that require high concentrations of protein, were performed with TMEGF45. The fully active TM fragments, TMEGF45 and TMEGF56 were expressed in *Pichia pastoris* yeast as previously described (White, Hunter et al. 1995). Both proteins were first purified by anion exchange chromatography (QAE Sephadex followed by HiLoad 26/10 Q Sepharose) equilibrated in 50 mM MES buffer, pH 6.5. A step gradient of 0, 15 and 30% buffer B (50 mM MES, pH 6.5, 1 M NaCl) was used, and the TM fragments eluted in the 30% fraction. This step was followed by HiLoad 16/60 Superdex 75 size exclusion chromatography (Pharmacia Biotech) in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. The protein was further purified and desalted by reverse-phase HPLC as described (Wood, Sampoli Benitez et al. 2000).

A 15 nucleotide single stranded DNA aptamer (5’-GGT TGG TGT GGT TGG-3’) that specifically binds at anion binding exosite I (ABE I) was purchased from Integrated DNA technologies, and purity was tested by MALDI-Tof mass spectrometry (Bock, Griffin et al. 1992). The DNA was resuspended in H$_2$O, divided into 19.5 µM/ml aliquots and lyophilyzed. Aliquots were stored at -20°C until use.

2. DAPAMe

DAPA (dansylarginine-N-[3-ethyl-1,5-pentanediyl]amine) is a specific active site inhibitor ($K_i=10^{-7}$M; $K_D=40 \times 10^{-9}$M) (Nesheim, Prendergast et al. 1979). When bound to thrombin, the fluorescence intensity and lifetime of the dansyl
moiety are increased three fold. For the sake of purity and cost effectiveness, we synthesized our own DAPAMe (dansylarginine-N-[3-methyl-1,5-pentanediyl]amine) (Figure 4.2). We used the extinction coefficient of 4010 cm$^{-1}$, 330nm reported for DAPA to quantify DAPAMe (Nesheim, Prendergast et al. 1979). DAPAMe was synthesized by coupling dansyl arginine with 4-methyl piperidine in a standard carbodiimide coupling reaction. To 0.6 ml of dry dimethylsulfoxide (in a reactivial) was added 75 mg dansyl arginine and 180 mg N, N' carbonyldiimidazole. After 10 min, 180 µL of 4-methylpiperidine was added and the reaction was allowed to stir for 4 hrs. The reaction was quenched by addition of 2 ml of 0.15M NaCl and the product was extracted into 3 ml and then 1 ml of ethylacetate. The product is yellow and it is possible to see that it is extracted into the ethylacetate. The ethylacetate solution was taken to dryness by evaporation under N$_2$, and the product was resuspended in 0.1% TFA, 10% CH$_3$CN and immediately purified on reversed phase HPLC using a gradient of 10 – 50% CH$_3$CN over 40 min. The product elutes at 20% (which is really 30% CH$_3$CN). Characterization by MALDI-TOF mass spectrometry shows a pure compound with a molecular weight of 489.3 g/mol.
3. Isothermal Titration Calorimetry (ITC).

Experiments were collected using a MicroCal VP ITC instrument. Samples were prepared by buffer exchanging bovine thrombin by HiLoad 16/60 Superdex 75 size exclusion chromatography into experimental buffer. Bis-Tris propane was used for most experiments because it is similar to Tris-HCl, yet has a broad buffer range of pH 6.3 to pH 9.5. Buffers used were 50 mM Bis-Tris Propane, 150 mM NaCl pH 6.5 and pH 7.4, and 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. Titrations were preformed
as described elsewhere (Wiseman, Williston et al. 1989), (Ladbury and Chowdhry 1996). In a typical experiment 19 injections of 15 µL each of ligand (70 µM) spaced 300 sec apart, were made into bovine thrombin (6.5 µM) or bovine thrombin plus ABE ligands in three fold molar excess (TMEGF56, 15mer) or five fold molar excess (TMEGF45) in the cell. Heats of dilution of the bovine thrombin into buffer were determined in separate experiments and subtracted prior to the analysis of the titration. The data were analyzed using the ORIGIN software supplied with the instrument according to a single binding site model.

C. RESULTS

1. DAPAMe inhibitor binds thrombin differently when ABE I is occupied.

We sought to find an inhibitor that binds with high affinity to the active site of thrombin for ITC measurements. DAPA (dansylarginine-N-[3-ethyl-1,5-pentanediyl]amine) was first synthesized by Neishem et al. as an inhibitor with fluorescent properties (Nesheim, Prendergast et al. 1979). We synthesized DAPAMe because 4-methyl piperidine was readily available. DAPAMe has methyl instead of an ethyl group (Figure 4.2). DAPAMe purity was determined by mass spectrometry and activity was compared to DAPA. The K_i of DAPAMe was determined to be 200 nM by thrombin inhibition assays. This agreed well with the published value for DAPA of 400 nM (Nesheim, Prendergast et al. 1979). In the first set of experiments, we compared DAPAMe binding to bovine thrombin alone to DAPAMe binding to the
thrombin-TMEGF45 complex. The experimental set up was as follows. Thrombin or thrombin plus a seven fold molar excess of TMEGF45 was placed in the cell. DAPAMe was titrated in until saturation was reached. The $K_d$ for thrombin binding to DAPAMe was $28.2 \pm 3.4 \times 10^{-9}$ M, which is similar to the results reported by Neshiem et al. of $K_d = 43 \times 10^{-9}$ M. Neshiem determined the $K_d$ by monitoring the change in DAPA fluorescence when thrombin was titrated into DAPA (Nesheim, Prendergast et al. 1979). The ITC measurements showed a slightly different $\Delta H$ when thrombin alone was titrated with DAPAMe as compared to when the thrombin TMEGF45 complex was titrated with DAPAMe.

**Table 4.1.** DAPAMe titration of thrombin alone or thrombin in complex with TMEGF45. Thrombin alone (6.5 µM) or in complex with a 7 fold molar excess of TMEGF45 was placed in the ITC cell. DAPAMe (70 µM) was placed in the syringe and used to titrate the solution to saturation. Experiments were performed in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 at 298K.

<table>
<thead>
<tr>
<th>Tris pH 7.4</th>
<th>Kd (M)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$(-T\Delta S)$ kCal/mol</th>
<th>$\Delta G$</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine thrombin</td>
<td>$28.2 \pm 3.4 \times 10^{-9}$</td>
<td>$-5.8 \pm .13$</td>
<td>$-4.7$</td>
<td>$-10.5$</td>
<td>1.1</td>
</tr>
<tr>
<td>bth + TM45</td>
<td>$26.7 \pm 1.1 \times 10^{-9}$</td>
<td>$-5.15 \pm .15$</td>
<td>$-5.39$</td>
<td>$-10.54$</td>
<td>.99</td>
</tr>
</tbody>
</table>
This difference was 0.65 kcal/mol and was found to be statistically significant by a standard t test (p= 0.044).

Table 4.2 shows the heat evolved from titrating thrombin with a single stranded DNA aptamer that also binds at ABE I and TMEGF45 and TMEGF56. There is no enthalpy change for TMEGF45 and TMEGF56, but the DNA aptamer has a large $\Delta H = -27.1$ kcal/mol. ITC of TMEGF45 was also performed by Abel Baerga-Ortiz in our lab (Figure 4.5) when characterizing a monoclonal antibody made to bind at ABE I and TMEGF456, which have the similar $K_D = 1.87$ nM and 1.64 nM respectively, but where the antibody has large favorable enthalpy change upon binding (Baerga-Ortiz, Bergqvist et al. 2004).

To further investigate the observed difference in $\Delta H$ of binding of DAPAMe to thrombin alone as compared to the thrombin-TMEGF45 complex, it was important to ensure that the thrombin in the cell was fully bound to TMEGF45. Although even a three-fold molar excess gave the same enthalpy change, all subsequent experiments were performed with a five-fold molar excess (Table 4.3).

Figure 4.5 and Table 4.4 summarize the data for the titration of DAPAMe into different complexes of thrombin. Bis-Tris propane proved to be a useful buffer because of its similarity to Tris-HCl and because its wide buffer range allowed us to use the same buffer at different pHs. Similar results were obtained using Tris-HCl or Bis-Tris propane at pH 7.5 indicating that there were no specific buffer effects on the thermodynamics of binding. The $K_D$ and therefore the $\Delta G$ for DAPAMe binding to thrombin was very consistent even when different ABE I complexes were formed. At pH 7.4, the $\Delta H$ of binding of DAPAMe to free thrombin (-5.75) was significantly
Figure 4.4. Crystal structure of thrombin (brown) bound to TMEGF456 solved by Fuentes-Prior et al., Nature 2001. Highlighted are the domains of TM; EGF4 (pink), EGF5 (gold), and EGF6 (gray). Active site residues are in green.
Table 4.2. Thermodynamic profile of ligands that bind to ABE I. Thrombin alone (4.5 µM) was placed in the cell and titrated with ABE I ligands (50 µM); ABE I aptamer, TMEGF45, or TMEGF56. The thrombin aptamer has a large favorable enthalpy change upon binding to thrombin whereas no enthalpy change is detectable when TMEGF45 or TMEGF56 bind to thrombin. Experiments were performed in 50 mM Bis-Tris propane, 150 mM NaCl, pH 7.4 at 298K.

<table>
<thead>
<tr>
<th>Ligand in syringe</th>
<th>Kd (M)</th>
<th>ΔH (kcal/mol)</th>
<th>-TΔS (kCal/mol)</th>
<th>ΔG (kcal)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine thrombin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thrombin aptamer</td>
<td>53.2 x 10⁻⁹</td>
<td>-27.1</td>
<td>17</td>
<td>-10.1</td>
<td>0.89</td>
</tr>
<tr>
<td>TM56</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TM45</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 4.5. Comparison of kinetic and thermodynamic measurements of binding of thrombin to TMEGF or to a monoclonal antibody that binds ABE1. Panels A and B are Biacore (SPR) traces of (A) the monoclonal antibody or (B) TMEGF456 binding to thrombin. Below each trace is the corresponding ITC thermograph. TMEGF45 has been used instead of TMEGF456. Data presented is from the paper by Baerga-Ortiz, et al. Protein Science 2004.
Table 4.3. Results from ITC experiments in which DAPAMe binding was measured. For each experiment, 6.5 µM thrombin was used alone or complexed with a different fold molar excess of TMEGF45 in the cell. The complex was then titrated with 70 µM DAPAMe in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4.

<table>
<thead>
<tr>
<th>BTP pH 7.4</th>
<th>Kd (M)</th>
<th>ΔH (kcal/mol)</th>
<th>-TΔS (kCal/mol)</th>
<th>ΔG (kcal)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine thrombin</td>
<td>26 x 10^{-9}</td>
<td>-5.1</td>
<td>-5.1</td>
<td>-10.4</td>
<td>1.1</td>
</tr>
<tr>
<td>bth + TM45 10x</td>
<td>27 x 10^{-9}</td>
<td>-5.1</td>
<td>-5.1</td>
<td>10.2</td>
<td>1.05</td>
</tr>
<tr>
<td>bth + TM45 7x</td>
<td>20 x 10^{-9}</td>
<td>-5.1</td>
<td>-5.4</td>
<td>-10.5</td>
<td>.95</td>
</tr>
<tr>
<td>bth + TM45 5x</td>
<td>27 x 10^{-9}</td>
<td>-5.15</td>
<td>-5.4</td>
<td>-10.6</td>
<td>.97</td>
</tr>
<tr>
<td>bth + TM45 3x</td>
<td>47 x 10^{-9}</td>
<td>-5.4</td>
<td>-4.8</td>
<td>-10.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>
different from the ΔH of binding of DAPAMe to thrombin complexed with TMEGF45 (-5.18, p=0.024), TMEGF56 (-5.0), and the ABE I aptamer (-5.0, p=0.023).

The same set of experiments was then performed at pH 6.5 (Figure 4.7, Table 4.5). Although small differences were observed in the K_D for binding of DAPAMe to thrombin, these were small enough as to not be significant thermodynamic differences. Interestingly, all of the complexes follow the same trend except thrombin-TMEGF45. The ΔH for thrombin alone binding to DAPAMe was even more favorable; -6.6 kcal/mol at pH 6.5 as compared to -5.8 kcal/mol at pH 7.4. Slightly more favorable ΔH of binding were also observed for the thrombin-TMEGF56 and the thrombin-aptamer complexes (Table 4.5). In contrast, the ΔH for binding of the thrombin-TMEGF45 complex to DAPAMe was slightly less favorable; -4.8 kcal/mol at pH 6.5 as compared to -5.15 at pH 7.4.
Figure 4.6. Isotherms titrated with DAPAMe in Bis-Tris propane, pH 7.4
Table 4.4. Comparison of isotherms in different buffer conditions. Buffer in top panel is 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. The buffer in the lower panel (BTP) is 50 mM Bis-Tris propane, 150 mM NaCl, pH 7.4.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Kd (M)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>-T$\Delta S$ (kCal/mol)</th>
<th>$\Delta G$ (kcal)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine thrombin</td>
<td>28.2 ± 3.4 x 10^{-9}</td>
<td>-5.8 ± .13</td>
<td>-4.7</td>
<td>-10.5</td>
<td>1.1</td>
</tr>
<tr>
<td>bth + TM45</td>
<td>26.7 ± 1.1 x 10^{-9}</td>
<td>-5.15 ± .15</td>
<td>-5.39</td>
<td>-10.54</td>
<td>.97</td>
</tr>
<tr>
<td>BTP pH 7.4</td>
<td>Kd (M)</td>
<td>$\Delta H$ (kcal/mol)</td>
<td>-T$\Delta S$ (kCal/mol)</td>
<td>$\Delta G$ (kcal)</td>
<td>N</td>
</tr>
<tr>
<td>Bovine thrombin</td>
<td>25.8 ± 3.0 x 10^{-9}</td>
<td>-5.7 ± .03</td>
<td>-4.7</td>
<td>-10.4</td>
<td>1.1</td>
</tr>
<tr>
<td>bth + TM45</td>
<td>33.4 ± 2.2 x 10^{-9}</td>
<td>-5.2 ± .11</td>
<td>-5.0</td>
<td>-10.2</td>
<td>1.2</td>
</tr>
<tr>
<td>bth + TM56</td>
<td>27.2 ± 1.2 x 10^{-9}</td>
<td>-5.0 ±</td>
<td>-5.3</td>
<td>-10.3</td>
<td>1.2</td>
</tr>
<tr>
<td>bth + aptamer</td>
<td>24.7 ± 1.5 x 10^{-9}</td>
<td>-5.0 ± .15</td>
<td>-5.1</td>
<td>-10.1</td>
<td>1.2</td>
</tr>
</tbody>
</table>
The $K_D$ and $\Delta G$ of DAPA binding to thrombin alone or any of the complexes is similar at pH 7.4. However, DAPAMe binding weakens significantly for thrombin alone and for all the complexes except the thrombin-TMEGF45 complex at pH 6.5.

These results show that binding a ligand at ABE I can affect the thermodynamics of binding of ligands at the active site of thrombin, and the effect can be seen by a thermodynamically coupled event. To test whether binding at the active site might affect the thermodynamics of binding at ABE1, we compared the binding of the aptamer to free thrombin and to thrombin inhibited with the covalent active site inhibitor PPACK (Figure 4.8). Indeed, there is a significant difference between the $\Delta H$ for thrombin -27.1 kcal/mol and PPACK-thrombin -19.3 kcal/mol while the $\Delta G$ remained relatively consistent (-10.1 kcal and -9.7 kcal). This experiment was repeated in Tris-HCl and the results were nearly identical. Binding of a small covalent inhibitor at the active site of thrombin caused a change at ABE I that could be quantitatively measured by an increased dissociation constant, from $K_D= 53 \times 10^{-9}$ for free thrombin to $K_D= 13 \times 10^{-9}$ for the PPACK-thrombin, and a change in the thermodynamic coupling from $\Delta H = -27$ kcal/mol, $-T\Delta S = 17$ kcal/mol, $\Delta G = -10.1$ kcal/mol for the free thrombin and $\Delta H = -19.3$ kcal/mol, $-T\Delta S = 9.6$ kcal/mol, $\Delta G = -9.7$ kcal/mol for PPACK-thrombin. This is direct evidence that communication from the active site to ABE I can alter the thermodynamics of binding at these two sites.
Figure 4.7. Thrombin alone and thrombin complexed with TMEGF45, TMEGF56, or ABE I aptamer titrated with DAPAm in Bis-Tris propane, pH 6.5.
Table 4.5. Comparison of binding thermodynamics at different pHs. Thrombin alone or thrombin in complex with ligand at ABE I was titrated with DAPAMe. The buffer is (BTP) is 50 mM Bis-Tris propane, 150 mM NaCl, pH 7.4, and pH 6.5.

<table>
<thead>
<tr>
<th>BTP pH 7.4</th>
<th>Kd (M)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$-T\Delta S$ (kcal/mol)</th>
<th>$\Delta G$ (kcal)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine thrombin</td>
<td>$25.8 \pm 3.0 \times 10^{-9}$</td>
<td>$-5.7 \pm .02$</td>
<td>$-4.7$</td>
<td>$-10.4$</td>
<td>$1.1$</td>
</tr>
<tr>
<td>bth + TM45</td>
<td>$33.4 \pm 2.2 \times 10^{-9}$</td>
<td>$-5.2 \pm .11$</td>
<td>$-5.0$</td>
<td>$-10.2$</td>
<td>$1.06$</td>
</tr>
<tr>
<td>bth + TM56</td>
<td>$27.2 \pm 1.2 \times 10^{-9}$</td>
<td>$-5.0 \pm$</td>
<td>$-5.3$</td>
<td>$-10.3$</td>
<td>$1.10$</td>
</tr>
<tr>
<td>bth + aptamer</td>
<td>$24.7 \pm 1.5 \times 10^{-9}$</td>
<td>$-5.0 \pm .15$</td>
<td>$-5.1$</td>
<td>$-10.1$</td>
<td>$1.06$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BTP pH 6.5</th>
<th>Kd (M)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$-T\Delta S$ (kcal/mol)</th>
<th>$\Delta G$ (kcal)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine thrombin</td>
<td>$76.5 \pm 5.0 \times 10^{-9}$</td>
<td>$-6.6 \pm .27$</td>
<td>$-3.1$</td>
<td>$-9.7$</td>
<td>$1.1$</td>
</tr>
<tr>
<td>bth + TM45</td>
<td>$45.2 \pm 10^{-9}$</td>
<td>$-4.8 \pm$</td>
<td>$-5.2$</td>
<td>$-10$</td>
<td>$1.2$</td>
</tr>
<tr>
<td>bth + TM56</td>
<td>$159 \pm 10^{-9}$</td>
<td>$-6.2 \pm$</td>
<td>$-3.0$</td>
<td>$-9.2$</td>
<td>$1.2$</td>
</tr>
<tr>
<td>bth + aptamer</td>
<td>$186 \pm 10^{-9}$</td>
<td>$-5.78 \pm$</td>
<td>$-3.3$</td>
<td>$-9.1$</td>
<td>$1.2$</td>
</tr>
</tbody>
</table>
**Figure 4.8.** Thrombin or PPACK-thrombin was titrated with the ABE I aptamer in 50 mM Bis-Tris propane, 150 mM NaCl, pH 7.4.

<table>
<thead>
<tr>
<th>Bovine thrombin</th>
<th>Kd (M)</th>
<th>ΔH (kcal/mol)</th>
<th>-TΔS (kCal/mol)</th>
<th>ΔG (kcal)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>thrombin aptamer</td>
<td>53.2 x 10^{-9}</td>
<td>-27.1</td>
<td>17</td>
<td>-10.1</td>
<td>0.89</td>
</tr>
<tr>
<td>PPACK-Bovine thrombin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thrombin aptamer</td>
<td>13.2 x 10^{-9}</td>
<td>-19.3</td>
<td>9.6</td>
<td>-9.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>
D. DISCUSSION

ABE I is a binding site for fibrinogen, TM, and hirudin, and a variety of DNA aptamers which are mostly small modifications of the core sequence discovered by Bock et al. (Bock, Griffin et al. 1992). The Fuentes-Prior crystal structure elucidated that it was Arg104, Arg106, Arg109, Tyr107, Ile114 (Arg73, Arg75, Arg77a, Tyr76, and Ile82 in chymotrypsin numbering) that were interaction sites for TM-binding and amide exchange experiments demonstrated solvent exclusion upon TM binding in these same residues (Fuentes-Prior, Iwanaga et al. 2000; Croy, Koepp et al. 2004). When thrombin is titrated with the ABE I aptamer, a favorable enthalpy change of -27.1 kcal/mol was measured. In contrast, no enthalpy change is observed for the binding of TM with thrombin. Remarkably, these two ligands bind to the same site on thrombin. In earlier work, Baerga-Ortiz et al also observed a large $\Delta H$ for binding of a monoclonal antibody to ABE1 (cite Baerga-Ortiz 2004). The difference in the thermodynamics of binding is only seen in the $\Delta H$ and not in the overall $\Delta G$. DAPAMe binds to thrombin alone or to thrombin-TM complexes with a similar $\Delta G$ but different $\Delta H$ revealing a compensation between entropy and enthalpy of binding. When the pH is decreased from 7.4 to 6.5, differences emerge between the different thrombin complexes. Whereas at pH 7.4, any ligand bound at ABE1 decreased the DH of binding a similar amount, at pH 6.5, binding weakened for all complexes except the thrombin-TMEGF45 complex. The weakened binding of DAPAMe to all the complexes is mainly due to a less favorable change in entropy upon binding, the enthalpy change actually becomes slightly more favorable at the lower pH. In
contrast, at pH 6.5, binding of DAPAME to the thrombin-TMEGF45 complex remains strong, with thermodynamic constants that are essentially the same as those observed at pH 7.4.

**Effect of binding PPACK at active site**

When PPACK-thrombin is titrated with aptamer, a different thermodynamic profile of binding is seen than with free thrombin. It is interesting to note that in all cases, only the $\Delta G$ remained consistent. The observation of compensation of $\Delta H$ and $\Delta S$ without an overall change in $\Delta G$ leads to the hypothesis that the allostery between the two sites is dynamic. Croy et al. showed that PPACK-thrombin had an unfolding temperature of 79°C whereas free thrombin unfolded at 57°C (Croy, Koeppe et al. 2004). Thus PPACK binding greatly stabilizes thrombin. The stabilization upon PPACK binding seems to be related to a decrease in dynamics because the PPACK-bound thrombin showed a decrease in solvent accessibility in ABE I and along a communication loop from the active site of thrombin by amide exchange experiments (Croy, Koeppe et al. 2004).

Taken together, these observations suggest that changes in backbone dynamics occur at ABE I the active site is occupied and *vice versa*. The traditional definition of allostery usually involves a ligand altering the free energy of binding for another ligand at a second site (Monod, Wyman et al. 1965; Koshland, Nemethy et al. 1966). Furthermore, allostery is often thought to involve conformational changes as well. The allostery observed in thrombin does not fit either of these two definitions. TM binds to free thrombin or to PPACK-thrombin with identical binding energy (Baerga-Ortiz 2000). Furthermore, the crystal structure of thrombin bound to TMEGF456 shows no
differences when compared to the PPACK inactivated thrombin structure solved by Bode et al. (Fuentes-Prior, Iwanaga et al. 2000) (Bode, Turk et al. 1992). The results of all of our isothermal titration calorimetry experiments uncovered an unusual thermodynamic coupling in which binding at one site changed the balance of $\Delta H$ and $T\Delta S$ but not the overall $\Delta G$. Thus, the allostery observed in thrombin has all the hallmarks of a new type of allostery, termed dynamic allostery, in which no large conformational changes are elicited by effector binding, instead a transmission of dynamic changes is observed. Enthalpy and entropy compensations, are manifested as only subtle changes in free energy (Tsai, del Sol et al. 2008). This type of allostery is now accepted to be operating in such exquisitely controlled enzyme systems as kinases (Popovych, Sun et al. 2006). We and others have already shown that TM is both providing a docking surface to "prime" protein C for activation, as well as altering the active site of thrombin to accept protein C as a substrate (Rezaie and Yang 2003; Koepe, Seitoa et al. 2005). At pH 6.5, a difference in all three ABE I ligands could be seen. This thermodynamic coupling difference may indicate how ligand specificity is communicated from ABE I to the active site of thrombin.
Chapter V

Thrombomodulin TMEGF456mini: Future Directions and Insights
A. Introduction

In the preceding chapters we have characterized the interaction between thrombin and TM through protein C and Biacore kinetic assays, fluorescence, and ITC. Through our studies we have seen that the 4th domain of TM has an allosteric effect on inhibitors at the active site as well as catalytic importance in the activation of protein C. What is not apparent, however, is how the 6th domain transfers its influence to the 5th domain. TMEGF456 aggregates at concentrations required for solution studies (Komives lab, unpublished). To investigate the relationship between the 5th and 6th domains, therefore, TMEGF56 was expressed and characterized. It was our hope that the solution structure and dynamics of TMEGF56 would be solved alone and bound to thrombin. The results of these experiments would reveal the effect that the 6th domain of TM has on the 5th domain and what is the structure of the 5th domain before, and upon binding to thrombin. As reported in Chapter II, HSQC data from the protein caused us to abandon our efforts for a solution structure and instead we used TMEGF56 to focus on kinetic and thermodynamic studies of the thrombin-TM interaction.

Tolkatchev et al. synthesized a 28 residue 6th domain peptide fragment spanning from Tyr422 to Gly449 and performed NMR 1D experiments to determine the structure of the peptide. (Tolkatchev, Ng et al. 2000). They found that the 1-3, 2-4 isomer of the peptide bound thrombin more tightly (K_D 80 µM) than other conformers. They were also able to concentrate the peptide to 0.5 mM. This led us to think that we could make a truncated version of TMEGF456 and still possibly retain
its full binding affinity to thrombin. We then designed TMEGF456mini, which terminates at Gly449 and has a C448S mutation since the sixth cysteine is missing in this truncation (see Figure 5.1). This chapter will detail the purification process and activity of our products. Also to be discussed are future directions for the project.

**Figure 5.1.** Schematic of TMEGF456mini. Residues in gray are absent. Cysteine 448 had been mutated to Serine to prohibit incorrect disulfide bond formation in the protein. EGF5 residues important for thrombin binding are in purple. Residues that participate in the calcium binding site are yellow. Note that Residues in the linker region between EGF5 and EGF6 are important to binding thrombin and calcium binding.
B. MATERIALS AND METHODS

1. Expression of TMEGF56

TMEGF456mini was subcloned from the TMEGF456 gene which was synthesized using optimized E. coli codons made by Chris White and expressed in Pichia pastoris similar to that which was described by White et al. (White, Hunter et al. 1995). DNA was amplified by PCR and then cloned into the pPIC9 vector. This step was necessary because the expression vector pPIC9K contains a second XhoI cleavage site in the kanamycin resistance gene. Retention of this XhoI at the KEX2 cleavage site is required for post-translational processing of the α-factor leader sequence used to direct secretion of the protein. The SacI–SalI fragment of pPIC9 containing TMEGF456mini gene was subcloned into pPIC9K to produce the final P. pastoris expression vector. The protein sequence begins at Glu346 and continues to the Gly449. Cys448 is the start of the 3rd disulfide bond in the 6th domain and was mutated to serine. In addition, the TMEGF56 protein produced by this vector contains histidine and methionine at the 5’ end; due to an NdeI site necessary for subcloning into a short shuttle vector pBKsh2. Plasmid DNA was transformed into P. pastoris SMD1168 cells, which had previously been proven to yield large quantities of TMEGF45 and expression was monitored as described previously (White, Hunter et al. 1995).

For purification from shake flasks, P. pastoris supernatant was first purified by anion-exchange chromatography (HiLoad 26/10 Q Sepharose) equilibrated in 50 mM MES buffer, pH 6.5. A step gradient of 0, 12%, and 30% buffer B (50 mM MES, pH 6.5, 1
M NaCl) was used, and the TM fragments eluted in the 30% fraction. This step was followed by reverse-phase HPLC as described (Wood and Komives 1999). Finally, TMEGF456mini was purified using size-exclusion chromatography (HiLoad 16/60 Superdex 75, Amersham/GE Healthcare) in 50 mM Tris-HCl, 150 mM NaCl, 2.5 mM CaCl₂, pH 7.4. For purification of TMEGF456mini from the fermentor, an initial purification step on QAE sephadex column was added using the same buffer as in the HiLoad Q step.

2. Protein C activation inhibition assay

To measure the thrombin-TM activation of protein C, 15 µL of active thrombin (0.004 mg/mL in TBS) were mixed with 10 µL of TMEGF456 (0.001 mg/mL in TBS) in 80 µL of TBS 0.1% BSA, 5 mM CaCl₂ in a 96-well plate for 10 min, allowing the thrombin-TM complex to form. After 10 min, 20 µL of 0.06 mg/mL human protein C (Haematologic Technologies, Essex Junction, VT) were added. After 20 min the reaction was quenched by addition of 40 µL of a solution of heparin (0.075 mg/mL, Calbiochem, San Diego, CA) and antithrombin III (0.070 mg/mL, Haematologic Technologies, Essex Junction, VT). The amount of activated protein C was determined using a chromogenic substrate, S2366 (Diapharma, West Chester, OH). An enzymatic unit is 1 nmol of activated protein C produced per min and was determined from a standard curve generated from activated protein C added to S2366 under the same conditions as the assay (White, Hunter et al. 1995).
3. Mass Spectrometry

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Voyager DE-STR instrument (Applied Biosystems) as previously described (Wood and Komives 1999). The matrix used was 10 mg/ml sinapinic acid (Sigma-Aldrich) dissolved in a solution containing 700 µL 2% TFA and 300 µL acetonitrile. A sample of TMEGF456mini was therefore deglycosylated using endoglycosidase H. Deglycosylation of 0.5 mg/ml protein was accomplished in 1 ml 100 mM potassium acetate buffer, pH 5.4, by incubation at 37°C overnight with 10 mU endoglycosidase H (recombinant from Boehringer-Mannheim). No differences in protein solubility were noticed with the deglycosylated protein.

C. RESULTS

1. Expression and Purification of TMEGF456mini.

Expression and purification was carried out in exactly the same fashion as TMEGF56 (White, Hunter et al. 1995), (Chapter 2). In the first step, a HiLoad Q column, TMEGF456mini elutes at the 30% NaCl step of the gradient, the same fraction that TMEGF56 and TMEGF456 elute. One major difference in the purification is that we have added an HPLC step; TMEGF456mini elutes slightly later than TMEGF56 on a RP-HPLC column at 37% acetonitrile instead of 35%. It is interesting to note that full length TMEGF456 has decreased activity when subjected to RP-HPLC, another indication that TMEGF456mini is different than either TMEGF56 or TMEGF456. Another major difference is seen in the size exclusion step of the purification. Almost all of the TMEGF456mini produced has a molecular
weight of 17 kD, instead of two distinct large peaks of different molecular weights as was the case for TMEGF56 (Figure 5.2). Purification from shake flasks yielded 1.2 mg/L of pure protein.

Table 5.1 summarizes the specific activities associated with each step of the purification process. The specific activity was $9.1 \times 10^5$ which was very close the value reported by Esmon et al. for human TM of $1 \times 10^6$ and the specific activity for rabbit TM (full length without the condroitin sulfate moiety) of $1.7 \times 10^6$ (Esmon, Esmon et al. 1982).

We are currently working out conditions for production of TMEGF456mini by fermentation. Usually fermentation increases protein expression dramatically by as much as 100 fold, a result seen in our lab as well as among other researchers (White, Hunter et al. 1995). While it remains to be seen what results of fermentation will be, we are optimistic. Ferментations are required to produce the quantities of protein needed for biophysical characterization and structure studies such as solution NMR and ITC. We can still however proceed with this project in the areas of kinetics and H/D exchange by mass spectrometry, both of which require much smaller protein quantities.

Mass spectra of TMEGF456mini are shown for the fully glycosylated protein and the deglycosylated form in Figure 5.3. The protein was deglycosylated by addition of endoglycosidase H, which cleaves at the $\beta 1 \rightarrow 4$ linkage between GlcNAc residues so that one GlcNAc is left and must be accounted for in the mass calculation. The fully glycosylated protein has a mass of $\sim 16900$ Da and has a remarkably sharp peak compared to other glycosylated TMs produced in yeast. This means that only a
few glycoforms have been made instead of the broad spectrum of forms we see in TMEGF45 and TMEGF56 (see Chapter 2 Figure 5.4). This peak is more characteristic of what we see in glycosylated proteins produced in mammalian systems such as the TMEGF56 we use for a standard from CHO cells. Deglycosylation yields a 12,017 Da product, which is exactly the expected mass. Deglycosylation reduces the mass by 4928 Da, meaning each substituted asparagine residue has approximately 2100 Da of mannose residues attached, which corresponds to about 10 mannose residues per N-linked oligosaccharide and is typical of branched mannose glycosylation (Bretthauer and Castellino 1999). This same amount of glycosylation was observed for TMEGF45 (White, Hunter et al. 1995).

Figure 5.2. Comparison of sized exclusion trace of TMEGF56 (A) and TMEGF456mini (B). The fraction with the highest specify activity for TMEGF456mini has is denoted by an arrow.
Table 5.1. Purification of TMEGF456mini. *1 unit of activity = 1nmol aPC produced per min. The specific activity of rabbit TM purified by the method of Esmon et al 1882 was $1.7 \times 10^6$.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (ml)</th>
<th>Activity /ml (U/ml)</th>
<th>Protein Mg/ml</th>
<th>Specific* Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Broth</td>
<td>1000</td>
<td>$2.9 \times 10^3$</td>
<td>0.4</td>
<td>$7.2 \times 10^3$</td>
</tr>
<tr>
<td>HiLoad Q</td>
<td>20</td>
<td>$6.5 \times 10^3$</td>
<td>1</td>
<td>$6.5 \times 10^4$</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>10</td>
<td>$1.54 \times 10^3$</td>
<td>0.9</td>
<td>$1.71 \times 10^5$</td>
</tr>
<tr>
<td>SEC</td>
<td>2.5</td>
<td>$3.55 \times 10^3$</td>
<td>0.39</td>
<td>$9.1 \times 10^5$</td>
</tr>
</tbody>
</table>
Theoretical MW = 11608.94 + 2 GlcNAc (204) = 12017 Da

**Figure 5.3** MALDI mass spectra of fully glycosylated TMEGF456mini (A) and the protein deglycosylated by endoglycosidase H (B) with major peak labeled.
D. Discussion and Future Directions

1. Protein C and thrombin binding kinetics

Determining the kinetics of the interaction by is the first order of business for our lab to investigate if indeed TMEGF456mini has the full binding power of TMEGF456. Kinetics will be determined by Protein C activation assay experiments to determine $K_m$ and $K_m$ as we have previously published and that has been described in Chapter 3. The second step will be SPR by Biacore by which we will determine the $K_d$ for the interaction. We have already formed a library of mutants that have mutations in the 4th domain of TM and affect thrombin binding and/or protein C activation (Beach, Koeppe submitted, Chapter 3). We can now extend our library with TMEGF456mini by testing double and triple mutants that effect $k_{cat}$ such as Y358F, M388L, both of which have increased specific activity in TMEGF456, to see if the effects are additive and we can make super active, or conversely combine D349N and D400E to make an inactive mutant.

2. Thermodynamics and Solvent Accessibility by ITC and H/D

H/D exchange by mass spectrometry experiments enabled us to explore the interfaces of the thrombin-TM interaction in many ways. TMEGF456mini has the full specific activity as TMEGF456, and is able to be purified by HPLC and concentrated to at least 0.5 mM without loss of activity. We are eager to begin work to see how the fully cofactor active and thrombin binding TM interacts with thrombin. Previous H/D MS experiments revealed differences in exchange at the active site when TMEGF45 vs. TMEGF56 were bound, however these experiments required a 7-fold excess of
TMEGF45 (Koepe, Seitova et al. 2005). It will be interesting to compare TMEGF456mini with TMEGF56mini to see if these differences are still present.

Some natural inhibitors and mimetic peptides are highlighted in Table 5.2. The plasma concentration of prothrombin is the highest of any of the blood factors and is estimated at 1.2µM, but the plasma concentration of active thrombin does not exceed 0.06-0.09µM (Machovich 1984). Thrombin is tightly regulated by a number of proteins, some of which bind directly to ABE I (fibrinogen, hirudin, TM) and ABE II (PAR I, heparin) (Esmon and Owen 1981; Machovich 1984; Fenton 1986; Esmon 2000). There is evidence showing binding ligands such as monoclonal antibodies, platelet receptor GP1bα, and heparin at ABE II causes changes at ABE I and the active site (Fredenburgh, Stafford et al. 1997; Colwell, Blinder et al. 1999; Verhamme, Olson et al. 2002). We have provided evidence that binding at ABE I changes the thermodynamics and solvent accessibility of the active site for small inhibitors. This information is of great importance for drug delivery and pharmaceutical researchers who would like to know how allostery potentially affects drug binding in vivo. We have contacted many different researchers and companies in the past and have not been able to obtain other tight binding non-covalent inhibitors for the active site until recently. We have now negotiated an agreement with a biotech company in England to test its novel direct thrombin active site inhibitors with TM. ITC provides a direct, fast, reproducible way to quantify the $K_d$ and $\Delta H$ of a reaction. We will also be able to test the effect of ABE II-binding through another single stranded DNA 29mer for ABE II created in the same manner as Bock et al. (Bock, Griffin et al. 1992).
Table 5.2. Amino Acid Alignment for Naturally Occurring Substrates of Thrombin.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>P6</th>
<th>P5</th>
<th>P4</th>
<th>P3</th>
<th>P2</th>
<th>P1</th>
<th>P1’</th>
<th>P2’</th>
<th>P3’</th>
<th>P4’</th>
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<tr>
<td>Fibrinogen</td>
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<tr>
<td>bovine (Aα)</td>
<td>Glu-Gly-Gly-Gly-Val-Arg</td>
<td>Gly-Arg-Val</td>
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<tr>
<td>human (Bβ)</td>
<td>Gly-Phe-Phe-Ser-Ala-Arg</td>
<td>Gly-His-Arg-Pro</td>
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<tr>
<td>PPACK</td>
<td>Phe--</td>
<td>Pro--Arg</td>
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<td>Protein C</td>
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<tr>
<td>human</td>
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<td>Leu--Ile--Asp--Gly</td>
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<tr>
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<td>Ile--Val--Asp--Gly</td>
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<tr>
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<td>Ser--Leu--Asn--Pro</td>
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<tr>
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</table>

*a Sequences were obtained from searching the gene databank at NCBI and from the CRC publication edited by Machovich. (Machovich 1984).
*b P# denotes the number of positions the residue precedes the P1 Arg.
*P’ denotes the number of positions the residue comes after the P1 Arg.
The two repulsive P3 and P3’ Asp residues in the protein C substrate are in bold.
3. Structure and Dynamics of the free and thrombin bound form of TM via solution NMR

To probe the interaction between the 5th and 6th domain of TM, we attempted to create TMEGF56. The peak dispersion in $^{15}$N-HSQC data acquired on the protein showed a large amount of overlap in the center of the spectra and a large variance of peak intensities indicating that the protein may be dynamic. The overlap issues were not overcome by any buffer changes and we abandoned that aspect of the project. TMEGF456mini gives us a sign of hope that we may someday have a solution structure and dynamics of TM with most of its 6th domain, including residues that have been found to play a role in thrombin affinity (Nagashima, Lundh et al. 1993). Although the piece of TM studied by Tolkatchev et al. was missing much of the 4th and 5th domains, it bound thrombin (Tolkatchev, Ng et al. 2000). In addition, the crystal structure shows interactions of thrombin with the N-terminal part of the 6th domain (Fuentes-Prior, Iwanaga et al. 2000). We are also optimistic that we will be able to measure differences in the dynamics of the 5th domain due to the presence of the 6th domain, something that has not been possible yet (Wood, Sampoli-Benitez et al. 2000; Wood, Prieto et al. 2005). The presence of a few dominate glycoform instead of a broad array of glycoforms will make the protein more homogeneous and better suited for NMR. Finally, we are optimistic that the improved binding affinity of TMEGF456mini will finally make dynamics studies on the thrombin-TM complex possible. It will be very interesting to see how the backbone dynamics of TM change upon binding to thrombin in a residue specific manner.
REFERENCES


Ye, J., N. L. Esmon, et al. (1991). "The active site of thrombin is altered upon binding to thrombomodulin. Two distinct structural changes are detected by fluorescence, but only one correlates with protein C activation." J. Biol. Chem. 266: 23016-23021.

