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**OXIDATION OF THROMBOMODULIN METHIONINE 388 IN CIGARETTE
SMOKERS**

**OXIDATION OF THROMBOMODULIN METHIONINE 388 IN CIGARETTE
SMOKERS**

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Chemistry

By

Samrat Bar Singh Thapa
Lyon College
Bachelor of Science in Biology, 2005

May 2013
University of Arkansas

ABSTRACT

This work tested the hypothesis that oxidation of methionine 388 in thrombomodulin is higher in cigarette smokers, and thus a likely contributor towards the hypercoagulable state in smokers. Thrombomodulin, a protein cofactor found on endothelial cell surfaces, regulates the activity of thrombin. Thrombin bound to thrombomodulin no longer converts fibrinogen to fibrin, but instead activates Protein C which, in turn, stops the coagulation cascade by inactivation of clotting factors. The oxidation of methionine 388 of thrombomodulin has been shown *in vitro* to dramatically decrease the anticoagulant cofactor activity of thrombomodulin. The blood of cigarette smokers is more prone to clot than that of non-smokers, a major factor in their premature deaths from cardiovascular disease. Cigarette smoke consists of many oxidizing species that impose oxidative stress on the body. These species include organic radicals and hydrogen peroxide, which can oxidize methionine. The fact that smokers are in a hypercoagulable state has been established, however the molecular origins of such a state have not been elucidated. Techniques were developed to isolate thrombomodulin cleared from the blood stream in urine, proteolytically digest it, and identify using mass spectrometry the peptide containing methionine 388 in both its oxidized and reduced forms. In many cases the oxidized version of the peptide was below the limits of detection in non-smokers and the reduced version was not detected in smokers. The intensity of these peaks in the mass spectra do not allow calculation of absolute percentages of oxidation because of differences in proton affinity of the two forms, but there is a very statistically significant difference ($P=0.002$ by Mann-Whitney Rank Sum test) in the apparent median reduced to oxidized ratios of >2.043 for non-smokers and of <0.308 for smokers. The much greater degree of oxidation of thrombomodulin methionine 388 in smokers should be examined for its contribution to smoking morbidity and mortality.

This dissertation is approved for recommendation
to the Graduate Council.

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Firstly, I would like to express my deepest gratitude to my research advisor Dr. Wesley E. Stites for taking me under his wings. Thank you for advising and guiding me, providing me the room to grow as a scientist, and mostly for your patience and great attitude. Looking back, if I had to redo things, I can't think of a scenario where I would not choose you as my advisor. I look forward to work with you on our elevator to Everest project.

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DEDICATION

This dissertation is dedicated to Dhruba Bar Singh Thapa LL.M. (McGill University, Montreal, Canada), Professor of International Law (Tribhuvan University, Kathmandu, Nepal), Visiting Scholar (Max Planck Institute, Heidelberg, Germany), Secretary of Ministry of Law and Justice (Government of Nepal), Dean of Institute of Law (Tribhuvan University, Kathmandu, Nepal), Election Commissioner (Government of Nepal), and my late grandfather.

I wonder, if you were still around....

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CHAPTER 1: DOES THE OXIDATION OF METHIONINE 388 IN THROMBOMODULIN
CONTRIBUTE TOWARDS THE HYPERCOAGUABLE STATE IN CIGARETTE
SMOKERS?

General introduction

Proteins are susceptible to oxidation and there are several consequences of that. Oxidation can lead to modification of side chain properties, dimerization of the protein, conformational changes, unfolding of the protein or loss of activity, cleavage of the protein backbone [1], effects on expression and gene regulation, and affect cell signaling [2]. Studies have shown higher levels of oxidized proteins are found with aging, oxidative stress, also with diabetes, atherosclerosis and neurodegenerative diseases [3-6]. Amino acids liable to oxidation [7, 8] are cysteine, methionine, tryptophan, phenylalanine, tyrosine, histidine, arginine, lysine, proline, and threonine. Cysteine and methionine stand out as the two amino acids that are most easily oxidized [3]. Cysteine can easily be converted to disulfide and methionine to methionine sulfoxide.

Aerobic organisms rely on oxygen for the production of ATP during oxidative phosphorylation. Oxygen is the ultimate electron acceptor during this biosynthesis, and water is one of the byproducts of this reaction. However, transfer of single electron to a molecule of oxygen results in production of superoxide anion, a highly reactive radical. The protonated superoxide radical can react with another molecule of superoxide anion, which produces hydrogen peroxide. Superoxide radical falls under a category of compounds called reactive oxygen species (ROS). Physiological levels of ROS are important for cellular functions like gene expressions, signal transduction, apoptosis and immune response [9]. However, high concentrations of ROS can damage biomolecules like DNA, lipids and proteins. *Superoxide dismutase*, an enzyme present in all aerobic organisms, along with *catalase*, a heme protein, converts the superoxide radical and hydrogen peroxide into water and molecular oxygen and act

as just one of the defense mechanisms against oxidative damage. However, oxidation of proteins still occurs, often at methionine.

Methionine is a non polar amino acid; along with cysteine they are the only two amino acids that contain sulfur. The sulfur atom exists as a thioether linkage in methionine. In the presence of reactive oxygen species (ROS), methionine is converted to methionine sulfoxide. In contrast to methionine, methionine sulfoxide's side chain is polar. The conversion of important methionine residues to sulfoxide within a protein, can lead alteration of structure and functionality [10]. The sulfoxide form of methionine can be converted back to methionine by naturally occurring methionine sulfoxide reductases (MSRs) [4, 11, 12]. It has been shown in Alzheimer's disease [13, 14] and aging, the physiological concentrations of MSRs are low, and they are unable to reduce all sulfoxides. This has led to speculation that oxidative stress and methionine oxidation contribute to the development of Alzheimer's disease [15].

Why might oxidation of a protein lead to such far-reaching consequences as Alzheimer's? Proteins are ubiquitous in all biological processes, and they play crucial roles in them. They are associated with enzyme catalysis, immune system, nerve impulses, motion, mechanical support, transport and storage among other processes that occur within a living organism. The remarkable range of activities proteins perform arises from their ability to fold into unique three-dimensional structures. This allows them to interact with wide range of molecules. Some of the noteworthy features of the native protein are: Polypeptides can fold in such a fashion, that distant amino acid residues on the polypeptide chain can be close in proximity to each other. Globular proteins are compact; they exclude almost all water from the inner hydrophobic core. The native protein is stabilized in the tertiary state by the following types of interactions. There are hydrophobic interactions, where hydrophobic side chains are

brought into close proximity to each other. Electrostatic interactions occur between the ionic groups. Hydrogen bonding, where hydrogen bonds are formed between the side chains and also between the polypeptide backbones. Covalent bonds, disulfide bonds formed between the 2 cysteine residues. If any of these interactions are disrupted, it could alter the structure of the protein, and consequently alters the functionality of the protein, which could lead to complications, or worse lead to development of diseases. Sick cell anemia demonstrates how a substitution of single amino acid can have drastic effect on the functionality of a protein. In sickle cell anemia, body produces a variant of hemoglobin, where sixth position of the β -globin gene codes for valine, instead of glutamic acid [16]. Could high levels of oxidation of a single side chain in the right protein have similarly profound consequences?

Cardiovascular diseases are the number one cause of death in the United States. The American Heart Association estimates that one out of three Americans die of cardiovascular death, and 80,700,000 people in the United States have one or more forms of cardiovascular disease [17, 18]. National Health and Nutrition Examination Survey (NHANES) reports 7.2% of Americans to have some type of cardiovascular diseases. This number comprised of 3.2% with coronary heart disease, 2.7% with stroke, and 2.0% with congestive heart failure, also many patients reported more than 1 condition [19].

Annually 437,900 people are killed by diseases caused by smoking, and 35% of those deaths are cardiovascular related [20]. Cigarette smoke is the major cause of pulmonary emphysema [21], bronchitis, myocardial infarction, and stroke as well as lung cancer. Four million people die every year from tobacco smoking related diseases worldwide. It has been estimated that 2 billion people use tobacco products [22]. Approximately 64.5 million people in

the USA are active smokers, between 1995-1999 the estimate of deaths that resulted from smoking and exposure to secondhand smoke was approximately 440,000 annually [23].

Cigarette smoke and oxidation

Cells and tissues are vulnerable to oxidative damage; however due to the proximity and function of the lung epithelial tissues, the components of the cigarette smoke makes them more vulnerable to oxidative damage. The epithelial cells of the lungs can be damaged by cigarette smoke from direct interaction of its components [24, 25].

Cigarette smoke is a complex mixture of over 4700 chemical compounds, including high concentrations of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [26-30]. Cigarette smoke contains epoxides, peroxides, nitric oxide (NO), nitrogen dioxide, peroxy nitrates, semiquinone, hydroxyl radical, and many other free radicals [29]. Besides the toxic components of cigarette smoke, lungs of cigarette smokers are further burdened by secondary oxidative stress triggered by ROS and RNS from cigarette smoke. It has been demonstrated that increased ROS and RNS levels leads to activation of transcription factors, signal transduction and gene expression of proinflammatory mediators which generates inflammation response. There are increased numbers of neutrophils and activated macrophages in the lungs of cigarette smokers [29, 30], and they are known to release large amount of hypochlorite, hydrogen peroxide and superoxide [31]. The inflammatory response and release of ROS under normal conditions is important in immune response. However, when the inflammation becomes chronic, the neutrophils and macrophages are perpetually activated, this creates severe oxidative damage to the cells and tissues of cigarette smokers [32], and that may predispose smokers to lung diseases.

Emphysema and cigarette smoke

Pulmonary emphysema (PE) is disease characterized by progressive and irreversible destruction of the lung parenchyma; it is a cause for lot of morbidity and mortality. It belongs to class of disease clinically classified as a chronic obstructive pulmonary disorder (COPD) [33-35]. In PE the air spaces distal to the terminal bronchiole are enlarged and alveolar walls are destroyed [36], as a result the lungs loses their elastic recoil force, which is required to drive the air out of the lungs. The volume airflow in the lungs is severely reduced due to loss of lung elasticity. PE can be subdivided into two types. Centrilobular emphysema, which is associated with cigarette smoke, is where there is dilation or destruction of bronchioles. Panlobular emphysema, which results from the deficiency of α 1-antitrypsin [36], is characterized by dilation and destruction of acinus. Studies have shown that approximately 40% of heavy smokers develop PE over the span of four decades [37].

α 1-Antitrypsin is a 52-kDa glycoprotein that protects the lungs from elastic damage. It is the major antiprotease of alveolar region; it provides protease/antiprotease balance. Neutrophil elastase (NE), a serine protease, is capable of destroying structural components of the alveolar wall of the lungs, α 1-Antitrypsin protects the lungs from NE. In people with hereditary deficiency of serum antitrypsin have low concentration of α 1-Antitrypsin in the blood, despite normal production by the liver. Due to low concentration of the enzyme in the blood, the required amount of α 1-Antitrypsin is unable to reach the lungs, resulting in unopposed activity of protease, leading to systematic destruction of the lungs. People with this hereditary deficiency usually develop PE.

The relationship between smoking and PE has been established by many studies. In one study, elastase inhibitory activity between smokers and non smokers were compared based on the lung fluid obtained from the donors, the cigarette smokers had two fold reduction in their anti elastase ability compared to the non smokers [38]. The methionine residues are oxidized to methionine sulfoxides in α 1-Antitrypsin found in broncho alveolar lavage (BAL) fluid [39]. In order to understand the molecular basis of lowered anti elastase activity in cigarette smokers Taggard et al. studied the effect of hydrogen peroxide, found in cigarette smoke, on the methionine residues found in α 1-Antitrypsin [40]. They found that the methionine residues were susceptible to oxidation. There are 9 methionine residues in α 1-Antitrypsin, however only 2 residues, methionine 351 and 358, are most susceptible to oxidation. Also, the oxidation of either Met351 or Met358 resulted in inactivation of α 1-Antitrypsin. They produced recombinant α 1-Antitrypsins, where one or both met residues were substituted with valine. The recombinant proteins with single substitutions were not easily inactivated as the wild types, and the double mutant was resistant to oxidative damage. They established that the oxidation of the 2 methionine residues takes away the anti elastase capacity of α 1-Antitrypsin. Combination of increased protease activity and proteases inactivation by oxidants have also been associated in diseases like cystic fibrosis, bronchopulmonary dysplasia, hyperoxia-induced lung damage, adult respiratory distress syndrome[40].

Hemostasis and thrombomodulin

The physiological process that stops bleeding, by formation of hemostatic plug, at the site of an injury while maintaining normal blood flow elsewhere in the circulation is referred as hemostasis [41]. The hemostasis can be divided into primary, secondary hemostasis and fibrinolysis [42]. Primary hemostasis is the primary response of the platelets towards an

endothelial injury. In primary hemostasis, plugging of the injury results when platelets are activated and they bind to the site of injury and with each other. Platelets circulate in the blood for approximately 10 days and their concentration ranges from 100 to 400 million per milliliter of blood [43]. Under normal physiological conditions they do not aggregate with each other nor do they adhere to the surfaces. In the event of injury, several platelet receptors in presence of their corresponding ligands activate the platelets, which results in platelets aggregation and adherence to the injured site.

Secondary hemostasis consists of the generation of thrombin and insoluble fibrin, and subsequent deposition of fibrin into and around the platelets on the site of injury. The fibrin forms a crosslinked mesh that strengthens and stabilizes the clot [41]. The initiation of secondary hemostasis involves coagulation cascade of serine proteases that results in cleavage of soluble fibrinogen by thrombin. The presence of anticoagulants like thrombomodulin and heparan sulfate proteoglycans prevents the activation of serine proteins cascade in normal blood vessels. In the event of injury to the blood vessels, the blood gets exposed to tissue factor, which activates factor VIIa [44], which subsequently activates factor X. The factor X can also be activated by factor IXa in presence of factor VIIIa. Prothrombin is activated by factor Xa in the presence of cofactor factor Va resulting in generation of thrombin [45]. Thrombin cleaves fibrinogen to produce insoluble fibrin. The coagulation cascade or waterfall model of proteolytic reactions was proposed in 1960s, and they are based on two complementary blood clotting models [46, 47]. This cascade has received general acceptance. The steps in the coagulation cascade look like Y-shape, with intrinsic and extrinsic pathway converging to form a common pathway at factor Xa. The coagulation cascade has been shown to be insufficient to predict bleeding tendency *in vivo* [48, 49]. In the past decade a cell based model of coagulation has been

proposed for the secondary hemostasis [48, 49]. This model consists of three phases: initiation, amplification, and propagation. During initiation there is production of prothrombinase on the cell surface the cells that express tissue factor, followed by production of low amounts of thrombin. During the amplification step thrombin activates the platelets and cofactors V and VII. In the propagation step, other platelets in the bloodstream are recruited on the site of injury, which leads to generation of large amounts of thrombin, which amounts production of fibrin to stabilize the platelet.

The third facet of hemostasis is fibrinolysis. The coagulant tendency of the primary and the secondary hemostasis is counteracted by fibrinolysis. Plasminogen circulating in the blood is cleaved to produce plasmin by endothelial bound tissue-type plasminogen activator. Plasmin subsequently degrades fibrin and destroys the fibrin-platelet plug [50, 51].

Blood coagulation is a delicate balance between anti thrombotic and pro thrombotic components, so the fibrin deposition and removal is tightly regulated, and defects in any of these components can either lead to thrombosis or excessive bleeding. Central to hemostasis is thrombin, a serine protease, which has created significant research interest, owing its ubiquity in hemostasis. As previously discussed, the end product of coagulation cascade is production of active thrombin, which catalyses the formation of fibrin from fibrinogen. Thrombin regulates the coagulation cascade through feedbacks. Besides its role in secondary hemostasis, thrombin also plays a vital role in primary hemostasis as an activator of platelets. Also, thrombin has long reaching effects on inflammation, cell survival, and cell proliferation [52]. It is very important to regulate the production of thrombin to maintain homeostasis.

Thrombomodulin, as the name indicates, modulates the activity of thrombin. Thrombin when bound to thrombomodulin activates protein C. Activated protein C proteolytically destroys coagulation factors Va and VIIIa and suppresses further thrombin formation. The binding of thrombin to thrombomodulin also activates the carboxypeptidase thrombin-activatable fibrinolysis inhibitor (TAFI) [53]. The activation of protein C and TAFI inhibits coagulation and fibrinolysis, making thrombomodulin key protein for the regulation of coagulation and fibrinolysis. Thrombomodulin is a protein cofactor that is found on the endothelial cell surfaces, at an approximate density of 50,000-100,000 molecules per cell [54], and it modulates the activity of thrombin [55, 56]. It was discovered by Esmon and Owen in the 1960's. The path to their discovery started with the evidence of circulating thrombin activated protein, activated protein C, and its precursor. Those findings led them to realize the necessity of a cofactor that activates thrombin mediated activation of protein C. In rats, it is found predominantly in the lungs when compared with kidney and liver [57]. Thrombomodulin, purified from human lung endothelial membrane preparations, had an apparent MW of 78,000 Da [55]. Encoded by an intronless gene, the mature single-chain glycoprotein in the human is 557 amino acids long. Structurally it has 5 distinct domains. It has a short cytoplasmic tail at the C-terminus, which anchors thrombomodulin. There are several potential sites for phosphorylation sites in the C terminus, in spite of that this region is not well conserved across different species [58]. The deletion of this tail in mice does not have any effect in development, survival, coagulation and inflammation [58]. There is a well conserved membrane-spanning domain, followed by a serine/threonine-rich domain with four potential sites for O-linked glycosylation. The serine/threonine region supports the attachment of chondroitin sulfate, which has 20 repeating disaccharide units and a terminal trisaccharide unit [59]. Adjacent to the serine/threonine-rich

region is a domain that has 6 epidermal growth factors (EGF)-like repeats, this domain is the best characterized. This region shows disulfide bonding pattern that is seen in a typical protein-protein interactions. The activation of protein kinase C and mitogen-activated protein kinase (MAPK) is linked to this domain for promotion of cell division on cultured vascular smooth muscle cells fibroblasts. The data shows that this domain is associated with atherogenesis and proliferation, although the clinical significance has not been established [60, 61]. The first two EGF-like repeats, which are furthest from the serine/threonine rich region, their function remains unknown. EGF-like repeats 3, 4, 5, and 6 [62-64] have been studied in detail by several groups and are essential for activation of Protein C by thrombin-thrombomodulin complex. The cofactor function for thrombomodulin requires the last three of six tandemly repeated EGF-like domains (EGF 4, 5, and 6), as well as a Ser/Thr-rich domain between EGF-like domain 6 and the transmembrane domain. The residues 1 through 222 comprises N-terminal region of thrombomodulin, which constitutes almost half of the extracellular portion of the protein. This region has two potential sites for N-linked glycosylation and it also shares similarities with C-type lectins. This lectin like domain has a compact hydrophobic core, two alpha-helices, two beta sheets and two disulfide bonds based on computer models [65]. The lectin like domain of thrombomodulin lacks carbohydrate recognition domain as well as Ca^{2+} binding site unlike other C-type lectins. The residues 155 through 222 of thrombomodulin might be associated with plasma membrane and this region is hydrophobic [66]. The lectin like domain is suggested to be globular and furthest from the plasma membrane making it in the prime position to interact with other molecules, this results are based on electron microscopy and computer models [67]. This region plays major role in cell adhesion, inflammation and cell proliferation but it lacks anti coagulant function.

Thrombin binds to thrombomodulin to activate Protein C which initiates the cascade that stops clotting, by inactivation of clotting factors Va and VIIIa [68]. The thrombin-thrombomodulin complex activates protein C 1,000-fold more when compared to activation of protein C by thrombin alone. Deficiency in protein C or activated protein C is well established as increasing the risk of thrombosis [69]. Low levels of thrombomodulin are a well established risk factor for heart disease [70, 71]. Without a doubt, thrombomodulin plays a key role in slowing or stopping clotting. It has been shown that thrombomodulin in complex with thrombin activates thrombin activatable fibrinolysis inhibitor (TAFI) by 1250 fold [72-75]. Activated TAFI is a plasma procarboxypeptidase B that stabilizes clot by down regulating fibrinolysis [76]. Activated TAFI catalyzes the removal of lysine residues from the C-terminal of fibrin [77, 78], resulting in elimination of plasminogen binding site of fibrin, subsequently plasminogen is unable to activate and prevention of fibrinolysis [79, 80]. This function of thrombomodulin might seem contradictory to its pro-fibrinolytic function previously discussed, but this highlights that thrombomodulin is a key regulator in coagulation, since it controls the formation and breakdown of clots.

Thrombomodulin is anchored on the luminal surface of the endothelium and as previously indicated, thrombomodulin lacking the cytoplasmic domain appeared normal [58]. Thrombomodulin undergoes endocytosis and degradation, and a significant amount of it is cleaved off and it is found in blood. Thrombomodulin found in blood are heterogeneous in size, due to differences in glycosylation [81, 82]. Four different fragments have been isolated from blood and seven fragments ranging in size from 12 to 100kDa after reduction of disulfides [83]. The concentration of thrombomodulin in serum and plasma are reported to be between 3 to 300 ng/mL [84]. In diseases like diabetes and lupus high levels of thrombomodulin in plasma have

been reported [85], this is believed to be a good marker for endothelial damage [53]. Studies have correlated high plasma thrombomodulin levels to be associated with a low risk of developing coronary heart disease [53].

As previously mentioned, thrombomodulin has 6 epidermal growth factor (EGF) like domains. Generally, EGF-like domains have 40 amino acid residues, and six cysteine residues that form three disulfide bonds [86]. Individual EGF like domains were synthesized and only the fifth domain bound to thrombin by itself [87]. The fourth and the fifth EGF like domains consists of 81 amino acids, and the fifth domain contains most of the residues that bind to thrombin. The EGF like domain 4 and 5 (TMEGF45) bind to thrombin more tightly than EGF 5 alone. TMEGF45 is the smallest fragment of thrombomodulin that when bound to thrombin can activate protein C. Addition of fourth and the fifth EGF-like domains separately, do not activate protein C, suggesting they work together in activation of protein C [88]. The k_{cat} values for protein C activation by the thrombin-TMEGF45 complex, calculated by binding kinetic studies and cofactor activity assays, shows TMEGF45 has full cofactor activity. The EGF like domain six increases the K_m value of thrombomodulin for thrombin by factor of ten, despite not altering the K_{cat} value of thrombin-thrombomodulin complex for protein C [89]. The EGF 4 and 5 of thrombomodulin is linked together by three residues, and one of them is methionine, which is the 388th residue in thrombomodulin (Met388) [90]. Figure 1.1 is thrombomodulin residues from all mammals sequenced as of the date of writing; Met388 is conserved in all the sequences.

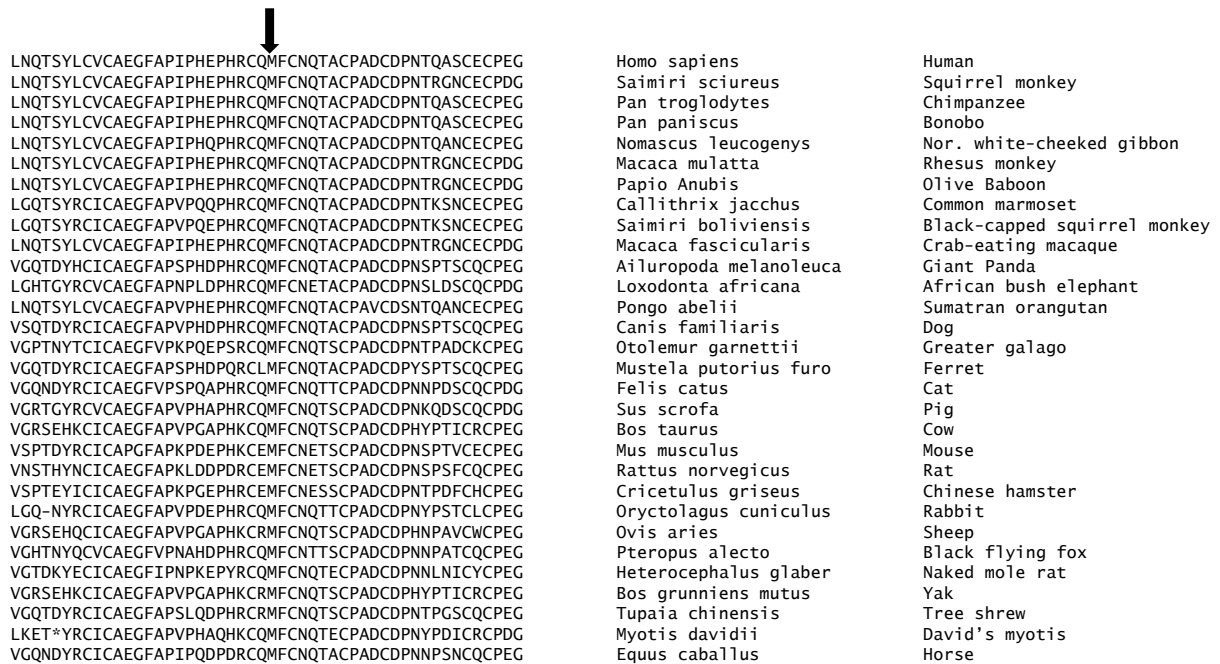


Figure 1.1: Alignment of region of interest of thrombomodulin amino acid sequences from different mammals. Methionine 388 is conserved in all these organisms.

The mutation of Met388 to any other residues, except leucine, decreases the anticoagulant cofactor activity of thrombomodulin [91]. More importantly, there is 76-90% loss of activity when Met388 is oxidized using H_2O_2 [92]. There are four other methionines in thrombomodulin, but their oxidation does not disrupt the ability to activate protein C [92]. The K_d of thrombomodulin thrombin interaction increases to 10.9 from 4.4nM, when a full length thrombomodulin had oxidized Met388 [93]. TMEGF45 with oxidized Met388 has 3.5 fold lower K_{cat} , and 3.3 fold higher K_m values when compared to wild type TMEGF45 based on protein C activation assays [94]. It must also be mentioned that clot stabilizing activation of TAFI by thrombin-thrombomodulin complex is unaffected by the oxidation of Met388 [95]. Since the activation of clot stabilizing activated TAFI, a procoagulant pathway, is unaffected by Met388 oxidation, while activation of protein C, an anticoagulant pathway, is suppressed by oxidation of

Met388. Although effects oxidation of Met388 on activation of protein C and TAFI were demonstrated *in vitro*, it seems that oxidation of Met388 leads hemostasis towards pro coagulation, and higher rates of formation of clots when significant amounts of Met388 is oxidized.

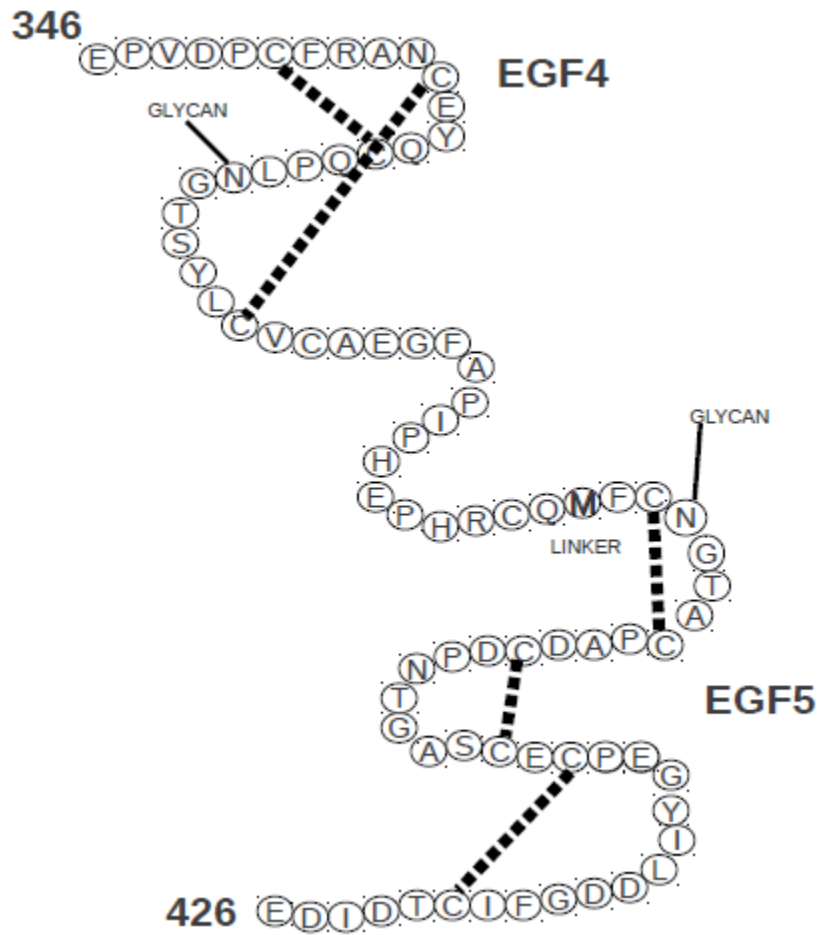


Figure 1.2: Schematic diagram of TMEGF45. It consists of 81 amino acids. The fifth domain contains most of the residues that bind to thrombin. TMEGF45 is the smallest fragment of thrombomodulin that when bound to thrombin can activate protein C.

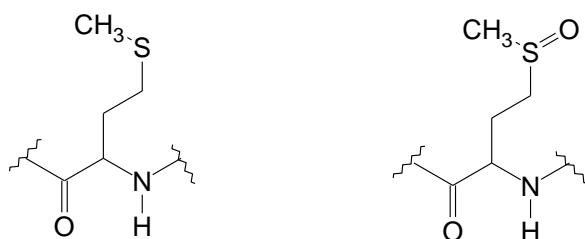


Figure 1.3: Structure of Methionine and Methionine Sulfoxide

The structures TMEGF45 and TMEGF456-thrombin complex have been investigated using NMR to understand the interactions of Met388 [94] in thrombomodulin. Met388 plays at least two structural roles. First, Met388 is important part of the fifth domain's hydrophobic region, and there is disruption of the hydrophobic interactions when the methionine is oxidized to methionine sulfoxide. Second, the interaction between the fourth and the fifth domain of thrombomodulin is mediated by methionine, and conversion to methionine sulfoxide has potential to disrupt the orientation of the two domains. There is a significant structural difference between oxidized and non oxidized forms in the fifth domain of thrombomodulin. Phenylalanine 376, located in the fourth domain, packs against the hydrophobic methionine but occupies a substantially different position when the hydrophilic sulfoxide form is present, making it a key part of the conformational switch. These structural changes bury several residues which interact with thrombin in the structure of the thrombomodulin-thrombin complex [96].

Is a decrease in thrombomodulin activity biologically relevant?

As mentioned previously, *in vitro* studies have shown that oxidation of Met388 leads to decrease of thrombomodulin anticoagulant cofactor activity. But is this decrease in activity biologically relevant? Could there be sufficient thrombomodulin in reserve, so that oxidation to some of the thrombomodulin is inconsequential? In a study conducted in mice, the intronless thrombomodulin gene was deleted to create a model for thrombotic disease [97]. The study found complete absence of thrombomodulin resulted in death of embryos at day 9.5, the death preceded before the functional cardiovascular system was assembled. In another study in mice, glutamate at position 387, which is the part of the linker region connecting EGF like domain four and five of thrombomodulin, was replaced with proline [98]. The Met388 is adjacent to glutamate 387, which make this study relevant to our hypothesis. This study found there was a decrease in the ability of mutant protein to activate protein C. The k_{cat} was 37% of wild-type, K_d for thrombin was down by a factor of 45, and K_m for protein C was increased by 1.6 fold. The amount of thrombomodulin purified, decreased by factor of three, possibly due to decreased amount of proteins expression on the cell surface or due to turnover increase. The cell culture experiments showed that mutant endothelial cells ability to activate protein C was reduced by 38 fold when compared to wild type cells. Despite the significant decrease in the activation of protein C in the recombinant animals, they were viable and reproduced normally. However, these animals showed cardiovascular diseases reminiscent symptoms, they had fibrin deposition and small blood clots in lungs, spleen and the heart [99]. The fibrin deposition was tenfold more in the recombinant mice when compared with normal mice at three to 6 months of age [100]. Myocardial infarction is a common and potentially fatal consequence of thrombosis resulting from imbalance of fibrin deposition and removal [101]. In the follow up to the recombinant

thrombomodulin study, it was shown that the recombinant mice were in hypercoagulable state with a tendency towards sepsis and thrombosis. Such conclusion was inferred from accelerated rate of platelet dependent thrombus formation after FeCl₃ induced endothelial injury was subjected to the carotid artery [102]. 80% of recombinant mice showed full thrombotic occlusion when subjected to FeCl₃ treatment, alternatively only 27% of the wild type mice showed such symptoms [102]. The carotid arteries of recombinant and wild-type mice were permanently blocked near carotid bifurcation by ligation. These animals recovered from surgery without any complications. The recombinant mice showed severe thrombotic occlusion which spanned over the entire length of the artery, whereas wild type mice showed thrombotic occlusion within 1mm from the ligation site [102]. When injected with lipopolysaccharide to induce infection, in recombinant mice resulted in much higher fatality compared to the wild-type, also in the mutant mice fatality occurred much earlier when injected with lipopolysaccharide [102].

Prolylcarboxypeptidase (PRCP) is an endothelial membrane-bound serine carboxypeptidase responsible for activation of bradykinin and angiotensin [103-105]. Both proteins regulate the vascular NO to provide protection from thrombosis. PRCP is indirectly involved in maintenance of normal blood pressure and reduction of thrombosis risk. PRCP polymorphism has been associated with hypertension and inflammation [106]. PRCP gene-trapped mice are hypertensive and are prone to faster thrombosis [107]. These mice have increased *in vivo* vascular ROS and uncoupled endothelial nitric oxide synthase (eNOS) and reduced expression of vascular thrombomodulin in their aorta [108-110]. The hypertensive and prothrombotic state in PRCP gene trapped mice was abrogated by antioxidant treatment. This shows the hypertensive and prothrombotic state arose from high levels of ROS in PRCP gene trapped mice. More importantly, the knockdown of PRCP by silencing RNA (siRNA) in Human

umbilical vein endothelial cells (HUVECs) resulted in increased uncoupled eNOS and 90% reduction in Protein C activation. The data obtained from thrombomodulin antigen showed no significant change ($p= 0.30$) in expression of thrombomodulin in siRNA knockdowns of PRCP HUVECs [107]. The thrombomodulin was expressed but it was unable to activate Protein C. The paper speculates that the inactivation of Protein C could be caused by ROS inactivation of critical methionine for functionality of thrombomodulin. This paper demonstrates that oxidation caused by high level of ROS prevents thrombomodulin from activation of Protein C in a semi *in vivo* environment (HUVECs).

The most common cardiovascular diseases seen in smokers are myocardial infarction and stroke. The blood of smoker is more prone to clot than non smoker; they are in hypercoagulable state. The narrowing of the arteries resulting from atherosclerosis increases the possibility of cardiovascular diseases [111], equally important is the hypercoagulable state seen in smokers. The fact that smokers have a hypercoagulable state has been established; however, despite intensive research the molecular origin of such a state has not been shown. Free radical mediated oxidative damage to the endothelium is considered to be an important factor for the development of cardiovascular diseases in smokers. Most of the research in this area has been focused on nitric oxide signaling impairment caused by oxidation [112-114]. As in emphysema, where oxidation of key methionine residues is the molecular basis of the disease, it is our belief that similar oxidation is taking place in the methionine 388 of thrombomodulin in cigarette smokers from the oxidation that occurs from smoking. We strongly believe that oxidation of methionine 388 in thrombomodulin is an important molecular factor leading to cardiovascular diseases in smokers.

As was mentioned earlier in this chapter, thrombomodulin is predominantly located in the lungs [115, 116] making it more vulnerable to oxidants found in cigarette smoke. The oxidants secreted by immune cells in the lungs in response to cigarette smoke, makes lung endothelium very oxidizing. It must be pointed out that oxidation of methionine in smokers and non smokers have not been measured so far, however the plasma levels of thrombomodulin in smokers and non smokers have been examined.

It has been shown that high levels of cigarette smoke exposure causes decrease in activated protein C, and there was direct correlation between the levels of protein C with the degree of cigarette smoke exposure [117]. This study found that activated protein C circulating in the blood were 23.3% lower in smokers than in non smokers. There could be several factors that contribute towards the low level of activated protein C in smokers, but based on the evidences we have seen so far; it seems very likely that methionine oxidation of thrombomodulin causes lower activation of protein C in smokers. Venous thromboembolism has been strongly linked to low levels of activated protein C [118, 119], also low levels of activated protein C are considered to be a risk factor for ischemic stroke [120]. The comparison of protein C levels in smokers and non smokers, and relation between thromboembolism and ischemic stroke study strengthens our case for a link between thrombomodulin oxidation and cigarette smoke as an important molecular cause of cardiovascular diseases.

CHAPTER 2: METHODS EXPLORED FOR QUANTIFICATION OF METHIONINE 388

OXIDATION IN HUMAN URINARY THROMBOMODULIN

Introduction

In the first chapter we hypothesized that the methionine 388 of thrombomodulin is more oxidized in cigarette smokers than in non smokers. We speculated that this could be the key molecular mechanism behind the prothrombotic state seen in smokers, hence, an important contributor towards the development of cardiovascular disease in smokers. In order to quantify the oxidation of methionine in thrombomodulin isolated from urine, a rapid method of isolation of the protein is necessary to process a reasonable number of samples and minimize any oxidation during processing. In this chapter we will discuss the method we have developed to quantitate the methionine 388 oxidation in thrombomodulin, as well as the other methods that were attempted.

Thrombomodulin is critically important for hemostasis [121-124]. As the name implies thrombomodulin serves to regulate the activity of thrombin. The thrombomodulin-thrombin complex activates protein C, and the activation results in degradation of the factors that promote clotting cascade [125]. The activated protein C or deficiency in protein C has been linked to increased risk of thrombosis [126-128]. Low levels of thrombomodulin have been established as a risk factor for heart diseases [71, 129, 130]. Thrombomodulin is also involved in activation of activatable fibrinolysis inhibitor (TAFI). Thrombin when complexed with thrombomodulin activates TAFI, activated TAFI stabilizes clot [76]. It is worth reflecting on this point that thrombomodulin controls the rate of formation of clot as well as its breakdown.

Thrombomodulin is anchored on the luminal surface of the endothelium [131, 132]. It is found in at much higher levels at the lungs of rats compared in kidney or liver [115, 133, 134]. The trans membrane helix and the C-terminal cytoplasmic domain of thrombomodulin anchors

the protein and they have not been demonstrated to have direct functional role to hemostasis. Mice lacking the cytoplasmic domain appeared normal besides the elevated levels of thrombomodulin in the plasma [58, 135]. Significant amounts of thrombomodulin is cleaved off from the surface and it circulates in the blood [136, 137]. The thrombomodulin circulating in the blood is not homogenous in size partly due to the differences in glycosylation [138, 139] and mostly due to variation in proteolysis of thrombomodulin. Four different protein fragments have been isolated from plasma, and following reduction of disulfides, seven fragments that range from 12 to 100 kDa in size have been isolated [121, 140, 141]. These fragments are able to activate protein C when complexed with thrombin, however their activity drops to 16-50% when compared to the membrane bound intact thrombomodulin [142]. The literature values for thrombomodulin in serum and plasma has been reported to be between 3 to 300 ng/ml [84, 121, 143, 144]. High concentrations of thrombomodulin are a good indicator of endothelial damage and have been reported in many diseases and conditions like lupus, preeclampsia, and diabetes [145].

In vitro oxidation of methionine 388 of thrombomodulin has been shown to lower the ability of thrombin-thrombomodulin complex to active protein C [146]. When the methionine 388 was substituted with leucine, the mutant was insensitive to oxidation and it activated protein C [147, 148]. The oxidation of methionine 388 in thrombomodulin has been shown to have no effect on TAFI activation by thrombomodulin-thrombin complex [149]. Since oxidation of methionine 388 promotes clotting by lowered activation of protein C and has no effect in the clot stabilizing by TAFI, methionine 388 oxidation of thrombomodulin in humans should be a factor promoting hypercoagulable state.

There is good reason to try to determine how much oxidation of this amino acid side chain actually occurs *in vivo*. The quantification of urinary thrombomodulin methionine 388 oxidation involves 3 three major steps: 1) purification of thrombomodulin from urine, 2) digestion of purified thrombomodulin 3) quantification of methionine oxidation. In this chapter we report several methods that were tested to arrive at a protocol to successfully perform the aforementioned three major steps.

Materials and Methods:

Purification of thrombomodulin

Detection of thrombomodulin by ELISA

The concentration of thrombomodulin in various fractions throughout the course of purification was followed using a commercially available sandwich enzyme linked immunoabsorbant assay (ELISA) kit for thrombomodulin (American Diagnostica, Greenwich, CT).

Urine sample collection

The University of Arkansas Institutional Review Board approved all experimental protocols using human subjects. Samples of the first morning urinate were collected by volunteers in 400 mL centrifuge bottles. Thiodiglycol (5 mL) and 0.5 M EDTA (1 mL, pH 8.0) were added to containers before sample collection. The collected samples were transported using an insulated bag containing an ice pack. The samples were generally transported to the laboratory within two hours of collection and stored at 4 °C for no longer than 2 hours before further processing to minimize the opportunity for oxidation. Typical volumes of the sample

from a donor ranged from 200-550 mL. Prior to processing, 1 mL of sample was removed and stored in -80°C for determination of the initial thrombomodulin concentration in the sample.

Jumbosep spin filter

Jumbosep spin filters were tested for the concentration of the urine sample. The Jumbosep centrifugal device (Pall) consists of sample reservoir, membrane filter insert, and the filtrate receiver. The sample reservoir has a maximum capacity of 60 mL. The membrane filters are constructed from modified polyethersulfone on polyethylene substrate, and they are low protein binding. A membrane filter with molecular weight cut off of 30 kd was used for purification. 60 mL of urine sample that was previously cooled at 4 °C, and centrifuged in Sorvall GS-3 rotor at 4,000 rpm for 30 min at 4 °C was added to the sample reservoir. The Jumbosep filter was centrifuged at 3500 rpm in a SH-3000 rotor for 30 minutes at 4 °C. After centrifugation for 30 minutes, the filtrate was removed and more urine sample was added to the sample reservoir and centrifuged. The previous step was repeated until entire urine sample was passed through the filter, and the final volume in the reservoir was 20 mL. Next, buffer exchange was performed to adjust pH and the ionic strength of the urine concentrate. 40 mL of 25 mM imidazole acetate at pH 5.5 was added to the sample reservoir, and the Jumbosep filter was centrifuged for 30 minutes. The buffer exchange was repeated two more times. The buffer exchanged concentrated urine sample was collected for downstream processing. Slurry of SP Sepharose FF was poured into a 12 mL Econo-Pac gravity-flow polypropylene column in a sufficient amount to pack a 1 ml bed volume. The column was equilibrated using 10 mL of 25 mM imidazole acetate, pH 5.5. Urinary protein concentrate following Jumbosep was passed through the column, and the column was washed with 3 mL of 25 mM imidazole acetate, pH 5.5. The flow through and the wash from the SP Sepharose column was collected in 50 mL conical

flask. Slurry of Q Sepharose FF was poured into a 12 mL Econo-Pac gravity-flow polypropylene column in a sufficient amount to pack 2 ml bed volume. This column was equilibrated using 10 mL of 25 mM imidazole acetate, pH 5.5. The pooled flow through and wash from the SP Sepharose was passed through the Q Sepharose column. The column was washed with 8 mL of 25 mM imidazole acetate, pH 5.5. Thrombomodulin was eluted by passing 8 mL of 0.5 M NaCl, 20 mM imidazole acetate, pH 5.5 through the column, elutes were collected in one mL fractions. The crude thrombomodulin typically eluted in the fourth through sixth fractions. The fractions containing thrombomodulin, tested using ELISA, were flash frozen in liquid nitrogen and lyophilized. The dried samples were stored at -20 °C or immediately subjected to HPLC purification.

Tangential flow filtration (TFF) system

Labscale Tangential Flow Filtration (TFF) System, manufactured by Millipore, was tested for the concentration of the urine sample. The Labscale TFF System has a graduated 500 mL acrylic reservoir with a retentate backpressure valve, feed and retentate pressure indicators, and a stirrer assembly. A Pelicon XL filter with 30 kd MW cutoff Biomax membrane was attached to the TFF system. The urine sample cooled at 4 °C, and centrifuged in Sorvall GS-3 rotor at 4,000 rpm for 30 min at 4 °C was poured into the TFF reservoir. The sample was concentrated until the urine sample was concentrated to the final volume of approximately 25 mL. 400 ml of 25 mM imidazole acetate buffer pH 5.5 was added to the concentrate, and this solution was concentrated to 20 mL to exchange the salts from the urine. The buffer exchange was repeated until the sample was clear. Slurry of SP Sepharose FF was poured into a 12 mL Econo-Pac gravity-flow polypropylene column in a sufficient amount to pack a 1 ml bed volume. The column was equilibrated using 10 mL of 25 mM imidazole acetate, pH 5.5. Urinary protein

concentrate following Jumbosep was passed through the column, and the column was washed with 3 mL of 25 mM imidazole acetate, pH 5.5. The flow through and the wash from the SP Sepharose column was collected in 50 mL conical flask. Slurry of Q Sepharose FF was poured into a 12 mL Econo-Pac gravity-flow polypropylene column in a sufficient amount to pack 2 ml bed volume. This column was equilibrated using 10 mL of 25 mM imidazole acetate, pH 5.5. The pooled flow through and wash from the SP Sepharose was passed through the Q Sepharose column. The column was washed with 8 mL of 25 mM imidazole acetate, pH 5.5. Thrombomodulin was eluted by passing 8 mL of 0.5 M NaCl, 20 mM imidazole acetate, pH 5.5 through the column, elutes were collected in one mL fractions. The crude thrombomodulin typically eluted in the fourth through sixth fractions. The fractions containing thrombomodulin, tested using ELISA, were flash frozen in liquid nitrogen and lyophilized. The dried samples were stored at -20 °C or immediately subjected to HPLC purification.

Ion exchange chromatography

The urine sample that was previously cooled at 4 °C was centrifuged in a Sorvall GS-3 rotor at 4,000 rpm for 30 min at 4°C. The supernate was then filtered using a bottle-top 0.22 µm polyethersulfone (PES) membrane filter to remove remaining particulates and cellular debris. Sufficient 0.25 M imidazole buffer, pH 6.0 was added to the urine filtrate to bring to a final concentration of 25 mM imidazole acetate. Next, the pH of the sample was adjusted to 6.0 by addition of 2 M acetic acid or 6 M NaOH depending on the urine sample. Typically after the addition of 0.25 M imidazole buffer, the pH of the samples ranged from pH 5.5-6.4. The pH of the sample was measured using a pH electrode. A slurry of Q Sepharose FF (GE Healthcare) was poured into a 50 mL Bio-Rad Econo column in sufficient amount to pack a 20 ml bed volume. A Fluid Metering, Inc (FMI) pump was connected to the Bio-Rad column to pump buffer and urine

sample through the Q Sepharose column at a constant speed of 5 mL/min. The column was first equilibrated with 60 mL of 25 mM imidazole acetate, pH 6.0. Next, the urine sample was passed through the column. The column was washed with 50 mL of 25mM imidazole acetate, pH 6.0 and then by addition of 20 mL of 25 mM imidazole acetate buffer, pH 6.0, 50 mM NaCl. Fractions containing thrombomodulin were eluted by addition of 50 mL of 25 mM imidazole acetate buffer, pH 6.0, 0.5 M NaCl. The 0.5 M NaCl fractions were collected in 15 mL conical tubes, 5 mL in each tube. The eluent after addition of 25 mL of 25 mM imidazole acetate buffer, pH 6.0, 0.5 M NaCl, tested positive for thrombomodulin. A solution of 0.2 M L-methionine (Sigma-Aldrich) was added to thrombomodulin containing fractions to a final concentration of 2 mM methionine. The fractions were transferred to 1.5 mL microcentrifuge tubes, typically 14 tubes, and flash frozen in liquid nitrogen. The frozen eluents in 1.5 mL microcentrifuge tubes were dried under vacuum in a *SpeedVac* concentrator system using medium heat (45°C).

Multiple urine samples were collected from each donor since there was usually not enough thrombomodulin isolated from a single sample to reliably observe it by mass spectrometry. Typically, three different urine samples were collected from each donor. These three samples were usually collected from each donor on consecutive days. Each sample was purified by ion exchange the very day it was collected. The thrombomodulin containing fractions were flash frozen, and were stored in -20 °C. The thrombomodulin containing HPLC fractions from different samples were pooled before chymotrypsin digestion.

Reverse phase HPLC purification.

The dried samples from Jumbosep filtration, TFF, or ion exchange were dissolved in minimum volume, generally 5 mL of total volume, of 18.2 megohm deionized water. The

dissolved sample was passed through a 25 mm syringe filter with 0.2 μ M PES membrane (VWR). The 4 sets of 1.375 mL redissolved sample were injected into reverse phase Waters HPLC system. An Atlantis dC18, 5 μ m particle, 4.6x250 mm column held at 58 °C and a 2996 photodiode array detector monitoring 214 nm and 254 nm was used. Upon injection, a gradient was run at 1 mL/min from 10 to 29% acetonitrile, 0.1% trifluoroacetic acid over 12 minutes, and then from 29% to 40% acetonitrile over the next 11 minutes, and then from 40% to 90% acetonitrile over the next 25 minutes. The eluents were collected in 1 mL fractions in 1.5 mL eppendorf tubes, to which were previously added 100 μ L of 200 mM Tris buffer, pH 8.0. The thrombomodulin containing fragments, identified by ELISA, eluted at 30 minute. The HPLC method for purification of thrombomodulin was developed by Dr. Jeffery Froude, former graduate student in Stites Lab. His method was used as a template to develop the current method. The two methods differ in their gradient profile, the new method provides a better resolution of the thrombomodulin containing peak. The thrombomodulin containing 1.5 mL microfuge tubes were flash frozen in liquid nitrogen, dried under vacuum in a *SpeedVac* concentrator system using medium heat. The HPLC Atlantis column was cleaned using 1.0 mL/min of 90% MeCN/0.1% TFA for 10 minutes, followed by equilibration to the starting conditions of 1.0 mL/min of 10% MeCN/0.1% TFA in preparation for the next injection.

Digestion of thrombomodulin

Digestion using manufacturer's protocol

The thrombomodulin containing fractions from the reverse phase chromatography was pooled and dissolved in approximately 100 μ L of 1X phosphate buffered saline (PBS; 1.46 mM

KH₂PO₄, 9.9 mM Na₂HPO₄, 2.68 mM KCl, 137 mM NaCl, pH 7.4). If the solution did not turn clear after the addition of the buffer, the sample was spun in a microcentrifuge (Galaxy 14D VWR) at 13K RPM for a minute. The supernatants were removed, and precipitates were dissolved in 75 µL of 1X phosphate buffered saline. Freshly prepared *tris*-carboxyethylphosphine (TCEP) was added to make the final concentration of 50 mM TCEP. 10 µL of 0.5 M sodium phosphate buffer pH 7.5 added to the sample, prior to addition of 500 units of PNGase F (New England Biolabs). The samples were incubated with gentle shaking at 37 °C for 2 hours to deglycosylate the sample. The protein was then digested at 30 °C for 8 hours after adding 4.1 µg of sequencing grade chymotrypsin (*Princeton* Separations) dissolved in 50 mM Tris-HCl, 1 mM CaCl₂, pH 8.0. The chymotrypsin was inactivated by placing the samples in a 100 °C dry bath for 30 seconds. The samples were immediately flash frozen, and stored in -20 °C until further processing.

Digestion using urea

The dried thrombomodulin containing HPLC fractions were redissolved in freshly prepared 150 µL 45 mM sodium phosphate buffer, 2.5 M urea pH 7.75. Freshly prepared 1 M TCEP was added to the final concentration of 10 mM. The sample was incubated in room temperature for 10 min. The sample was placed in a 65 °C heat block for 10 min, then immediately cooled on ice. 50 units of PNGase F (2 µL) was added to the sample and incubated at 37 °C for 4 hours. 5 µg of sequencing grade chymotrypsin was dissolved in 5 µL of 50 mM Tris-HCl pH 8.0, 1 mM CaCl₂. The dissolved chymotrypsin was added to the sample and incubated in room temperature for 4, 12, or 36 hours.

Alternatively, thrombomodulin digestion was conducted using minimal volume of 45 mM sodium phosphate buffer, 8 M urea pH 7.75. The volume of buffer used ranged from 100 – 150 μ L. Freshly prepared TCEP was added to the final concentration of 10 mM. The sample was placed on 100 °C heat block for 20 min, then immediately cooled on ice. The concentration of urea was decreased from 8 M to 4 M by addition of 45 mM sodium phosphate buffer, pH 7.75. 50 units of PNGase F was added to the sample and incubated at 37°C for 4 hours. 10 μ g of sequencing grade chymotrypsin was added to the sample and incubated in room temperature for 24 hours.

Digestion using acetonitrile

The dried thrombomodulin containing HPLC fractions were resuspended in 100 μ L of 80% acetonitrile/20% 50 mM Tris-HCl/10 mM CaCl₂ pH 8.0 buffer solution. Freshly prepared 0.1 M TCEP was added to the final concentration of 10 mM. The sample was incubated in room temperature for 10 minutes. 50 units of PNGaseF was added and incubated at 37 °C for 1 hour. 5 μ L of fresh sequencing grade chymotrypsin (1 μ g/ μ L) was added and incubated for 4- 24 hours. This experiment was repeated with acetonitrile concentration at 20, 40, and 50%.

Digestion using Invitrosol

The Invitrosol LC/MS Protein Solubilizer (Invitrogen Corporation) was purchased to assist in thrombomodulin digestion. This proprietary surfactant blend claimed to solubilize hydrophobic proteins, not interfere with protease activity, and be compatible with reverse-phase high pressure liquid chromatography separations of digested peptides. The dried thrombomodulin containing HPLC fragments were reconstituted in 90 μ L of 25 mM ammonium bicarbonate, pH 8.0. 10 μ L of 5X Invitrosol was added, and sonicated for 10 min. The solution

was heated for 15 min at 60°C, and then cooled to room temperature by placing it on ice. Freshly prepared 0.1 M TCEP was added to the final concentration of 10 mM. The solution was incubated for 20 min at room temperature. 50 units of PNGaseF was added and incubated at 37 °C for 4 hour. 5 µL of fresh sequencing grade chymotrypsin (1µg/µL) was added and the sample was incubated for 12 hours at room temperature. The supernatant was removed and injected into the waters HPLC.

Digestion using RapiGest

RapiGest SF (Waters Corporation) is a reagent used to enhance the in-solution digestion of proteins. It enhances the digestion of proteins by making the proteins more soluble in solution. 0.1 % (w/v) solution of RapiGest SF was prepared by dissolving 1 mg of lyophilized RapiGest SF in 1 mL of 50 mM ammonium bicarbonate buffer, pH 8.0. The dried thrombomodulin containing HPLC fragments were dissolved in 50 µL of 0.1 % RapiGest SF solution. Freshly prepared 0.1 M TCEP was added to the final concentration of 10 mM. The solution was heated for 10 min at 100°C, and then cooled to room temperature by placing it on ice. 50 units of PNGaseF was added and incubated at 37 °C for 4 hour. 5 µL of fresh sequencing grade chymotrypsin (1µg/µL) was added and the sample was incubated for 12 hours. TFA was added to the final concentration of 0.5%, and incubated at 37°C for 30 minutes to cleave the RapiGest SF. The sample was centrifuged at 13, 000 rpm for 10 minutes. The supernatant was removed and injected into the HPLC.

Digestion using RapiGest and EDTA

The dried thrombomodulin containing HPLC fractions were dissolved in 100 µL of 0.2% RapiGest in 50mM Bis-Tris, 4 mM EDTA, pH 6.0 buffer. The sample was sonicated for 1 hour.

Sufficient 0.35 M TCEP was added to make the final TCEP concentration of 20 mM. The sample was incubated at 60 °C for 30 minutes. 100 units of PNGaseF was added and incubated at 37 °C for 4 hours. 5 µL of fresh sequencing grade chymotrypsin (1 µg/µL) was added and the sample was incubated for 12 hours at room temperature. The supernatant was removed and injected into the HPLC. The reaction was quenched and RapiGest decomposed with addition of TFA to final concentration of 25% TFA. The sample was injected into the HPLC.

Quantification of methionine oxidation

Alkylation of cysteine by fluorescent reagent

The alkylation of cysteine by fluorescent reagent to thrombomodulin isolated from urine was performed for identification and quantification of our peptide. The three potential advantages of alkylation are increased sensitivity, fewer peaks detected by the fluorescent detector and, finally, alkylation prevents disulfide bond reformation. Synthetic peptide, AIPHEPHRCQMF, was alkylated with 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS)[150, 151]. 1, 5-IAEDANS has excitation wavelength of 336 nm and an emission wavelength of 490 nm.

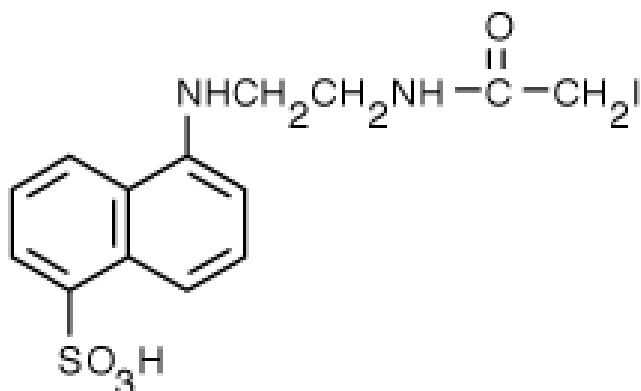


Figure 2.1: Chemical structure of 1, 5-IAEDANS, molar mass is 434.25 g, excitation wavelength is 340 nm, and emission wavelength is 460 nm [152, 153].

The 2 μ L of 10 mg/mL synthetic peptide stock solution was dissolved in 198 μ L 0.1 M Tris pH 8.0 that had 50 mM *tris*-carboxyethylphosphine (TCEP). The sample was incubated for 10 minutes at room temperature. Next, 5 fold molar excess of 1,5-IAEDANS over total thiols was added. The samples were incubated for 8 hrs. 10 fold molar excess of 2-mercaptoethanol (2-ME) over 1,5-IAEDANS was added to the sample after alkylation [154]. The sample was injected into the Waters ACQUITY UPLC System, BEH 130 C18 1.7 μ m 2.1x150 mm column that was heated to 45°C, 0.4 mL/min flow rate. The PDA detector monitored at 214nm and fluorescence (FLR) detector excitation wavelength was 340 nm, and emission was monitored at 460 nm). The initial solvent composition 10% MeCN/0.1% TFA, and it was transitioned to 20% MeCN/0.1% TFA in a convex curve gradient over two minutes. Over the next 2.5 minutes, a linear gradient from 20% MeCN/0.1% TFA to 42% MeCN/0.1% TFA was run.

Determination of methionine 388 oxidation ratio by UPLC

Following chymotrypsin digestion, the thrombomodulin fragments were injected into a Waters Acquity UPLC System with the detector monitoring 214 nm. The BEH 130 C18 1.7 μ m 2.1x150 mm reverse phase column was heated to 45 °C and a 0.2 mL/min flow rate was used to separate the peptide mixture. The eluent was started at 10% MeCN/0.1% TFA and transitioned to 20% MeCN/0.1% TFA over two minutes. Over the next 2.5 minutes, a linear gradient from 20% MeCN/0.1% TFA to 42% MeCN/0.1% TFA was run. The methionine and methionine sulfoxide forms of the peptide APIPHEPHRCQMF eluted at 11.5 and 22.2 minutes, respectively. The peptide APIPHEPHRCQMF (synthesized by SIGMA Genosys) in the methionine and/or methionine sulfoxide forms was co-injected to confirm peak position. After the run, the column was cleaned with 0.20 mL/min of 90% MeCN/0.1% TFA for 8 minutes followed by 5 minutes equilibration to 0.2 mL/min of 10% MeCN/0.1% TFA.

Mass spectrometry analysis of methionine 388 oxidation

The presence of oxidized and reduced forms of peptide in the chymotrypsin digested thrombomodulin was confirmed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The MALDI analysis was performed by the Statewide *Mass Spectrometry* Facility located at the *University of Arkansas*. The pure samples were mixed with the MALDI matrix, 2, 5-dihydroxybenzoic acid (DHB) (Sigma Aldrich). MALDI-TOF mass spectra were obtained on a Bruker Ultraflex II (Bruker Daltonic GMBH, Bremen, Germany) time-of-flight mass spectrometer operated in the positive-ion reflectron mode. The accelerating voltage, delayed extraction time, and laser power were adjusted to optimize sensitivity and resolution for ions between m/z 500 – 4000.

Recombinant TMEGF 456

To conduct a study of the oxidation of methionine in the thrombomodulin of smokers and non-smokers we were successful in purifying thrombomodulin from urine, however the quantities were not sufficient to study the oxidation occurring during individual steps and effects of additives to minimize oxidation occurring during the processing. Methionine 388 is found between EGF 4 and 5 of thrombomodulin, and this region is sufficient to activate protein C. Recombinant fragment EGF 456 of thrombomodulin was expressed using *Pichia Pastoris* to produce large quantity of recombinant protein. Fragments of human thrombomodulin were expressed in the *Pichia pastoris* expression system. The construction of the *P. pastoris* expression system containing the gene TMEGF456 has been described by White *et al.* [62] and the expression system was the kind gift of Dr. Komives. The recombinant protein was produced using *P. pastoris* strain SMD1168, as this strain lacks protease A. As a result of deficiency of protease A, this strain is deficient in carboxypeptidase Y and protease B1. Due to absence of proteases in this strain, the yield is typically much higher. The transformants of this strain can grow on the histidine deficient medium, since it is defective in dehydrogenase gene (*his 4*), this makes selection of transformants easier. The plasmid vector pPIC9K was used for insertion of synthetic gene for TMEGF456. Along with Met 388, the TMEGF456 fragment expressed in *P. pastoris* contains His and Met residues at the N-terminus.

P. pastoris strains were typically grown on YPD-agar plates (2% (w/v) dextrose, 2% (w/v) peptone, 1% (w/v) yeast extract, and 2% (w/v) agar) or YPD medium (1% (w/v) yeast extract, 2% (w/v) dextrose, and 2% (w/v) peptone.). Single colony was selected from YPD-agar plate, and it was inoculated to a sterile flask which contained 10mL solution of YPD. This culture was placed in a shaker (300 rpm) for 24h at 30 °C. The YPD solution was centrifuged for

10 min at 3000 rpm using Sorvall GS-3 rotor. The cells were collected and resuspended in YPD solution containing 15% glycerol, and aliquots of 1 mL were placed in sterile 1.5 eppendorf tubes. The tubes were flash frozen using liquid nitrogen and stored at -80 °C, these 'stocks' were used to inoculate the fresh cultures. The selection of a single colony, inoculation of YPD solution, and preparation of storage of 'stocks' was done by Dr. Jeffery Froude, a former graduate student from our lab.

4 L of BMGY media was prepared. The BMGY media had composition of 1% (w/v) ammonium sulfate, 1% (w/v) casamino acids, 3.4 g/L yeast nitrogen base (without ammonium sulfate and amino acids), 2 mL/L biotin solution, 2% (v/v) 1 M potassium phosphate buffer pH 6.0 and 1% (v/v) glycerol. The BMGY solution was sterilized by passing through the 0.2 µm bottle top filter. The 0.2 g/L of stock solution of biotin was prepared by dissolving in 0.02 M potassium hydroxide solution, and passing it through 0.2 µm PES filter, and were stored in -20 °C. 2 L culture flasks were covered with four layers of cheese cloth and two layers of aluminum foil, and were autoclaved for 30 min at 121 °C. Approximately 320 mL of sterilized BMGY media were aseptically transferred to the 2 L sterile flasks.

A sterile 150 mL baffled flask was used to prepare the starter culture of *P. pastoris*. 10 mL of sterile BMGY media and 2 mL of frozen stock culture was added to the flask, and was placed in a shaker (3000 rpm) at 30 °C for 12 hrs. 1mL of starter culture was added to the 2 L culture flasks containing BMGY media. After 24 hrs of growth, the aluminum foil was removed from the culture flasks, cheese cloth was not removed, and was continued to incubate for additional 24 hrs. Following 2 days of incubation, the culture was transferred to 400 mL centrifuge bottles and centrifuged at 2500 rpm for 10 min using Sorvall GS-3 rotor. The supernatant was discarded, and the cell pellets were redissolved in BMMY media. BMMY media

composition is identical to BMGY, except 2 % methanol is used instead of 1 % glycerol. The BMMY suspended cells were placed in the culture flask and covered with four layers of cheesecloth. The flasks were incubated in the shaker (300 rpm, 30 °C) for 36 hrs. Upon completion of the induction phase, the culture was centrifuged at 3000 rpm for 45 min. The supernatant was collected and disodium EDTA was added to make the final concentration 5 mM. The supernatant was passed through the 0.22 µm bottle top filter to remove the particulates. A prefilter was used along with the bottle top filter to prevent the premature clogging of the 0.2 µm filter.

The purification of the supernatant was based on anion exchange chromatography using Q-sepharose FF. The pH of the supernatant was adjusted to 6.5 by addition of sodium hydroxide. This step is crucial for the TMEGF456 to bind to the anion exchange column. Two buffers were prepared for the ion exchange, buffer A (50 mM Tris, 1 mM EDTA) and buffer B (50 mM Tris, 1M NaCl, and 1 mM EDTA). The anion exchange column was prepared by packing q-sepharose FF into an empty 200 mL glass column. 40 mL bed volume of q-sepharose ff was loaded into the glass column. The column was attached to the FMI pump attached with solvent inline filter. The column was equilibrated with 5 bed volumes of buffer A. The flow rate was set to 5 mL/min. Following equilibration the filtered supernatant was passed through the column at the rate of 5mL/min. Since this part of the procedure took several hours to complete, the ion exchange was performed in the cold room at 4 °C. The column was washed with 3 column volumes of buffer A. The TMEGF456 was eluted by step gradient with buffer B through the column. The proteins eluted after the gradient was changed to 30% buffer B. The fractions containing TMEGF456 were verified using protein C activation assay. The active fragments were concentrated down to 2 mL volume using Centricon plus-70 centrifugal filter units with 10 kDa molecular weight

cutoff (Millipore). The concentrated fractions were injected into a reverse phase Waters HPLC system. The HPLC system contained Vydac dC18, 5 µm particle, 300 Å 4.6x250 mm column held at 58 °C. Upon injection, a gradient is run at flow rate of 1 mL/min from 10 to 26% acetonitrile, 0.1 % trifluoroacetic acid over 8 minutes, followed by 26% to 40% acetonitrile over the next 14 minutes and finally from 40% to 90% over the next 25 minutes.

Results and Discussion:

Purification of thrombomodulin

Thrombomodulin is anchored on the luminal surface of the endothelium and as previously indicated, thrombomodulin lacking the cytoplasmic domain appeared normal. Thrombomodulin undergoes endocytosis and degradation, and a significant amount of it is cleaved off and it is found in blood. The concentration of thrombomodulin in serum and plasma are reported to be between 3 to 300 ng/mL [84]. The thrombomodulin is removed through urine. Several groups have previously developed methods to isolate thrombomodulin from urine. The methods they developed were not intended for repeated and rapid purification of thrombomodulin from urine. They also used large volumes of pooled urine sample to isolate thrombomodulin, for example, Jackson et al, started from 38 L of urine to isolate thrombomodulin. The previously developed methods were not applicable for our study, since we needed quick method of thrombomodulin isolation from urine. The need for rapid processing stems for our need to prevent oxidation occurring during processing and also to compare levels of oxidation occurring in different individuals.

Thrombomodulin concentration in urine was assayed by using IMUBIND Thrombomodulin ELISA kit by American Diagnostica Inc. This is a sandwich ELISA which employs two monoclonal antibodies, first antibody recognizes the EGF1-EGF2 region of thrombomodulin, and second antibody recognizes EGF5-EGF6 region. At the end of the assay, the concentrations of thrombomodulin were determined by measuring the solution absorbance at 450 nm using a Safire microplate reader by Tecan. Typical volume of morning urine obtained from a donor was 200-500 mL. The urine from the morning was chosen since they have more volume, and they are higher concentration of thrombomodulin. The average concentration of thrombomodulin in urine before any processing was 3-8 ng/ml, based on ELISA assay.

As mentioned in the materials and methods section, several methods were tested for purification of thrombomodulin from urine prior to purification using HPLC. Regardless of the method, after collection, urine samples were cooled in 4 °C for no more than 2 hours, and centrifuged in Sorvall GS-3 rotor at 4,000 rpm for 30 min at 4 °C. The samples were stored at 4 °C because the cooling produces precipitate.

Tangential flow filtration (TFF) System

Labscale Tangential Flow Filtration (TFF) System, manufactured by Millipore, was tested for the concentration of the urine sample. In regular filtration, particles that are too big to pass through the membrane are retained on the membrane surface, and small particles pass through the membrane. As the filtration progresses, the large particles accumulate on the surface of the membrane, this lowers the surface area of the membrane, and small particles do not pass through the membrane. In TFF system, solution is pumped tangentially along the surface of the membrane, unlike normal filtration where the flow of the solution is normal to the plane of the

membrane. The applied pressure forces the smaller molecules through the filter and the molecules that are too big to pass through the membrane are swept away by the tangential flow.

Jumbosep spin filter

Jumbosep spin filters were tested for the concentration of the urine sample. The Jumbosep centrifugal device (Pall) consists of sample reservoir, membrane filter insert, and the filtrate receiver. The membrane filters are constructed from modified polyethersulfone on polyethylene substrate, and they are low protein binding. A membrane filter with molecular weight cut off of 30 kd was used for purification. The urine sample that was previously cooled at 4 °C was concentrated followed by buffer exchange, passed through SP Sepharose FF and Q Sepharose FF, and thrombomodulin was eluted. We compared the amount of thrombomodulin retained in the concentrate after the purification using Jumbosep and TFF system. The urine sample was split into two equal parts and they were run simultaneously. The ELSIA test showed that there was ten times higher concentration of thrombomodulin in retentate from Jumbosep compared to TFF system. We did not incorporate the concentration step in our final purification method, however if we incorporate a concentration step in the future, we will use Jumbosep.

Q sepharose purification

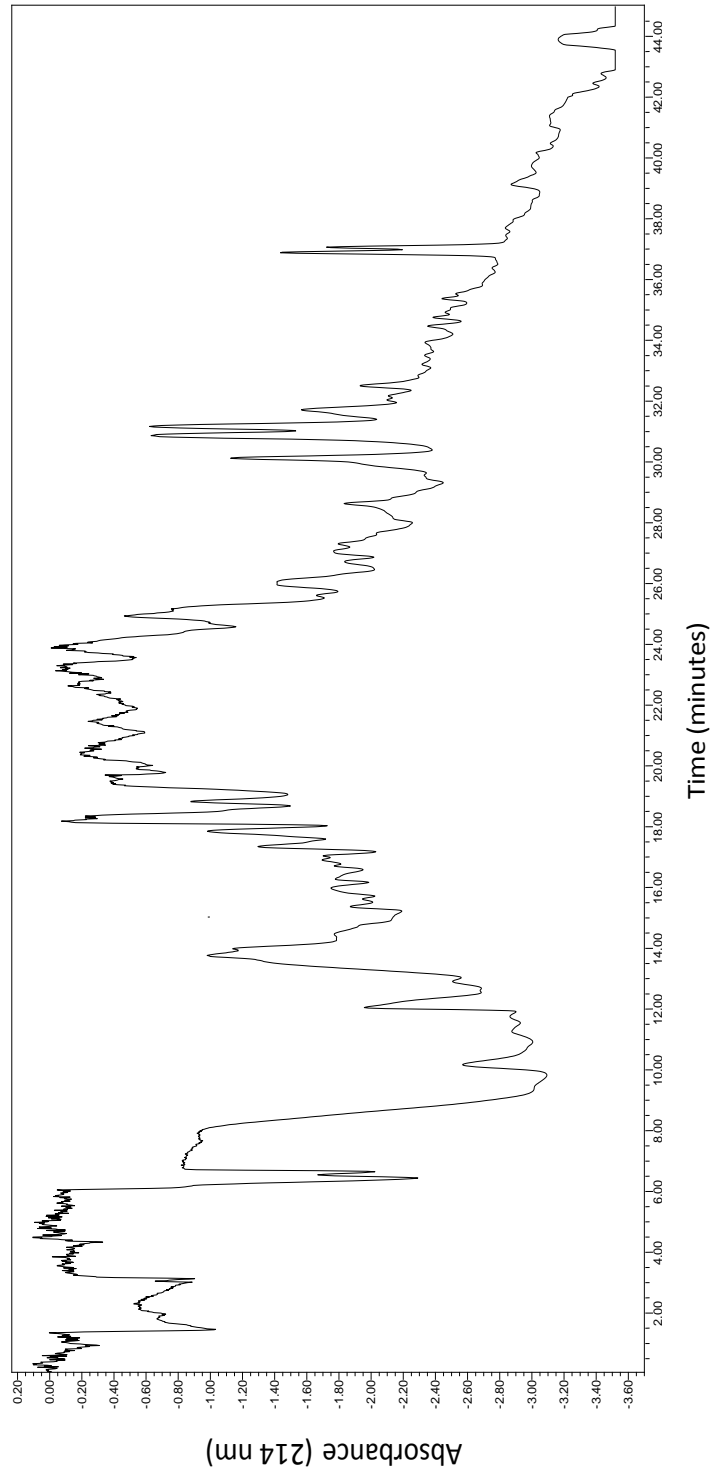
The urine sample that was cooled at 4 °C and centrifuged in Sorvall GS-3 was passed through the polyethersulfone 0.2 µm bottle top vacuum filter from corning. These hydrophilic filters are low binding to proteins and they remove particulates during filtration. The removal of particulates is necessary prior to the downstream ion exchange; their presence will clog the Q Sepharose column and lower the column life. Ideally, buffer exchange would be an appropriate step for the urine sample to undergo so the ionic strength of the various samples is constant.

Buffer exchange with high volumes of samples would be time consuming; instead 0.25 M imidazole acetate at pH 6.0 was added to make final concentration 25 mM imidazole acetate. Thrombomodulin is a very acidic protein; the pI of the protein is approximately 4.8. The pH of the urine sample was adjusted to 6.0 to make the protein negatively charged. Next, the urine sample was passed through a 50 mL Bio-Rad column containing 20 mL bed volume of Q Sepharose FF using FMI pump. The column was previously equilibrated with 3 bed volumes of 25 mM imidazole acetate at pH 6.0. The negatively charged proteins along with thrombomodulin bind to the ion exchange column. As previously mentioned, thrombomodulin is not found intact in urine, instead it is fragmented. Fortunately, all the thrombomodulin fragments are uniformly acidic throughout its length, making the ion exchange purification of its various fragments simple. The column is washed with 25 mM imidazole acetate at pH 6.0, and proteins are eluted using 25 mM imidazole acetate, 50 mM NaCl at pH 6.0, and 25 mM imidazole acetate, 0.5 M NaCl at pH 6.0. Eluents following the addition of 25 mM imidazole acetate buffer, pH 6.0, 0.5 M NaCl was collected in 15 mL conical tube, 5 mL in each tube. 15 mL eluent, after addition of 25 mL of 25 mM imidazole acetate buffer, pH 6.0, 0.5 M NaCl, tested positive for thrombomodulin. Solution of 0.2 M L-methionine was added to thrombomodulin containing fractions to make final concentration 2 mM methionine. Methionine was added as a sacrificial molecule to absorb oxidants that might be occurring during the downstream processing. The thrombomodulin containing fractions dried using medium heat, under vacuum, using the *SpeedVac* concentrator system. The *SpeedVac* surface that was accessible to light was covered with aluminum foil to prevent photooxidation. The dried samples were reconstituted into 18.2 megaohm pure water.

Reverse phase HPLC purification

Thrombomodulin containing fragments from Q Sepharose were dried using SpeedVac under vacuum, and reconstituted in 18.2 megaohm pure water, passed through the 0.2 μ M spin filter, and were injected into the HPLC. A shows the HPLC chromatogram obtained from the injection of 1.4 mL sample of 27 year old male donor. Figure 2.2 B is the magnification of Figure 2.2 A, and it spans the area where thrombomodulin elutes, namely at 30 minute. The total amount of thrombomodulin isolated from a single urine sample after the HPLC purification was 0.05–0.1 μ g, which is not sufficient to compare the relative oxidation of methionine 388 oxidation. Multiple urine samples were collected from an individual donor to increase the amount thrombomodulin collected following HPLC purification. Typically 2-3 urine samples were collected from a donor within consecutive days, 0.15-0.25 μ g thrombomodulin was collected after the samples were pooled.

A.



B.

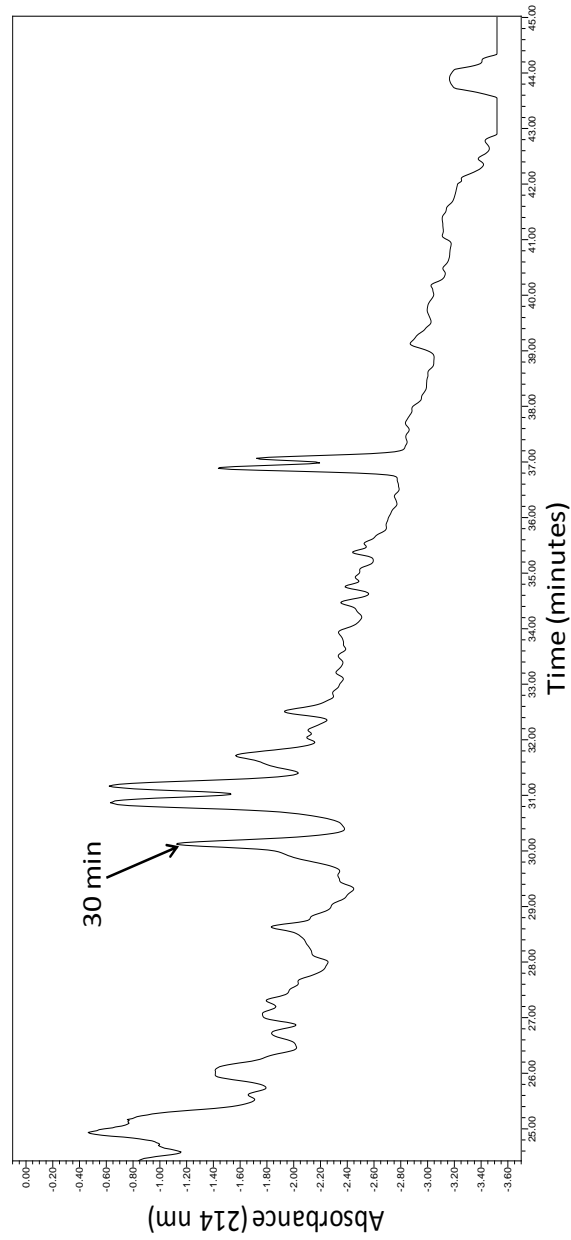


Figure 2.2 A&B: Final purification of thrombomodulin by reverse phase HPLC. The following sample, a final purification of a male non-smoker urinary thrombomodulin, contained thrombomodulin in the peak at 30 minute. Figure 2.2 B is a selected area from Figure 2.2 A.

Oxidation during processing

Oxidation that could be occurring during the purification of thrombomodulin has always been a concern. Several steps were taken to minimize the oxidation that was occurring during processing. During the lyophilization step, the samples were shielded from light to prevent photo oxidation of methionine. The transparent regions of the speedvac were covered with aluminum foil. Thiodiglycol was added to the urine samples prior to collection. It was added as a scavenger molecule to prevent methionine oxidation. 5 mL of thiodiglycol was added to the centrifuge bottle before the samples were collected. 1 mL of 0.5 M EDTA pH 8.0 was added to the centrifuge bottles prior to urine collection. EDTA is a chelator[155], and it has been shown to slow Fenton oxidation[156, 157]. Following the elution of thrombomodulin from the Q-sepharose FF column and reverse phase HPLC, free methionine was added to make the final concentration to 2 mM. This was added as sacrificial molecule to prevent oxidation of methionine 388 during processing.

Final method for the purification of thrombomodulin

The urine sample was cooled at 4 °C, centrifuged in Sorvall GS-3, and passed through the polyethersulfone 0.2 µm bottle top vacuum filter. The pH of the sample was adjusted to 6.0, and the sample was passed through a column containing 20 mL bed volume of Q Sepharose FF. The protein was eluted using NaCl gradient, and dried under vacuum, and injected into the reverse phase HPLC. There was ten times the amount of thrombomodulin retained after the purification using Jumbosep compared to TFF system. The purification using 20 mL bed volume of Q Sepharose FF purified 1.5 fold more thrombomodulin compared to Jumbosep, however Q Sepharose FF was without any preconcentration step. Besides the low yield of thrombomodulin,

the concentration using Jumbosep or TFF was also accompanied by other drawbacks. The purification and buffer exchange of 400 mL of urine sample required on average 6 hours, which increased the likelihood of our protein being oxidized. Also, it required frequent buffer changes and manipulation of the sample, so was quite labor intensive. On the other hand, the processing time using Q Sepharose FF typically was just 2-3 hours and, equally importantly, gave a higher yield of thrombomodulin.

Digestion of thrombomodulin

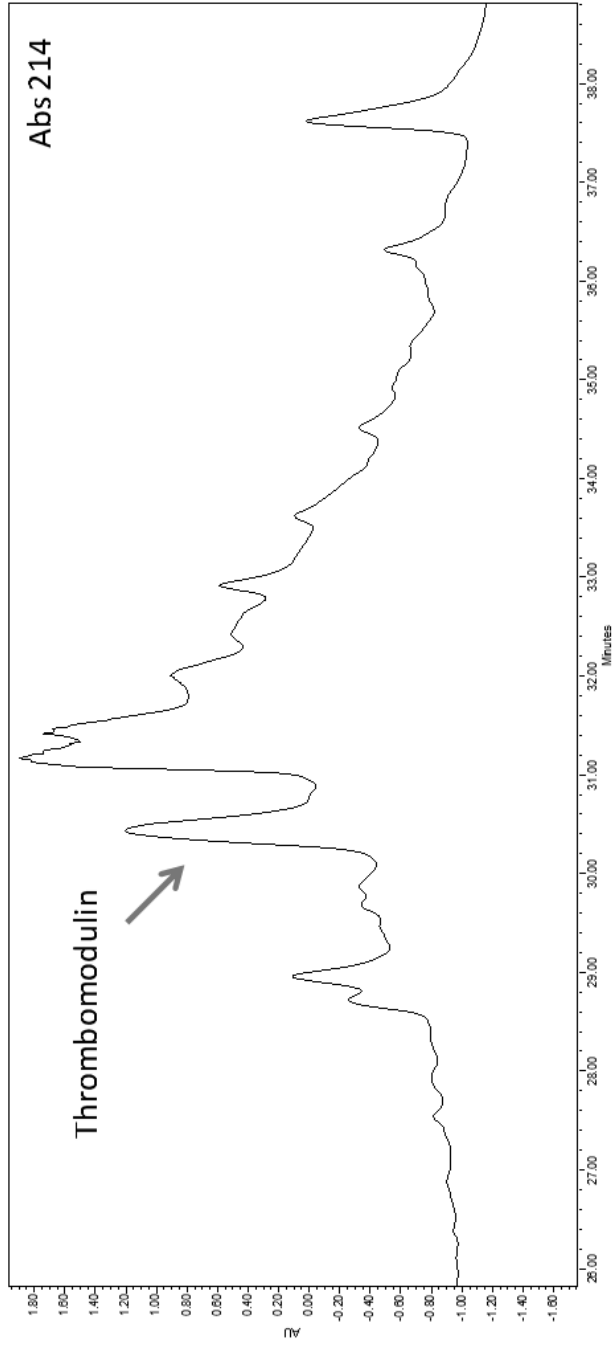
The linear chains of amino acids fold into a specific structure to carry out their functions. The folded protein in its native state is stable under physiological conditions. The forces behind the 3D folded structure of proteins are following: 1) Hydrogen bonds [158]. 2) Van der Waals interactions. 3) Backbone angle preferences. 4) Electrostatic interactions. 5) Hydrophobic interactions [159-161]. Collectively these forces can be referred to as forcefields [162]. In order for a protein to undergo complete digestion, the forcefield associated with the folded protein must be disrupted to unfold the protein. The folded protein imposes steric hindrance to the protease, as a result the protease is unable to access and ultimately cleave the protein. In most proteomics studies it is assumed that the digestion of proteins is driven to completion, and such assumption is correct for most proteins. However, many proteins are resistant to proteolysis, and they do not undergo complete digestion. There are several factors that affect the protein digestion, including but not limited to ineffective denaturation due to poor protein solvation, lack of localization of enzyme, short reaction time, and the competing presence of other proteins in the digestion [163]. Effective digestion of purified thrombomodulin was crucial to quantify the

reduced and the oxidized peptides ratios to quantify the methionine oxidation. We monitored the degree of thrombomodulin digestion by chymotrypsin by injecting the digested sample into the HPLC. The degree of digestion was calculated by comparing the area of the peak occupied by thrombomodulin in HPLC chromatogram before and after chymotrypsin digestion (Figure 2.3).

Digestion using manufacturer's protocol

Using the manufacturer's protocol in which the HPLC purified dried thrombomodulin fraction was reconstituted in minimal volume of 200 mM Tris-HCl pH 8.0, reduced using TCEP, deglycosylated, and digested for 2 hrs using 4 Units of chymotrypsin dissolved in 50 mM Tris-HCl, 1mM CaCl₂, pH 8.0 only digested 15 % of the total thrombomodulin. Until this point, we were under the assumption that the digestion using chymotrypsin went to completion, hence we focused towards creating a method in the Acquity UPLC that would provide a PDA chromatogram that gave us resolved oxidized and the reduced peaks following digestion of thrombomodulin. Little did we know, only 15% of the thrombomodulin underwent complete digestion after 2 hr reaction with chymotrypsin (Figure 2.3). Following this discovery, the priority for this project was to create a digestion method that would effectively digest thrombomodulin to the extent that would allow us to detect the oxidized and the reduced forms of peptide using the quantification of methionine techniques that will be discussed further in this chapter.

A.



B.

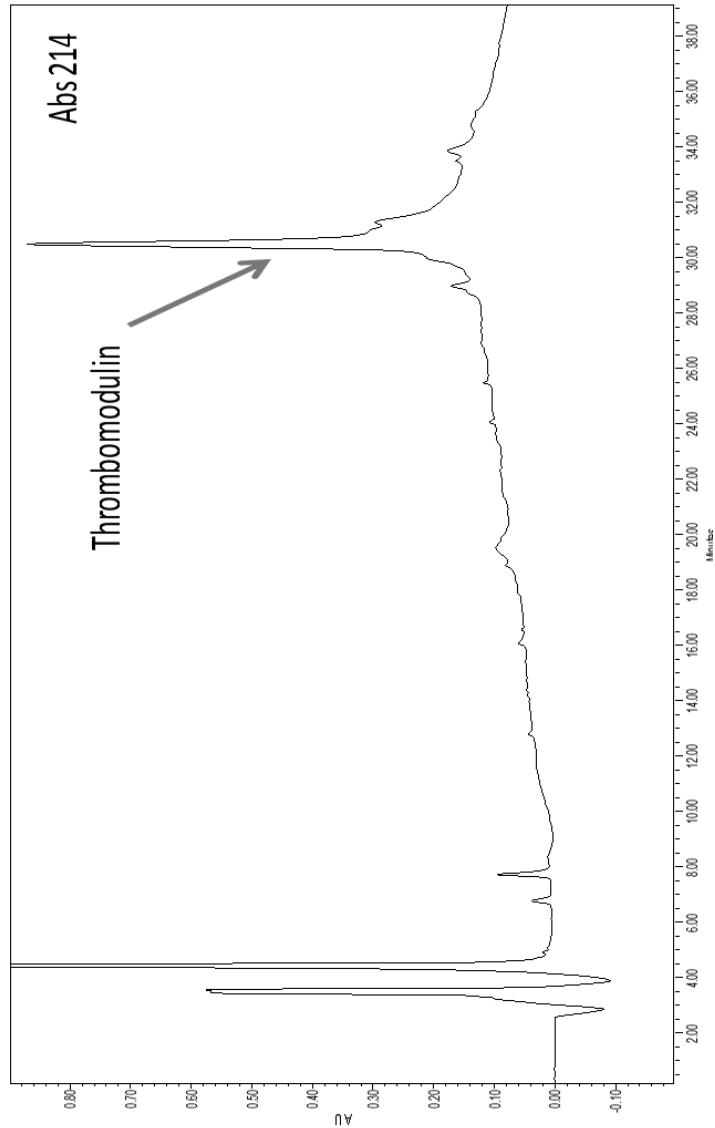


Figure 2.3 A&B: HPLC chromatogram of purified thrombomodulin prior to chymotrypsin digestion (A). HPLC chromatogram of 2 hr chymotrypsin digested thrombomodulin (B). Two chromatograms were used to monitor the extent of digestion. The efficiency of various digestion protocols were tested using this method.

Digestion using urea

Protein digestion using urea is still the most widely used technique to solubilize proteins in a solution digestion. The mechanism of protein denaturation by urea is believed to begin with attachments of urea to histidine, and then to the amide groups and positively charged amino acid and, which results in disruption of the hydrogen bonds, followed by water and urea solvation [164, 165]. Over the time, urea in water releases isocyanate, which can carbamylate primary amines, hence urea was freshly prepared. Also, upon heating, urea can degrade into ammonium cyanate, which can lead to the carbamylation of the primary amines. Based on the manufacturer's manual, chymotrypsin retains 100% enzymatic activity in up to 4M urea. In the experiments where 8M urea was used, the concentration of the urea was halved by dilution using the appropriate buffer prior to addition of chymotrypsin. The thrombomodulin digestion using 2.5 M and 8 M urea did not enhance the digestion compared to the initial method.

Digestion using acetonitrile

Changing the solvent in which the digestion is probably the most convenient way to facilitate the solubilization of the protein, and increased solubility can potentially increase the rate of digestion by protease. Trypsin digestion of protein in acetonitrile has been used in some proteomics studies [166, 167]. Trypsin digestion was conducted on myoglobin and chicken ovalbumin using organic solvent [167]. These proteins are resistant to proteolysis unless they are subjected to thermal or chemical denaturation prior to digestion. These proteins are easily digested in organic solvent, efficient digestion occurred within 5 min [167]. The autolysis of the protease is a concern when using organic solvents, but the proteolytic activity of trypsin is not affected even in 80% acetonitrile solution. Also, since these protocols did not use chaotropes or

surfactants, it makes downstream MS or HPLC analysis easier. The use of chymotrypsin to digest thrombomodulin in an organic solvent was attempted achieve complete digestion. Chymotrypsin in water miscible organic solvents like methanol, ethanol, 1-propanol, 2-propanol and acetonitrile shows enhanced stability and catalytic activity [168]. In the presence of 20% acetonitrile in buffer, the enzyme activity of chymotrypsin increased up to 6 fold. Accordingly, thrombomodulin was dissolved in acetonitrile in 50 mM Tris-HCl/10 mM CaCl₂, pH 8.0 buffer. Multiple experiments where the concentration range of acetonitrile was varied between 20%-50% were conducted. Unfortunately, this method did not enhance the digestion compared to the initial method.

Digestion using Invitrosol

Invitrosol is a proprietary surfactant from Invitrogen. It keeps hydrophobic proteins in the solution and it has no effect on the activity of protease. More importantly, it is compatible with HPLC and MS analysis, unlike most surfactants that interfere with such instruments. The use of Invitrosol to enhance the digestion of thrombomodulin from urine did not result in significant improvement in digestion. Experiments using organic solvent along with Invitrosol did not enhance the digestion compared to the initial method.

Digestion using RapiGest

RapiGest SF is an acid labile surfactant. It can be immediately be degraded from solution by addition of acid. It decomposes into dodeca-2-one and sodium-3-(2,3-dihydropropoxy) propanesulfonate. The first product is insoluble in water and second is highly soluble in water, so it is not retained in reverse phase HPLC. This feature makes the use of this surfactant attractive, since it does not interfere with the downstream analysis like HPLC and MS. RapiGest SF

improves solubility of protein in a solution and is an enzyme friendly surfactant for in-solution protein digestion. Proteases are resistant to denaturation in RapiGest SF solution. Trypsin has 100% activity in 0.1% RapiGest SF solution, and 87% activity in 0.5% RapiGest solution [169]. The proteins unfold in RapiGest SF solution and the proteolytic sites are exposed for digestion. The globular proteins, which are typically difficult to digest, can be digested within minutes using RapiGest SF [170]. This surfactant also aids in deglycosylation of proteins. However, the use of RapiGest SF to digest the thrombomodulin isolated from urine did not enhance the digestion compared to the initial method.

Digestion using EDTA

The thrombomodulin EGF domains 5 and 6 are the primary binding site for thrombin [171, 172], while EGF4 of thrombomodulin is necessary for the thrombin-thrombomodulin mediated protein C activation [173, 174]. In studies using recombinant thrombomodulin, the thrombin-thrombomodulin mediated activation of protein C shows a bell shaped dependence on Ca^{2+} concentration. Ca^{2+} binds to EGF3 and EGF6 region of thrombomodulin, the binding affinity is almost 15 fold higher in the EGF6 region based on Ca^{2+} analysis of dialyzed recombinant thrombomodulin [175]. There is a higher proportion of β -sheet and more structured conformation seen in the EGF domains that are saturated with Ca^{2+} . The ordered conformation resulted in decreased efficiency of trypsin to cleave the C-loop of EGF6. We alternatively hypothesized the partial digestion of thrombomodulin might be due to Ca^{2+} stabilization.

Proteins can be converted into apoproteins by addition of chelators like EDTA and EGTA. Ca^{2+} binds to thrombomodulin EGF3 and EGF6, and the binding of Ca^{2+} results in formation of secondary structures in these regions. The stabilization induced by Ca^{2+} binding is

known to make thrombomodulin resistant to protease digestion [175]. The simple method to expedite the digestion of thrombomodulin might be by removal of Ca^{2+} . Thrombomodulin purified from urine was digested using chymotrypsin in presence of 10 mM EDTA. Upon completion of digestion, the digested sample was injected into reverse phase HPLC to assess the degree of digestion. The HPLC peaks corresponding to thrombomodulin were no longer seen in the chromatogram, indicating complete digestion of thrombomodulin. Sample of digested thrombomodulin in presence of EDTA was sent for MS analysis. The MS did not identify peptides corresponding to 1562 or 1578 m/z. The results were puzzling, HPLC indicated the digestion had gone to completion; however MS did not confirm the presence of the methionine 388 containing peptide. The experiment were repeated several times, however the results consistently remained the same. The results were puzzling until we found the following paper, “The CBP/p300 TAZ1 domain in its native state is not a binding partner of MDM2” [176].

Zinc-binding proteins comprise one of the largest classes of protein and they are very important for the gene expression. This class of protein includes transcription factors of the DNA, co-activators, RNA polymerase and chromatin-modifying and -remodeling enzymes [177]. Many zinc-binding proteins need Zn^{2+} to fold into their 3D structure, and for participation in their intermolecular interactions. In the mentioned study [176], the zinc domain of transcriptional proteins CBP (CREB-binding protein) and p300 were studied. The zinc binding domain when treated with EDTA resulted in denaturation of the protein. They attempted to restore the native structure of the protein by addition of excess Zn^{2+} following EDTA denaturation, this did not result in refolding of the protein, suggesting irreversible denaturation by EDTA. More significantly, their results showed that under EDTA induced denaturation the proteins formed non specific interactions resulting in aggregation of proteins. They concluded that the EDTA

mediated denaturation leads to irreversible denaturation and aggregation of the proteins. Based on these findings, we believe that EDTA induced denaturation and aggregation resulted in disappearance of thrombomodulin peak in the HPLC chromatogram. The pursuit of thrombomodulin digestion using EDTA was discontinued.

Final method for the digestion of thrombomodulin

The dried thrombomodulin containing fractions from the reverse phase chromatography were pooled and dissolved in approximately 100 μ L of 1X phosphate buffered saline (PBS). The samples that were cloudy prior to digestion were spun in a microcentrifuge (Galaxy 14D VWR) at 13K RPM for a minute. This undissolved material proved to contain the bulk of the thrombomodulin. Fortunately, after the supernatant was removed, the precipitate readily dissolved in 75 μ L of 1X PBS. Fresh *tris*-carboxyethylphosphine (TCEP) was added to make the final concentration of 50 mM TCEP. 10 μ L of 0.5 M sodium phosphate buffer pH 7.5 added to the sample, prior to addition of 500 units of PNGase F (New England Biolabs). The samples were incubated at 37°C for 4 hours to deglycosylate the sample. The protein was then digested at 30°C for 8 hours after adding 4.1 μ g of sequencing grade chymotrypsin (Princeton Separations) dissolved in 50 mM Tris-HCl, 1mM CaCl₂, pH 8.0. The chymotrypsin was inactivated by placing the samples in a 100°C dry bath for 30 seconds. This method achieved thrombomodulin digestion which resulted in detection of both reduced and oxidized peptides by MALDI-TOF. This method was incorporated into the final purification and qualitative analysis of thrombomodulin.

Quantification of methionine oxidation

The quantification of urinary thrombomodulin methionine 388 oxidation involves 3 three major steps: 1) purification of thrombomodulin from urine, 2) digestion of purified thrombomodulin 3) quantification of methionine oxidation. We have discussed the first two steps, in this section we will discuss the methods attempted to complete the final step.

Alkylation of cysteine by fluorescent reagent

Cysteine is the most reactive residue commonly found in proteins and peptides. In its unprotonated state, the thiol group is strong nucleophile and will react with a wide variety of reagents. The alkylation of cysteine side chain was attempted for two reasons. First, the alkylation of cysteine prevents reformation of disulfide bonds which subsequently prevents the formation of the secondary structure hence protein remains in the denatured state. And second, our hope was to introduce a fluorescent reporter group on the peptide AIPHEPHRCQMF. Several peptides are formed when thrombomodulin is digested by chymotrypsin, not all of these peptides have cysteine. Only the peptides that have cysteine will have the covalently bonded fluorescent reporter group, when the digested and alkylated sample is injected into an HPLC with fluorescent detector, only the fluorophores will be detected by the detector. The attachment of fluorophore eliminates the noise from non fluorophore peptides, and in theory would increase the resolution and sensitivity of detection of the oxidized and the reduced peptide.

Reaction of the sulfhydryl group of cysteine with α -haloacids or their amide derivatives is the most common method used to alkylate cysteine. The iodo compounds is preferred over other halides since iodide is a much better leaving group, hence the reaction is much faster. 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS) [150] was used for

alkylation of cysteine in thrombomodulin. Reaction of a cysteine side chain with 1, 5 IAEDANS produces dansylated cysteine on the protein (Figure 2.4). 1, 5-IAEDANS has a peak excitation wavelength of 336 nm and a peak emission wavelength of 490 nm. The extinction coefficient of the dye is 5700.

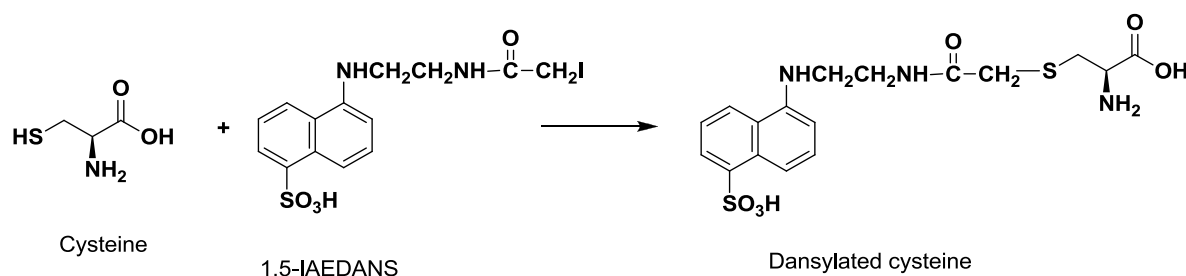


Figure 2.4: Alkylation of cysteine using 1, 5-IAEDANS.

Before attempting to alkylate the cysteine residues in thrombomodulin, the alkylation using 1, 5-IAEDANS was attempted in the synthetic peptide APIPHEPHRCQMF. The 2 μ L of 10 mg/mL synthetic peptide stock solution was dissolved in 198 μ L 0.1 M Tris-Cl pH 8.0 containing 50 mM freshly prepared *tris*-carboxyethylphosphine (TCEP). The sample was incubated, and 5 fold molar excess (over total thiols) of freshly prepared 1, 5-IAEDANS was added. Nitrogen was flushed over the surface of the liquid and the microcentrifuge tube was sealed. The sample was incubated for > 4 hrs at 37 $^{\circ}$ C. Following alkylation, 10 fold molar excess of 2-mercaptoethanol (2-ME) over 1,5-IAEDANS was added to the sample [154]. The sample was injected into the Waters ACQUITY UPLC System, BEH 130 C18 1.7 μ m 2.1x150 mm column, and Fluorescence (FLR) detector (excitation wavelength 340 nm, and emission wavelength 490 nm). The methionine and methionine sulfoxide forms of the peptide APIPHEPHRCQMF eluted at 2.25 and 2.5 minutes, respectively. The alkylated oxidized and

reduced peptides eluted at 2.35 and 2.6 minutes, respectively. Figure 2.5 is a UPLC chromatogram of 4 μL injection of 0.1 $\mu\text{g}/\mu\text{L}$ synthetic peptide alkylated using 1, 5-IAEDANS for 16 hrs at 37 $^{\circ}\text{C}$, and Figure 2.6 is UPLC FLR chromatogram of the same injection.

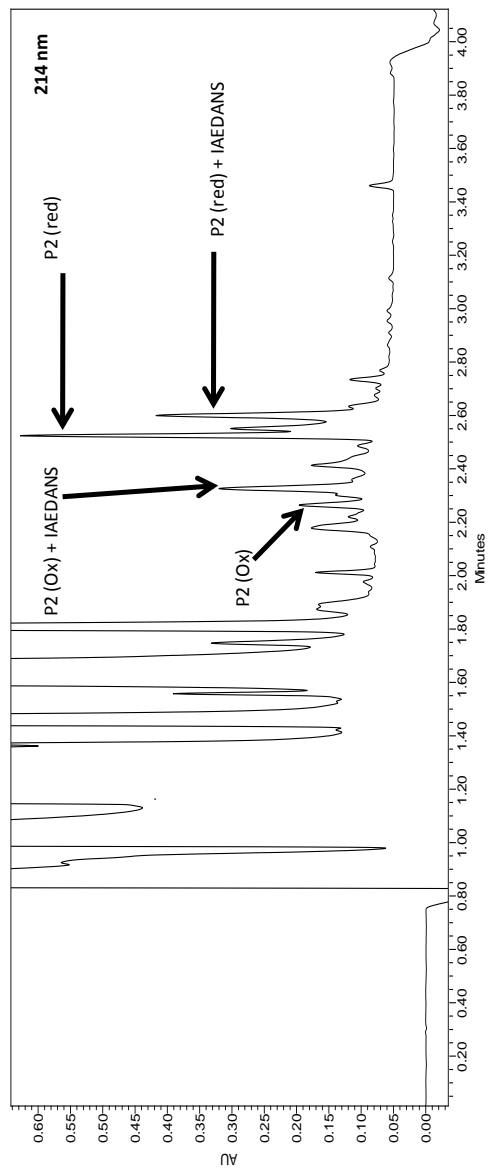


Figure 2.5: Acquity UPLC chromatogram of IAEDANS labeled synthetic APIPHEPHRCQMF and APIPHEPHRCQMoxF at 214nm. Two forms of non alkylated peptide APIPHEPHRCQMF eluted at 2.25 and 2.5 minutes, respectively. The alkylated oxidized and reduced peptides eluted at 2.35 and 2.6 minutes, respectively.

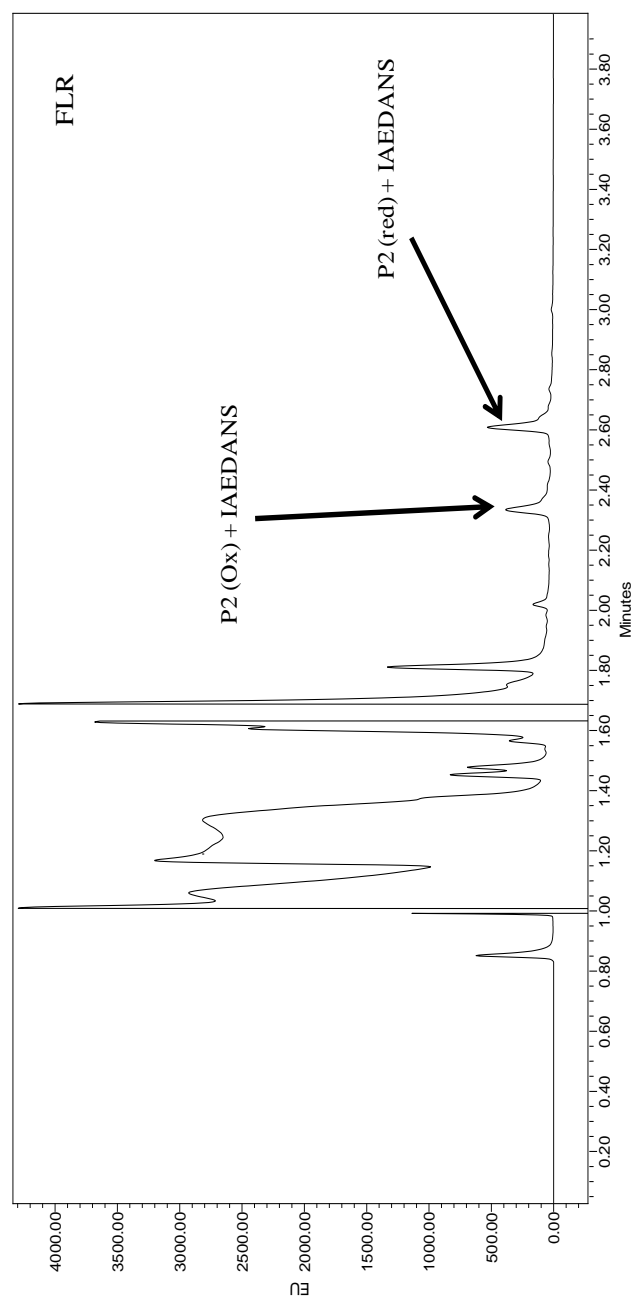


Figure 2.6: UPLC FLR chromatogram of IAEDANS labeled synthetic peptide AIPHEPHRCQM_{ox}F and AIPHEPHRCQMF. The two peptides eluted at 2.35 and 2.6 minutes, respectively.

The cysteine alkylation by 1,5-IAEDANS was attempted on urinary thrombomodulin purified by HPLC. The protocol used to alkylate the synthetic peptide was used to alkylate the purified thrombomodulin. The alkylated thrombomodulin was digested using chymotrypsin, and subsequently injected into the Acquity UPLC. Based on alkylation experiments performed using the synthetic peptide, the elution time of the alkylated oxidized and the reduced peptide was discovered. The FLR detector did not detect alkylated oxidized and the reduced peptide following alkylation using 1,5-IAEDANS and digestion using chymotrypsin. We hypothesized the absence of the alkylated peptides in FLR chromatogram resulted from ineffective alkylation, which was mostly likely due to stubbornness of thrombomodulin to denature. This method was not incorporated in quantification of methionine 388 oxidation, because significant alkylation of thrombomodulin could not be achieved that would produce alkylated peptide FLR signal. In retrospect, the difficulty may have been the lack of digestion of thrombomodulin, so revisiting this labeling procedure may be worthwhile.

Determination of methionine 388 oxidation by UPLC

Acquity UPLC offers increased resolution, sensitivity and speed compared to HPLC. It uses small particle columns (1.7 μm – 1.8 μm), which improves system efficiency by producing better resolution, and also lowering the analysis time. This system is designed to minimize band spreading resulting from the column and also from the instrument. Several UPLC methods were developed to separate the synthetic peptide mixture (APIPHEPHRCQM_{ox}F and APIPHEPHRCQMF). New methods were created by changing the gradient, temperature, flow rate, and column. After numerous experiments we developed our most effective UPLC method for purification. Using this method the oxidized and the reduced peptides in the synthetic peptide

mixture eluted at 12 and 21 minute respectively, they were 9 minutes apart from each other (Figure 2.7).

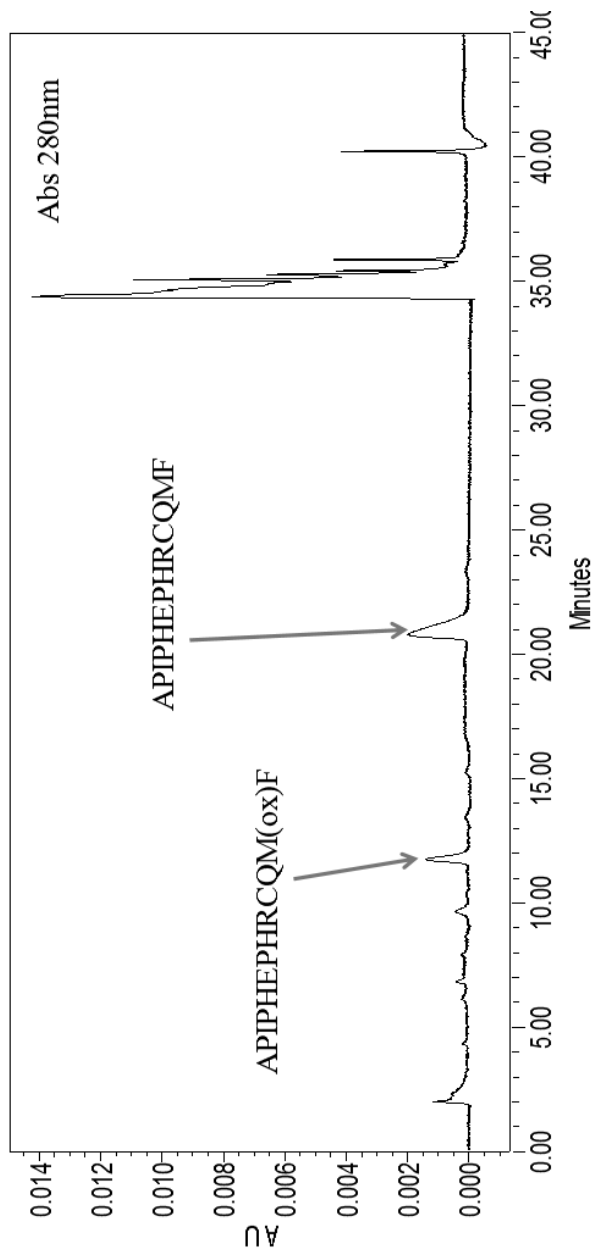


Figure 2.7: Acquity UPLC chromatogram of the commercial peptide mixture. APIPHEPHRCQM(ox)F elutes at 12 min and APIPHEPHRCQMF elutes at 21 min.

The ratio of oxidized and reduced peptide within the synthetic peptide can be calculated using the UPLC chromatogram simply by dividing the area occupied by the oxidized by area occupied by the reduced peptide. Using this approach we should be able to calculate the ratio of the reduced and the oxidized peptide in a chymotrypsin digested thrombomodulin sample. The ratio of reduced and the oxidized peptide within a sample is equivalent to the ratio of reduced and the oxidized methionine in a sample of thrombomodulin purified from urine.

The HPLC purified dried thrombomodulin following chymotrypsin digestion was injected in the UPLC. The elution of the oxidized and the reduced peptide was mapped using the synthetic peptides, they elute at 12 and 21 min respectively. Figure 2.8 is UPLC chromatogram of 25 μ L injection of chymotrypsin digested thrombomodulin of 26 year old non smoker donor.

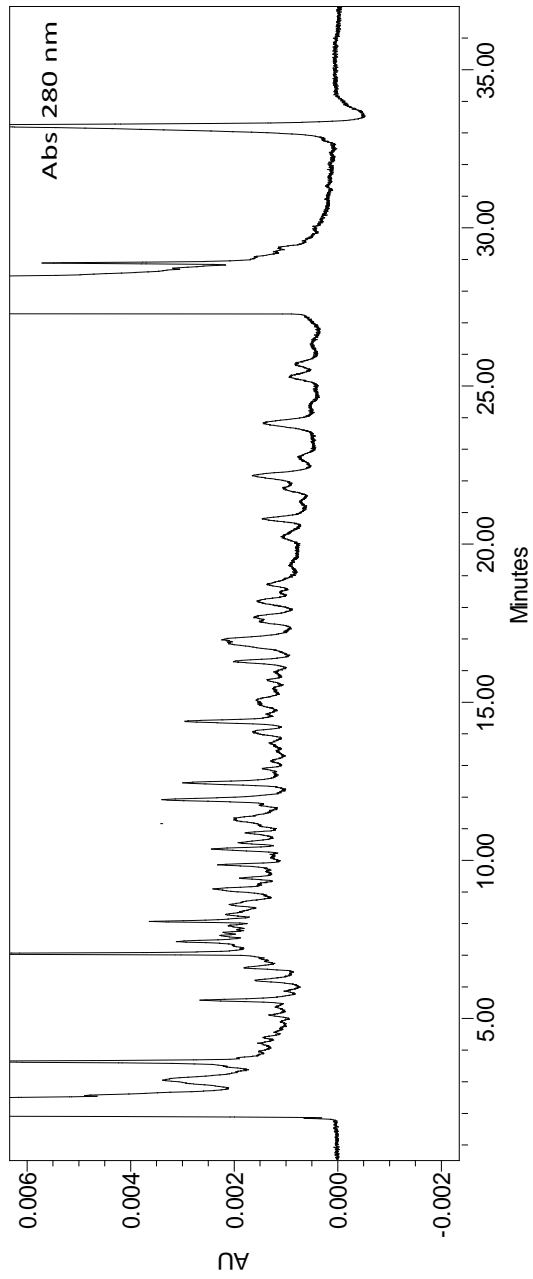


Figure 2.8: UPLC chromatogram of chymotrypsin digested urinary thrombomodulin of 26 year old non smoker donor.

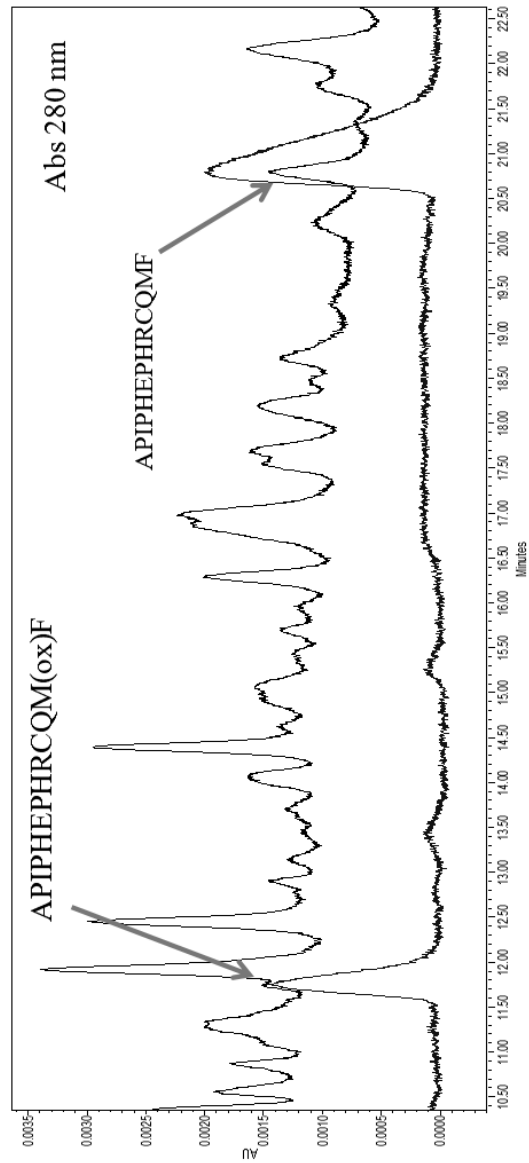


Figure 2.9: Overlay of UPLC chromatograms. Top chromatogram is of chymotrypsin digested urinary thrombomodulin of a 26 year old Caucasian male non smoker, and the bottom chromatogram is of synthetic peptide mixture.

Figure 2.9 is overlay of UPLC PDA chromatogram of chymotrypsin digested urinary thrombomodulin and synthetic peptide mixture. As seen in the figure the oxidized and the reduced peaks of the chymotrypsin digested urinary thrombomodulin is not resolved, especially the oxidized peptide. Much time and resources were devoted to develop methods to resolve the two peaks, however the resolution necessary to calculate the relative area occupied by the reduced and the oxidized peaks could not be achieved.

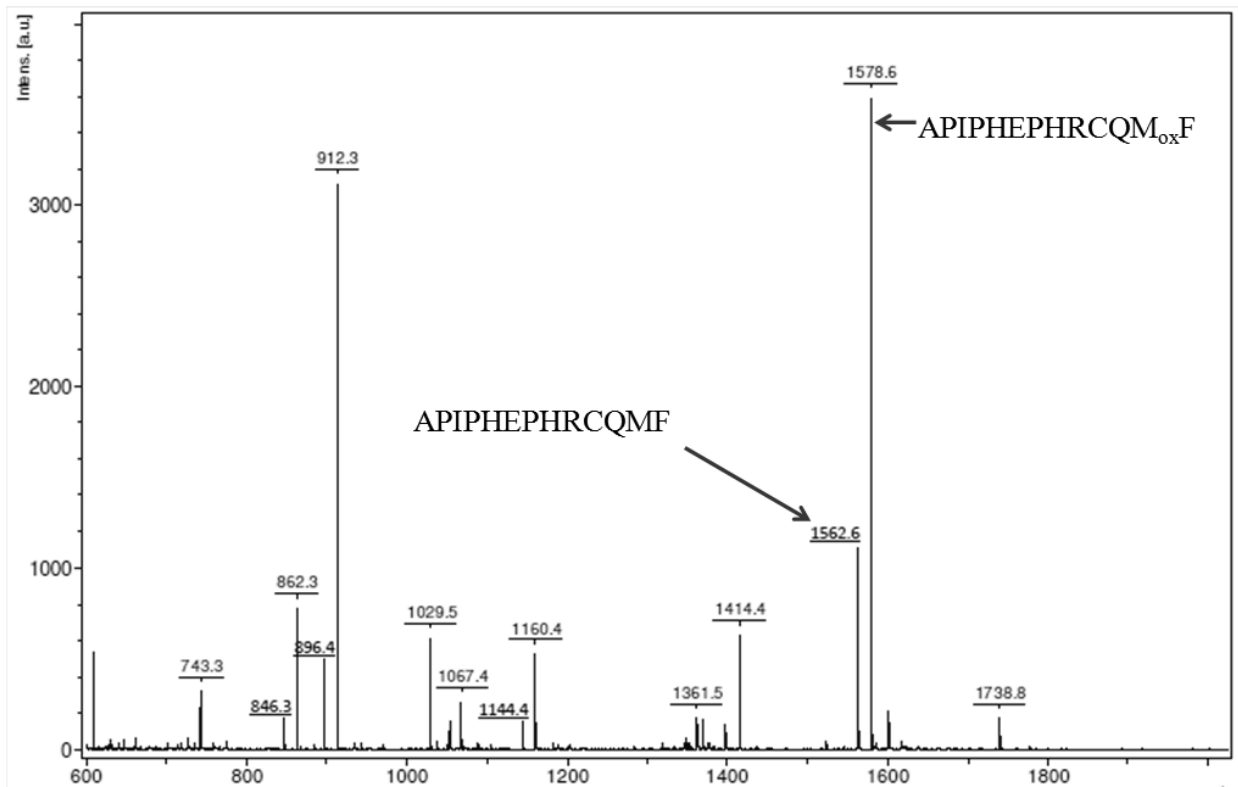


Figure 2.10: MALDI-TOF spectra of chymotrypsin digested thrombomodulin purified from urine. The sample was collected from a 28 year old male smoker. The oxidized and the reduced forms of peptide are present in this sample.

Mass spectrometry analysis of methionine 388 oxidation

Matrix-assisted laser desorption ionization (MALDI) is a powerful technique for mass spectrometry of peptides and proteins [178]. This technique provides fast and accurate acquisition of molecular mass and purity. The digested thrombomodulin was sent for MALDI analysis in Mass Spectrometry Facility located at the University of Arkansas. Figure 2.10 is MALDI-TOF spectra of chymotrypsin digested urinary thrombomodulin from a 28 year old male smoker. The isotopically averaged mass of reduced (AIPHEPHRCQMF) and oxidized (AIPHEPHRCQMoxF) peptides is 1562.73 and 1578.74 Da respectively. Both forms of peptides were detected by MALDI-TOF, and the intensity of the oxidized peptide was three fold more compared to its counterpart. This technique can be used for semi-quantitative test for the two forms of peptide.

Final method for the quantification of methionine 388 oxidation

The chymotrypsin digested thrombomodulin alkylation using 1,5-IAEDANS did not result in sufficient alkylation of the peptides, and they could not be visualized in the FLR detector. Separation of reduced and the oxidized peptides using Acquity UPLC could not produce sufficient resolution to quantify the oxidation of methionine. The MALDI TOF injection of digested thrombomodulin confirmed the presence of two forms of peptides and also provided their relative intensities. This intensity of the peptides was used for the quantification of the methionine oxidation, the details of this method will be covered in the following chapter.

Conclusion

In this chapter we have discussed techniques that were used to for 1) purification of thrombomodulin from urine, 2) digestion of purified thrombomodulin 3) quantification of methionine oxidation. The cooled urine sample centrifuged in a Sorvall GS-3 rotor, filtered using 0.22 μm PES membrane filter, passed through Q Sepharose FF, and reverse phase HPLC provided maximum yield. The digestion of purified thrombomodulin where the samples were washed in 1XPBS buffer prior to addition of chymotrypsin was the most successful compared to other methods. The fluorophore and Acquity UPLC system could not be used for quantification of methionine oxidation. MALDI-TOF was used for quantification of methionine oxidation; this topic will be discussed in detail in chapter 3. This procedure works well, but it is worth considering how it might be improved in the future.

Oxidation that might be occurring during the purification of thrombomodulin would not invalidate a finding that one population had a higher level of oxidation than another, but would complicate comparison of absolute levels of oxidation. Based on oxidation experiments using synthetic peptide, we believe significant oxidation is not occurring during processing. A 300 μL of synthetic peptide APIPEPHRCQMF was divided into three 100 μL aliquots, the first aliquot was control. The second aliquot was dried under medium heat (45°C) for 4 hours in SpeedVac, and upon drying the synthetic peptide was reconstituted in distilled water to make the final volume 100 μL . The third 100 μL aliquot was allowed to sit in room temperature for 4 hours, which is the time samples are subjected to chymotrypsin digestion and which is also approximately the time it takes for a sample to be centrifuged and passed through the Q Sepharose FF column. All three samples were separately injected into the Acquity UPLC and the ratio of oxidized and the reduced form of APIPEPHRCQMF for each sample was calculated.

The ratio of reduced and oxidized peptide remained constant in all three samples. The calculation was performed comparing the relative area occupied by reduced and oxidized peak in the UPLC PDA chromatogram at 214nm.

Although this experiment was reassuring, steps were taken to minimize oxidation of protein during processing. During the lyophilization step, the samples were shielded from light to prevent photo oxidation of methionine. Thiodiglycol was added to the urine samples prior to collection. Thiodiglycol was added as a scavenger molecule to minimize methionine oxidation [179, 180]; it was added to the urine sample container before collection. EDTA was also added to the sample container prior to urine collection. EDTA is a chelator [155, 181], and it has been shown to slow Fenton oxidation [156, 157], and would also inactivate any metal dependent proteases that might be present [182, 183]. Following the elution of thrombomodulin from the Q-sepharose FF column and reverse phase HPLC, free methionine was added as a sacrificial molecule to minimize oxidation. None of these additives used to minimize oxidation were used for the synthetic peptide oxidation experiments. Oxidation of synthetic peptide dissolved in water may not mimic the behavior of protein in urine, because latter is a complex solution composed of cells, proteins and salts. However, the methionine in the synthetic peptide is fully exposed to the solvent, and methionine 388 in thrombomodulin is partly buried in the protein's hydrophobic core, which might make it less susceptible to oxidation than the synthetic peptide. While we are reasonably confident that oxidation of the protein is not occurring, experimental proof of this would be useful.

In the future, experiments can be designed to test the competency of each of the mentioned steps to minimize oxidation during processing. It is possible to produce large quantities of the recombinant TMEGF 456 using *Pichia pastoris*. As previously mentioned,

methionine 388 is found between EGF 4 and 5 of thrombomodulin. The recombinant TMEGF 456 could be subjected to the urine purification protocol. Recombinant TMEGF 456 could be purified containing only single additives, followed by purification using all additives. The recombinant TMEGF 456 purified without any additives would serve as the control. Successful completion of this experiment will tell us the approximate oxidation that occurs during the processing of urine sample. It should be pointed out that the minimization of oxidation during the processing would be advantageous for this research; however, it is not critical for its success. Since all the urine samples are subjected to the same purification method, the oxidation that could be occurring during processing should be the similar for all of them. When the methionine oxidation levels in smokers and non smokers are calculated, the oxidation that occurred during their respective purification, assuming they are equal, cancels out each other.

The thrombin affinity chromatography was the very first method used to isolate thrombomodulin and it has been used in some other studies [184-186]. Dr. Deepika Talla, a former graduate student in our lab, had examined a method based on thrombin affinity chromatography. However, this method failed to produce significant thrombomodulin from urine apparently due to the following reasons. First, papers using thrombin affinity used some other steps prior or after to finish cleaning up the thrombomodulin, so it was not as simple in practice as it appears in theory. Second, depending upon the glycosylation state of thrombomodulin there is variance towards its affinity for thrombin column [187]. Third, and most importantly, Komives' group confirmed that thrombomodulin oxidation reduces thrombin affinity by a factor of 10. [94] In our preliminary work, thrombin affinity columns, even used in fairly large excess, "leaked" some thrombomodulin and this thrombomodulin was presumably more oxidized. This

method was therefore abandoned. However, might some other form of affinity purification work?

Antibodies might work, and should be explored in the future. Aptamers are *in vitro* selected oligonucleotides that have high affinity and specificity towards their target [188, 189]. They are selected in a process called systematic evolution of ligands by exponential enrichment (SELEX), selection process involves screening for single stranded oligonucleotides sequences based on its binding affinity towards the target of interest [189-193]. DNA aptamers that bind to thrombin have been developed to capture thrombin using affinity phase stationary phase chromatography [193]. Thrombin was captured on stationary phase that had thrombin aptamer covalently attached to it. Proteins were loaded into the column, the non specific proteins were washed and bound thrombin was eluted using 20 mM Tris buffer containing 8 M urea, pH 7.3, at 50 °C. Our current protocol requires 2-3 samples of urine to be collected from each donor to isolate sufficient thrombomodulin to detect the peptide of interest in MALDI-TOF. Thrombomodulin binding aptamers could be developed and covalently bound to the stationary phase to conduct affinity based chromatography. However, the greatest promise of aptamers is to skip the purification of the protein entirely.

It is possible that aptamer that are specific to each of the oxidized and the reduced forms of thrombomodulin could be developed. Previously, our group attempted to raise antibodies specific for the reduced and oxidized forms of thrombomodulin. This was unsuccessful, possibly because of the high homology in this region of thrombomodulin between different mammalian species. If successful in developing aptamers for the oxidized and reduced form of thrombomodulin, one can imagine using them in an ELISA-like test of the amounts of each form

in urine or blood. Such a test would be a great timesaving device, minimize the chances of oxidation, and would require much less sample.

CHAPTER 3: IS METHIONINE 388 OF THROMBOMODULIN MORE OXIDIZED IN
CIGARETTE SMOKERS?

Introduction

The blood of a smoker is more prone to clot than a non smoker; they are in hypercoagulable state. The narrowing of the arteries resulting from atherosclerosis increases the possibility of cardiovascular diseases [111], equally important is the hypercoagulable state seen in smokers. Cardiovascular diseases are the most common cause of death in smokers. The most common cardiovascular diseases seen in smokers are myocardial infarction and stroke. The fact that smokers have a hypercoagulable state has been established [194-197]; however, despite intensive research the molecular origin of such a state has not been shown. Free radical mediated oxidative damage to the endothelium is suggested to be an important factor for the development of cardiovascular diseases in smokers [112]. We hypothesized that oxidation of methionine 388 of thrombomodulin results from smoking. We further hypothesize that oxidation of methionine 388 in thrombomodulin is an important molecular factor leading to cardiovascular diseases in smokers.

To understand why we feel thrombomodulin oxidation is important, one must first understand the role of this regulatory protein. Thrombin when bound to thrombomodulin activates protein C. Activated protein C proteolytically destroys coagulation factors Va and VIIIa, thus suppressing further thrombin formation. The thrombin- thrombomodulin complex also activates the carboxypeptidase thrombin-activatable fibrinolysis inhibitor (TAFI) [198]. The activation of protein C and TAFI inhibits coagulation and fibrinolysis, making thrombomodulin a key protein for the regulation of coagulation and fibrinolysis.

Thrombomodulin is found on endothelial cell surfaces [55, 56]. It was discovered by Esmon and Owen in the 1960's. In rats, it is found predominantly in the lungs when compared

with other vascular organs like kidney and liver [57]. Thrombomodulin, purified from human lung endothelial membrane preparations, had an apparent MW of 78,000 Da [55]. Encoded by an intronless gene, the mature single-chain glycoprotein in the human is 557 amino acids long. Structurally it has 5 distinct domains. It has a short cytoplasmic tail at the C-terminus, which anchors thrombomodulin [58]. The deletion of this tail in mice does not have any effect in development, survival, coagulation and inflammation [58]. There is a well conserved membrane-spanning domain, followed by a serine/threonine-rich domain with four potential sites for O-linked glycosylation. The serine/threonine region supports the attachment of chondroitin sulfate, which has 20 repeating disaccharide units and a terminal trisaccharide unit [59]. Adjacent to the serine/threonine-rich region is a domain that has 6 epidermal growth factors (EGF)-like repeats, this domain is the best characterized. This region shows disulfide bonding pattern that is seen in a typical protein-protein interactions. The first two EGF-like repeats, which are furthest from the serine/threonine rich region, remain unknown as to their function. EGF-like repeats 3, 4, 5, and 6 [62-64] have been studied in detail by several groups and are essential for activation of Protein C by the thrombin-thrombomodulin complex. The cofactor function for thrombomodulin requires the last three of six tandemly repeated EGF-like domains (EGF 4, 5, and 6), as well as a Ser/Thr-rich domain between EGF-like domain 6 and the transmembrane domain. The residues 1 through 222 comprises N-terminal region of thrombomodulin, which constitutes almost half of the extracellular portion of the protein. The residues 155 through 222 of thrombomodulin might be associated with plasma membrane and this region is hydrophobic [66].

Thrombin binds to thrombomodulin to activate Protein C, which stops clotting by inactivation of clotting factors V and VIII [68]. The thrombin-thrombomodulin complex

activates protein C 1,000-fold more when compared to activation of protein C by thrombin alone. Deficiency in protein C or activated protein C is well established as increasing the risk of thrombosis [69]. Low levels of thrombomodulin are a well established risk factor for heart disease. Without a doubt, thrombomodulin plays a key role in slowing or stopping clotting.

It has also been shown that thrombomodulin in complex with thrombin activates thrombin activatable fibrinolysis inhibitor (TAFI) by 1250 fold [72]. Activated TAFI is a plasma procarboxypeptidase B that stabilizes clot by down regulating fibrinolysis [76]. Activated TAFI catalyzes the removal of lysine residues from the C-terminal of fibrin, resulting in elimination of plasminogen binding site of fibrin, subsequently plasminogen is unable to activate and prevention of fibrinolysis [79, 80]. This function of thrombomodulin might seem contradictory to its pro-fibrinolytic function previously discussed, but this highlights that thrombomodulin is a key regulator in coagulation, since it controls both the formation and breakdown of clots.

Thrombomodulin is anchored on the luminal surface of the endothelium and as previously indicated, thrombomodulin lacking the cytoplasmic domain appeared normal. Thrombomodulin undergoes endocytosis and degradation, but a significant amount of it is cleaved off and it is found in blood. Thrombomodulin found in blood is heterogeneous in size, due to differences in glycosylation [81, 82] and proteolysis. Four different fragments have been isolated from blood and seven fragments ranging in size from 12 to 100kDa after reduction of disulfides [83]. The concentration of thrombomodulin in serum and plasma are reported to be between 3 to 300ng/mL [84]. In diseases like diabetes and lupus high levels of thrombomodulin in plasma have been reported, this is believed to be a good marker for endothelial damage [198].

Studies have correlated high plasma thrombomodulin levels to be associated with a low risk of developing coronary heart disease [198].

The fourth and the fifth EGF like domains of thrombomodulin consists of 81 amino acids in together, and the fifth domain contains most of the residues that bind to thrombin [87]. The EGF like domain 4 and 5 (TMEGF45) bind to thrombin more tightly than EGF 5 alone. TMEGF45 is the smallest fragment of thrombomodulin that when bound to thrombin can activate protein C. Addition of fourth and the fifth EGF-like domains separately, do not activate protein C, suggesting they work together in activation of protein C [88]. The k_{cat} values for protein C activation by the thrombin-TMEGF45 complex, calculated by binding kinetic studies and cofactor activity assays, shows TMEGF45 has full cofactor activity. The EGF like domain six increases the K_m value of thrombomodulin for thrombin by factor of ten, despite not altering the K_{cat} value of thrombin-thrombomodulin complex for protein C [89].

The EGF 4 and 5 of thrombomodulin is linked together by three residues, and one of them is methionine, which is the 388th residue in thrombomodulin (Met388). Met388 in thrombomodulin is conserved in bovine, mouse and human. The mutation of Met388 to any other residues, except leucine, decreases the anticoagulant cofactor activity of thrombomodulin [91]. More importantly, there is 76-90% loss of activity when Met388 is oxidized using H_2O_2 [92]. There are four other methionine residues found in thrombomodulin, but their oxidation does not disrupt the ability to activate Protein C [92]. The K_d of thrombomodulin thrombin interaction increases to 10.9 from 4.4nM, when a full length thrombomodulin had oxidized Met388 [93]. TMEGF45 with oxidized Met388 has 3.5 fold lower K_{cat} , and 3.3 fold higher K_m values when compared to wild type TMEGF45 based on protein C activation assays [94]. It must also be mentioned that clot stabilizing activation of TAFI by thrombin-thrombomodulin complex

is unaffected by the oxidation of Met388 [95]. Since the activation of clot stabilizing activated TAFI, a procoagulant pathway, is unaffected by Met388 oxidation, while activation of protein C, an anticoagulant pathway, is suppressed by oxidation of Met388, there is no question that methionine oxidation favors coagulation. Although the effects of oxidation of Met388 on activation of protein C and TAFI were demonstrated *in vitro*, it seems likely that oxidation of Met388 *in vivo* would lead hemostasis towards coagulation.

Our hypotheses state that the oxidation of methionine 388 in thrombomodulin is higher in smokers when compared to non smoker. It is our belief that this oxidation is a key molecular cause of the prethrombotic state in smokers and thus a critical factor in the development of thrombosis and premature death in smokers. The case for relationship between smoking and oxidation of thrombomodulin as an important player in cardiovascular diseases has also been published by the Fernández group [117]. The circulating levels of protein C was compared in smokers and non smokers, activated protein C was 23% lower in smokers than nonsmokers. One of the possible causes of low levels of protein C could be due to the reduced thrombomodulin activity in smokers, probably due to methionine 388 oxidation. We have successfully purified significant quantities of thrombomodulin from urine from multiple donors, both smokers and non smokers, and quantified the relative oxidation levels in them.

Materials and Methods

The University of Arkansas Institutional Review Board approved all experimental protocols using human subjects. Samples of the first morning urinate were collected by volunteers in 400 mL centrifuge bottles. Thiodiglycol (5 mL) and 0.5 M EDTA (1 mL, pH 8.0) were added to containers before sample collection. The collected samples were transported using an insulated bag containing an ice pack. The samples were generally transported to the laboratory within two hours of collection and stored at 4°C for no longer than 2 hours before further processing to minimize the opportunity for oxidation. Typical volumes of the sample from a donor ranged from 200-550 mL. Prior to processing, 1 mL of sample was removed and stored in -80°C for determination of the initial thrombomodulin concentration in the sample. The samples were centrifuged in a Sorvall GS-3 rotor at 4,000 rpm for 30 min at 4°C. The samples were then filtered using a bottle-top 0.22 µm polyethersulfone (PES) membrane filter to remove remaining particulates and cellular debris. Sufficient 0.25 M imidazole acetate buffer, pH 6.0 was added to the urine filtrate to bring to a final concentration of 25 mM imidazole acetate. Next, the pH of the sample was adjusted to 6.0 by addition of 2 M acetic acid or 6 M NaOH depending on the urine sample. Typically after the addition of 0.25 M imidazole acetate buffer, the pH of the samples ranged from pH 5.5-6.4. The pH of the sample was measured using a pH electrode. A slurry of Q Sepharose FF (GE Healthcare) was poured into a 50 mL Bio-Rad Econo column in a sufficient amount to pack a 20 mL bed volume. A Fluid Metering, Inc (FMI) pump was connected to the Bio-Rad column to pump buffer and urine sample through the Q Sepharose FF column at a constant speed of 5 mL/min. The column was first equilibrated with 60 mL of 25 mM imidazole acetate buffer, pH 6.0. Next, the urine sample was passed through the column. The column was washed with 50 mL of 25 mM imidazole acetate buffer, pH 6.0 and then by

addition of 20 mL of 25 mM imidazole acetate buffer, pH 6.0, 50 mM NaCl. Fractions containing thrombomodulin were eluted by addition of 50 ml of 25 mM imidazole acetate buffer, pH 6.0, 0.5 M NaCl. The 0.5M NaCl fractions were collected in 15 mL conical tubes, 5 mL in each tube. The eluent after addition of 25 mL of 25mM imidazole acetate buffer, pH 6.0, 0.5 M NaCl, tested positive for thrombomodulin. A solution of 0.2 M L-methionine (Sigma-Aldrich) was added to thrombomodulin containing fractions to a final concentration of 2 mM methionine. The fractions were transferred to 1.5 mL microcentrifuge tubes, typically 14 tubes, and flash frozen in liquid nitrogen. The frozen eluent in 1.5 mL microcentrifuge tubes was dried under vacuum in a *SpeedVac* concentrator system using medium heat.

Reverse phase HPLC purification

The dried samples from ion exchange were pooled and dissolved in minimum volume, generally 5 mL of total volume, of 18.2 megaohm deionized water. The dissolved sample was passed through a 25 mm syringe filter with 0.2 μ M PES membrane (VWR). The redissolved sample was injected into a reverse phase Waters HPLC system. An Atlantis dC18, 5 μ m particle, 4.6x250 mm column held at 58°C and a 2996 photodiode array detector monitoring 214 nm and 254nm was used. The maximum volume that could be injected into Atlantis dC18, 5 μ m particle, 4.6x250 mm column per run is 1.4 mL. The total volume of the sample to be injected into the HPLC was 5 mL, 4 different HPLC runs had to be performed to purify entire sample. Upon injection, a gradient was run at 1 mL/min from 10 to 29% acetonitrile, 0.1% trifluoroacetic acid over 12 minutes, and then from 29% to 40% acetonitrile over the next 11 minutes, and then from 40% to 90% acetonitrile over the next 25 minutes. Eluent was collected in 1 mL fractions in 1.5 mL microcentrifuge tubes, to which were previously added 100 μ L of 200 mM Tris buffer, pH 8.0. The thrombomodulin containing fragments, identified by ELISA, typically eluted at 30

minute. Thrombomodulin containing 1.5 mL microfuge tubes were flash frozen in liquid nitrogen, dried under vacuum in a *SpeedVac* concentrator system using medium heat. The HPLC Atlantis column was cleaned using 1.0 mL/min of 90% MeCN/0.1% TFA for 10 minutes, followed by equilibration for 15 minutes to the starting conditions of 1.0 mL/min of 10% MeCN/0.1% TFA in preparation for the next injection.

Digestion of thrombomodulin

The dried thrombomodulin containing fractions from the reverse phase chromatography were pooled and dissolved in approximately 100 uL of 1X phosphate buffered saline (PBS; 1.46 mM KH_2PO_4 , 9.9 mM Na_2HPO_4 , 2.68 mM KCl, 137 mM NaCl, pH 7.4). Based on the previous attempts with chymotrypsin digestion of thrombomodulin after HPLC purification, we observed the samples that were cloudy did not undergo digestion with the protease. The samples that were cloudy prior to digestion were spun in a microcentrifuge (Galaxy 14D VWR) at 13K RPM for a minute. The supernatant was removed. The precipitate was dissolved in 75 μL of 1X phosphate buffered saline, and was used for digestion. Freshly prepared *tris*-carboxyethylphosphine (TCEP) was added to make the final concentration of 50 mM TCEP. 10 μL of 0.5 M sodium phosphate buffer pH 7.5 added to the sample, prior to addition of 500 units of PNGase F (New England Biolabs). The samples were incubated at 37°C for 4 hours to deglycosylate the sample. The protein was then digested at 30°C for 8 hours after adding 4.1 μg of sequencing grade chymotrypsin (*Princeton Separations*) dissolved in 50 mM Tris-HCl, 1 mM CaCl_2 , pH 8.0. The chymotrypsin was inactivated by placing the samples in a 100°C dry bath for 30 seconds. The samples were immediately flash frozen, and stored in -20°C until further processing.

Mass spectrometry analysis of methionine 388 oxidation

The semi quantification of oxidized and reduced forms of peptide in the chymotrypsin digested thrombomodulin was conducted by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The MALDI analysis was performed by the Statewide *Mass Spectrometry* Facility located at the *University of Arkansas*. The pure samples were mixed with the MALDI matrixes. The two types of matrixes used were alpha-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) (Sigma Aldrich). MALDI-TOF mass spectra were obtained on a Bruker Ultraflex II (Bruker Daltonic GMBH, Bremen, Germany) time-of-flight mass spectrometer operated in the positive-ion reflectron mode. The accelerating voltage, delayed extraction time, and laser power were adjusted to optimize sensitivity and resolution for ions between m/z 500 – 4000.

UPLC injections of synthetic peptide

The stock solution of the synthetic peptide was diluted using 18.2 megohm water to make following concentrations: 100, 10, 1, and 0.1 mM. Injections of 25 μ L of each of these solutions were performed on the Waters ACQUITY UPLC system, detector monitoring 214nm. A BEH 130 C18 1.7 μ m 2.1x150 mm, reverse phase column heated to 45°C, 0.2 mL/min flow rate was used to separate the peptide mixture. The eluent was started at 10% MeCN/0.1% TFA and transitioned to 20% MeCN/0.1% TFA in a linear gradient over two minutes. Over the next 25 minutes, a linear gradient from 20% MeCN/0.1% TFA to 90% MeCN/0.1% TFA was run.

Results and Discussion

This study was undertaken to quantify the relative oxidation of methionine 388 of thrombomodulin from smokers and non smokers. Cigarette smoke is a complex mixture of chemical compounds, including high concentrations of reactive oxygen species and reactive nitrogen species [12]. Also important is the secondary oxidative stress triggered by ROS and RNS from cigarette smoke. The lungs of cigarette smoker are exposed to primary and secondary oxidation from cigarette smoke. Since thrombomodulin is predominantly found in the lungs, we hypothesize that in smokers it is subjected to significant levels of oxidation from cigarette smoke.

There are several challenges associated with quantifying the oxidation of thrombomodulin purified from urine. The thrombomodulin is cleaved into fragments prior to its circulation in the urine and combined with the heavy mass of the fragments make direct quantification impossible. Also, the thrombomodulin is heterogeneous in size due to differences in glycosylation [81, 82]. Peptide mass fingerprinting provides a significantly easier alternative compared with working with bigger protein fragments. In this technique the protein of interest is cleaved, and the mass of the resulting fragments is measured using mass spectrometer.

Chymotrypsin digestion of thrombomodulin results in the fragmentation of the protein into several peptides. Among the peptides of digest, the peptide APIPHEPHRCQMF is the prime interest for the purposes of this study. This peptide is composed of residues 377-389 of thrombomodulin. The second to the last methionine residue corresponds to the 388th residue. In the naturally occurring thrombomodulin this peptide can exist in two different forms; APIPHEPHRCQMF and APIPHEPHRCQM_{ox}F. The former is a relatively non polar peptide and

its isotopically averaged mass is 1562.73 Da. The latter is more polar than its counterpart, and its mass is 1578.74 Da.

Depending on the state of methionine 388 of thrombomodulin, the chymotrypsin digestion should result in differences in the resulting peptides. If the methionine 388 is exclusively present in the reduced state, the digestion will only produce APIPHEPHRCQMF. In the case of methionine completely oxidized to methionine sulfoxide, the digestion results in exclusive presence of APIPHEPHRCQM_{ox}F. If the methionine 388 is partially oxidized the resulting digest will have presence of both oxidized and the reduced forms of the peptide. The presence of either forms of the peptide can be confirmed by MALDI-TOF. The MALDI-TOF measurement also provides the relative signal intensity of the peptides in the sample. The intensity of the signal from each peptide is proportional to its concentration in the sample. Comparing the intensity of different peptides can be problematic though, because each peptide has a different tendency to ionize and thus be detected. Nevertheless, the intensity of reduced and oxidized peptide in MALDI-TOF measurement can be used to calculate the approximate ratio of oxidized and reduced forms of APIPHEPHRCQMF in thrombomodulin. Figure 3.1 is MALDI-TOF spectra of digested thrombomodulin purified from urine. This urine sample was collected from a 28 year old male smoker. The intensity of the signal from the reduced peptide is approximately three fold higher than that of the oxidized form in this particular sample.

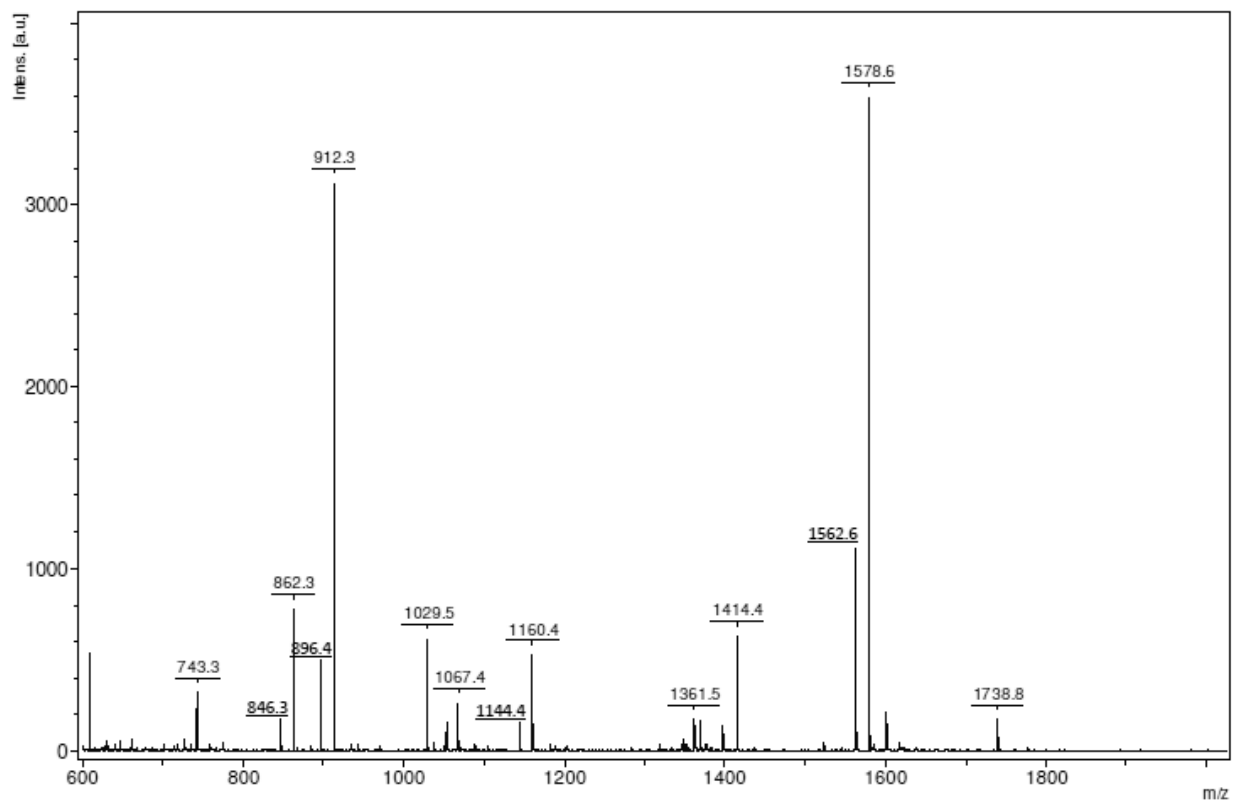


Figure 3.1: MALDI-TOF mass spectra of chymotrypsin digested thrombomodulin purified from urine. Urine sample was collected from a 28 year old male smoker.

UPLC injections of synthetic peptide

Acquity UPLC offers increased resolution, sensitivity and speed when compared to HPLC. It uses small particle columns (1.7 μm – 1.8 μm), which improves system efficiency by producing better resolution, and also lowering the analysis time. This system is designed to minimize band spreading resulting from the column and also from the instrument. The stock solution of the synthetic peptide APIPEPHRCQMF (Sigma) was diluted with 18.2 megohm water to make 100, 10, 1, and 0.1 mM solutions. The commercial peptide is mixture of both oxidized and reduced peptides, APIPEPHRCQM_{ox}F and APIPEPHRCQMF. 25 μL of each of

the diluted solutions were injected into the Waters ACQUITY UPLC System. This system is much more suitable for quantitative analysis than MALDI. The accurate ratio of reduced and oxidized peptide in each of the solution can be obtained by using the UPLC. The relative area occupied by the reduced and the oxidized peptide in the UPLC chromatogram can be used to calculate the ratio of the peptides in each solution. Figure 3.2 is the Acquity UPLC chromatogram of 25 μ L injection of 1 mM synthetic peptide. The ratio of reduced and oxidized peptide in 0.01 M synthetic peptide solution is 3.26.

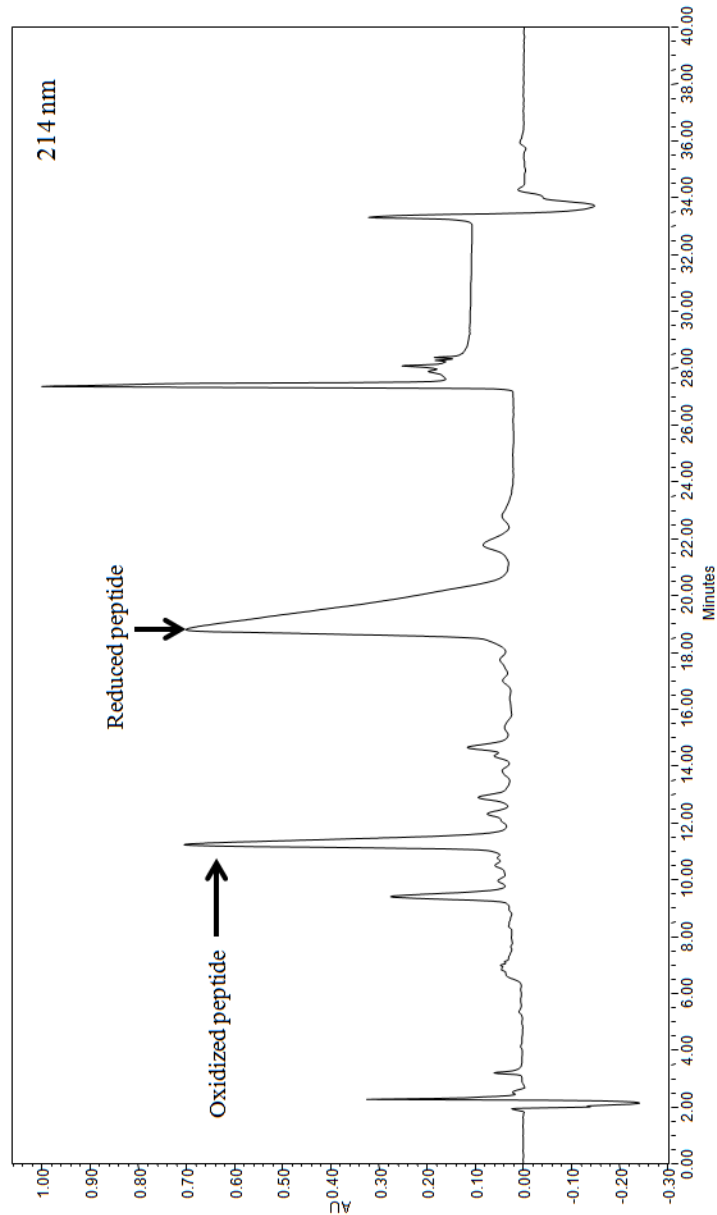


Figure 3.2: Acquity UPLC chromatogram of 25 μ L injection of 1 mM synthetic peptide. The oxidized and reduced peptide elute at 11.2 and 18.7 min respectively. The area occupied by the oxidized and the reduced peptide is 23.47% and 76.53%. The synthetic peptide purity was >85%, hence the presence of other peaks in this chromatogram.

Peptide concentration(mM)	Area of reduced peak (%)	Area of oxidized peak (%)	Ratio of peptides
100	76.49	23.51	3.25
10	76.46	23.54	3.25
1	76.53	23.47	3.26
0.1	76.58	23.42	3.27

Table 3.1: Ratio of the reduced and oxidized peptides in different concentrations of synthetic peptide, acquired using a Waters Acquity UPLC.

Table 3.1 shows the ratio of the reduced and oxidized peptide in 100, 10, 1, and 0.1 mM solutions of the synthetic peptide. The ratio of reduced and oxidized peptide in each solution was calculated using the relative area occupied by each peptide in the UPLC chromatogram at 214 nm absorbance. Since sulfoxide does not absorb significantly at this wavelength, the amount of material present in each peak should be directly proportional to the peak areas. In the UPLC chromatogram of 100 mM synthetic peptide, the reduced and oxidized peptide occupied 76.49% and 23.51% area respectively. Based on that information, the ratio of reduced and oxidized peak in the 100 mM solution was calculated to be 3.25. The mean ratio for the four different solutions was 3.25 with a standard deviation of 0.01.

We attempted to use Acquity UPLC to directly quantify the oxidation of methionine 388 of thrombomodulin purified from urine. The synthetic peptide mixture was injected into the UPLC to map the peptide. Under these conditions, the oxidized and reduced peptide elute at 12 and 21 min respectively (Figure 3.3).

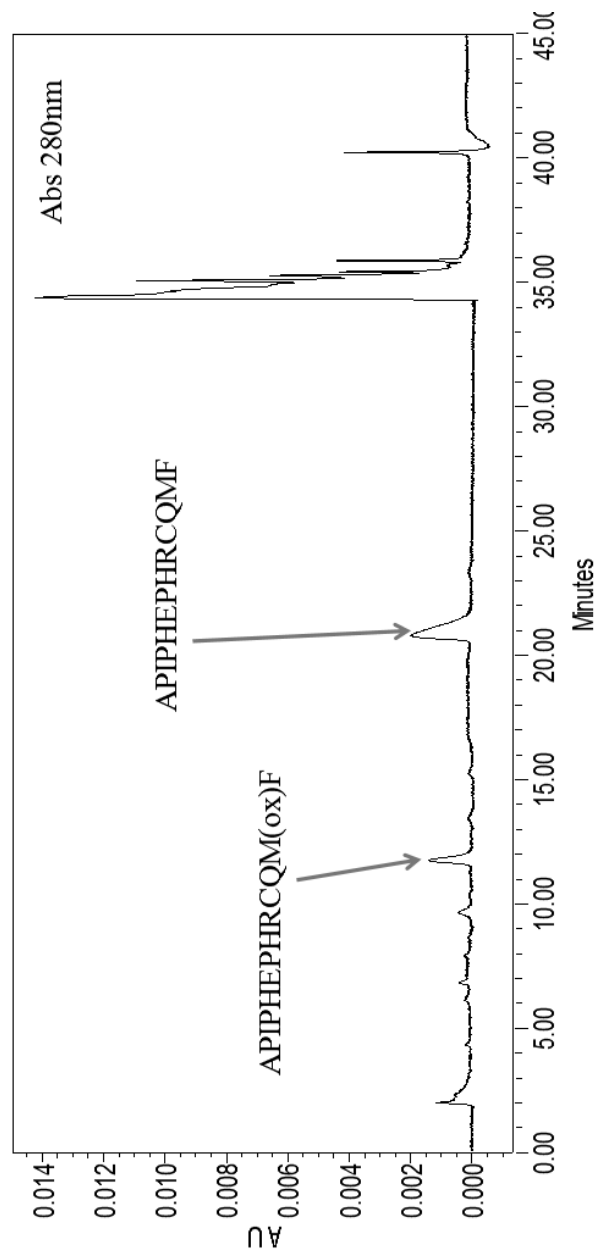


Figure 3.3: Acquity UPLC chromatogram of the commercial peptide mixture. AIPHEPHRCQM(ox)F elutes at 12 min and AIPHEPHRCQMF elutes at 21 min.

The chymotrypsin digested urinary thrombomodulin was injected into the UPLC system. Unfortunately, there were several contaminating peptides that eluted very near or even co-eluted with the peptide of interest. Despite trying numerous elution profiles, the resolution necessary to calculate the relative area occupied by the oxidized and the reduced peptide often could not be achieved using the UPLC. The digested samples had slightly different elution times from the synthetic peptides and co-injected samples had slightly different elution times still. These problems were exacerbated when of the two peaks was only minimally present. It was, in those cases, difficult to be sure which peak was the one of interest. It is possible that a good UPLC-MS system could overcome these difficulties, but attempts to interface our UPLC directly with a mass spectrometer failed because none of the mass spectrometers available to us had high enough scan rates. Peaks were often not detected by MS. The use of UPLC to calculate the methionine oxidation was therefore discarded.

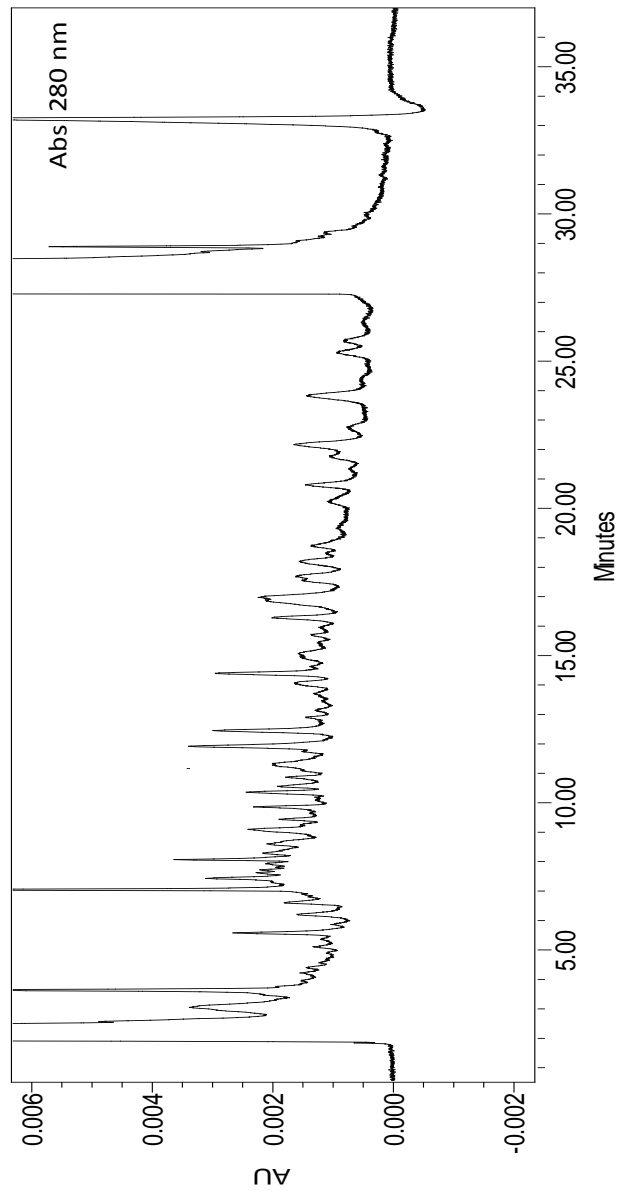


Figure 3.4: UPLC chromatogram of chymotrypsin digested urinary thrombomodulin of a 26 year old Caucasian male non smoker. The two forms of peptide elute at 12 and 21 min respectively, notice the contaminating peaks at those times.

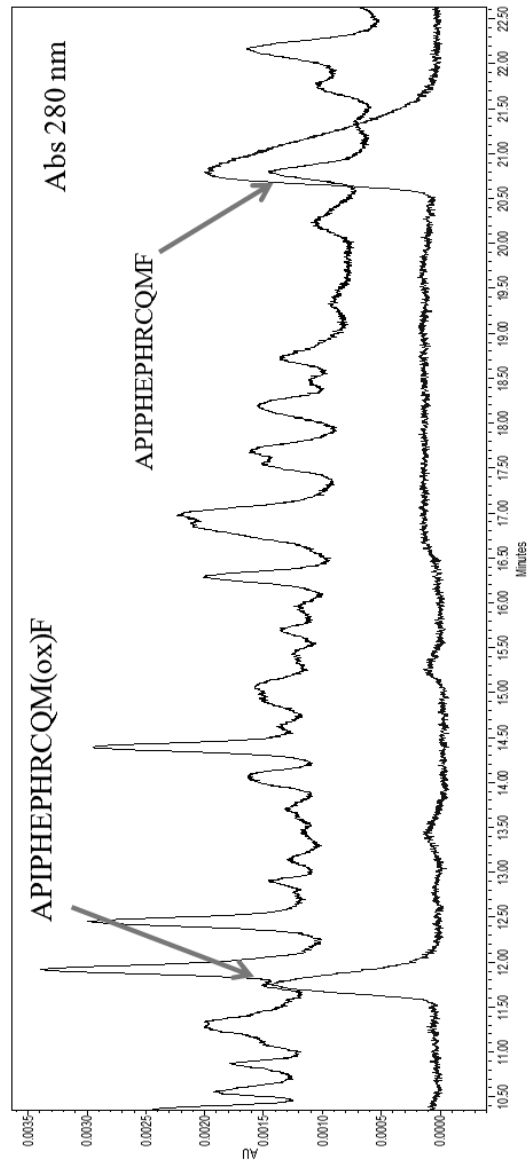


Figure 3.5: Overlay of UPLC chromatograms. Top chromatogram is of chymotrypsin digested urinary thrombomodulin of a 26 year old Caucasian male non smoker, and the bottom chromatogram is of synthetic peptide mixture.

Accuracy of quantification by MALDI

Matrix-assisted laser desorption ionization (MALDI) is a powerful technique for mass spectrometry of peptides and proteins [178]. This technique provides fast and accurate acquisition of molecular mass and purity. Some of the advantages offered by this instrument are follows: It is very easy to operate and it is tolerant towards the presence of buffers, salts and other additives. It can be used for analysis of compounds with a MW over 300 KDa [199]. It ionizes the analytes very softly; usually only $M+H^+$ and a small amount of $M+2H^+$ ions are generated making analysis of mixtures feasible. Briefly, solution of analyte is mixed with the matrix (2,5-dihydroxybenzoic acid) directly on a target, and evaporation produces crystals which are a mixture of the analyte and matrix. The laser UV pulse is directed on the matrix/analyte crystals, matrix ionizes and transfers charge to the analyte, and the ionized matrix and analyte vaporize and expands into gas phase. Despite its popularity with analysis of proteins and peptides, this technique is considered impractical for the analysis of analytes that have low mass and for quantitative analysis [200]. The presence of excess matrix can blanket the low m/z signal in the spectrum that corresponds to the analyte with low mass.

Quantitative analyses are not generally attempted in MALDI because there is not a uniform distribution of the sample and the matrix on the surface of the target. This creates a variability of the analyte within the matrix, and as a result there is significant variability in the peak intensities, baseline, and noise level seen in MALDI spectra from the same sample [200]. Another challenge in conducting quantitative studies of complex sample using MALDI is the competitive ionization and ion suppression. Some analytes have greater affinity for charge than others and they are more competitive in obtaining available proton. For example, peptides that terminate in arginine have higher affinity in charge when compared to peptide that ends with

lysine [201]. However, for our purposes the difference in ionization efficiency of different components in the sample is less important, since we are only interested in two versions of a single peptide. Also, to enhance the quantitative ability of MALDI, alpha-cyano-4-hydroxycinnamic acid (CHCA) was used as a matrix to produce an ultrafine homogenous sample-matrix composition. It showed better sensitivity and reproducibility compared to traditional dried droplet preparations using 2,5-dihydroxybenzoic acid (DHB) [202, 203].

The accuracy and the precision of MALDI-TOF can be directly tested using synthetic peptide APIPHEPHRCQMF. The commercial peptide is mixture of both oxidized and reduced peptides, APIPHEPHRCQM_{ox}F and APIPHEPHRCQMF. The stock peptide was diluted using 18.2 megohm water to prepare 100, 10, 1, and 0.1 mM solution of the synthetic peptide. These solutions were sent for MALDI analysis. For each sample, three different sample-matrixes were prepared, and each of the sample/matrix was subjected to approximately 5000 shots. Table 3.2 shows averaged intensity values from these 5000 shots for reduced and oxidized peptide in 100, 10, 1, and 0.1 mM solutions of the synthetic peptide. MALDI of 1 μ L (5 pmol) of 0.01 mM synthetic peptide did not produce a detectable signal for oxidized peptide.

Peptide concentration (mM)	Intensity of Reduced Peptide (A.U)	Intensity of Oxidized Peptide (A.U)	Ratio of Intensities (red/ox)
100	14000	1000	14
10	12500	1500	8.3
1	5400	1000	5.4
0.1	2300	250	9.2

Table 3.2: The average MALDI spectra intensities of reduced and oxidized synthetic peptide in four different solutions. Three sample-matrixes were mixed for each solution, and each sample-matrix was subjected to approximately 5000 shots. The table provides average values from these shots (A.U = arbitrary units). The fourth column is the ratio of the second and the third column. MALDI of 1 μ L of 0.01 mM solution of peptide did not produce a signal for oxidized peptide.

The 100, 10, 1, and 0.1 mM solutions of the synthetic peptide were prepared by serial dilution of the stock solution of the synthetic peptide. The ratio of reduced and oxidized peptides in each of the solutions should be same even after the dilutions. The fourth column on Table 3.2 provides the ratio of the two forms of peptides in each of the solutions, the mean of the ratios is 9.2, and standard deviation is ± 3.6 . Clearly, the Acquity UPLC system is much more suitable for quantitative analysis compared to MALDI and, when it was used to measure the mean ratio of reduced and oxidized peptide in each solution (Table 3.1), the calculated mean was 3.25. With such low accuracy and precision, the use of MALDI for quantification of two forms of peptide in a sample is challenging. MALDI overestimates the amount of the reduced form and the measurement is much more variable. However, if the differences in thrombomodulin methionine 388 oxidation in cigarette smokers and non smokers are large enough, these weaknesses do not prevent detection of dissimilarities.

Relative methionine oxidation in smokers and non smokers

There are a few definitions we use to classify our donors. A cigarette smoker is someone who has been smoking for at least the past six months, smokes a minimum of 5 cigarettes per day, and does not smoke anything other than tobacco (e.g. marijuana). A non-smoker is someone who has not smoked in over six months and does not use any other form of tobacco and is not heavily exposed to secondhand smoke. A never-smoker is a non-smoker that has never smoked. A secondhand smoker is an otherwise non-smoker who is heavily exposed. A former smoker is someone who has not smoked for least six months.

We wanted to minimize the variables that are linked with oxidation in this research. For example, a donor who has diabetes would be under increased oxidative stress, and such condition

would increase the uncertainty of our results. The donors were carefully screened to eliminate the ones who could be under greater oxidative stress than normal. Potential donors with known history of cardiovascular diseases, diabetes, high blood pressure, hyperhomocysteinemia, severe arthritis, renal failure, current use of anti-coagulants, recent surgery, extreme use of over-the-counter anti-oxidant vitamins, chronic allergies or infection, and pregnancy were excluded from this study.

In this study a smoker donor was matched with non-smoker control, and the difference in methionine 388 oxidation between the two was measured. It was very important to minimize the variables that affect oxidation in the smoker and the control. In our study smokers were matched with controls based on age, gender, and race. We understand that the oxidation of thrombomodulin could be affected by multitude of factors such as diet, differences in expression of repair proteins, variability in expression of thrombomodulin, and many other possibilities, and it would be impossible to eliminate all variables between the smokers and the control.

The oxidation of methionine 388 of thrombomodulin was studied in six non smokers (Table 3.3) and 13 smokers (Table 3.4). In the two out of six non smokers the MADLI spectra did not report the presence of oxidized peptide. The average ratio of reduced and oxidized peptides in four non smokers was 1.5. The concentration of reduced peptide was higher. Conversely, six of the thirteen smoker subjects did not have reduced peptide in the MALDI spectra (Table 3.4). The average ratio of reduced and oxidized peptide for the seven smokers was 0.58. The ratio of oxidized peptide was higher in cigarette smokers. The reduced/oxidized ratio and percent oxidation in Tables 3.3 and 3.4 must not be taken literally. We know that MALDI tends to either overestimate the level of reduced peptide or underestimate the level of oxidized peptide or some combination of both. In other words both non-smokers and smokers are likely to be actually

much more oxidized than the values in the two tables indicate. However this systematic error is similar for both populations. While we cannot say exactly how much the oxidation levels differ, we can examine whether the differences observed are statistically significant.

The presence of completely reduced peptides in two non smokers, and presence of completely oxidized peptide in six smokers and the relative ratio of the two forms of peptides in smokers and non smokers clearly indicate that the methionine 388 of thrombomodulin in cigarette smoker is significantly oxidized in smokers. In order to perform the t-test, we revisited each of the MALDI spectra of digested thrombomodulin and recorded the noise level. The noise level was multiplied by the factor of 2.5. This value was assigned for the peptide which was not detected in the MALDI spectra. This, of course, is a very conservative estimate for the amount of the peptide that was not detected and most of the actual values are likely much less than this.

The ratio of the reduced to oxidized peptide MALDI data from smokers and non smokers passed the Shapiro-Wilk test for normality ($P=0.068$) and an equal variance test ($P=0.111$). Statistical comparisons were performed using Student's t-test ($t= 3.72$, $P=0.001$). Thus, there is a very statistically significant difference between thrombomodulin methionine 388 oxidation in smokers and non smokers, despite using very conservative estimates.

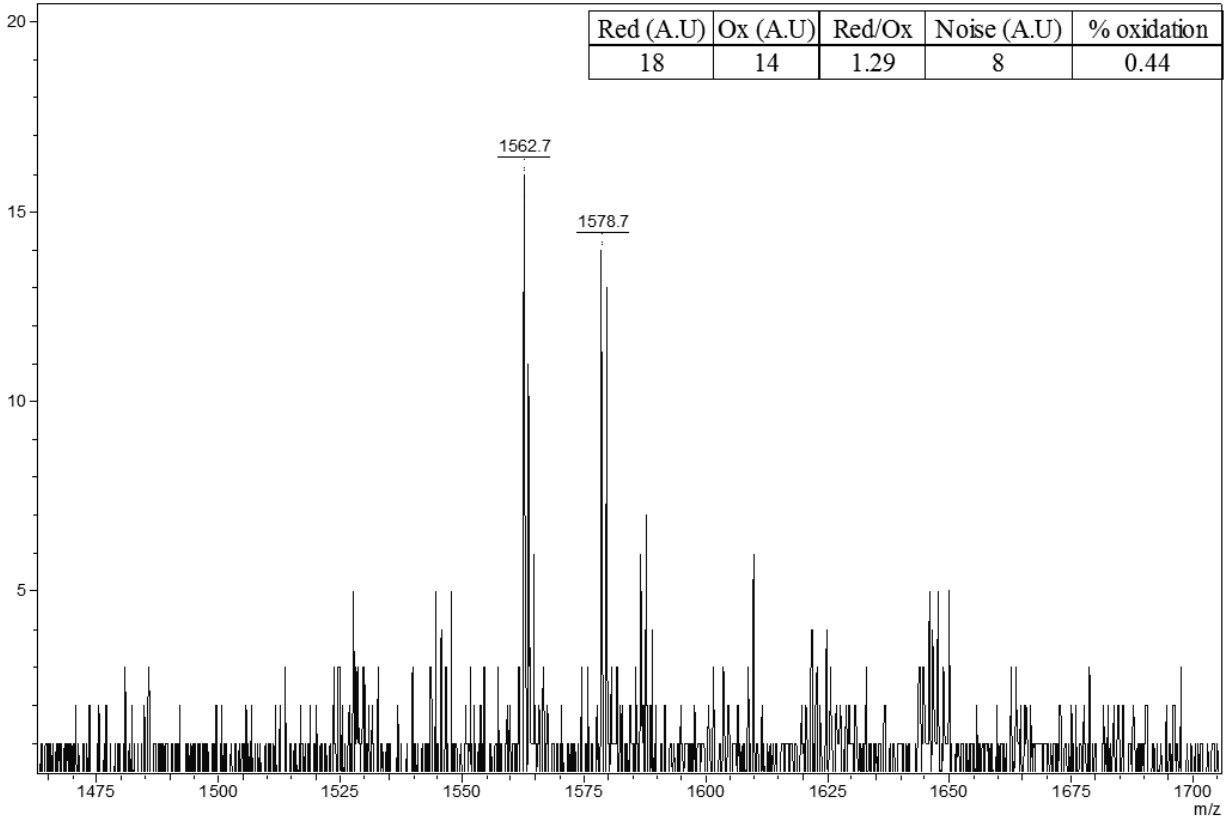


Figure 3.6: MALDI-TOF spectra of chymotrypsin digested urinary thrombomodulin of 25 year old caucasian male never smoker (Donor code: NS 36, Table 3.3). The spectrum shows presence of both reduced and oxidized peptides. The signal to noise ratio is greater than 10 for both forms of peptide.

Donor Code	Red (A.U)	Ox (A.U)	Red/Ox	Age	Gender	Race	Smoker	Noise (A.U)	% Oxidation
NS 02	92	30	3.1	52	M	C	Never	5	0.25
NS 11	12	18	0.7	32	F	C	Never	8	0.60
NS 23	812	< 325	2.5	24	M	C	Never	130	< 0.28
NS 28	98	< 87.5	1.1	30	M	C	Never	35	< 0.47
NS 36	18	14	1.3	25	M	C	Never	8	0.44
NS 40	94	99	0.9	28	M	C	Non, 4 years	51	0.51

Table 3.3 : MALDI spectra values of oxidized and reduced peptides in non smokers. NS = Non smoker, ND = Not Detected, Red = Reduced, Ox= oxidized, A.U. = arbitrary units, M = male, F = female, C = Caucasian

Donor Code	Red (A.U)	Ox (A.U)	Red/Ox	Age	Gender	Race	Smoker	Daily intake	Noise (A.U)	% Oxidized
CS 19	< 25	1150	0.02	29	M	SEA	8 yr	10	10	> 0.97
CS 116	< 7.5	141	0.05	39	M	C	10 yr	14	3	> 0.95
CS 119	550	1775	0.31	22	M	C	2.5 yr	7	90	0.76
CS 123	< 325	522	0.62	60	M	C	30 yr	7	130	> 0.62
CS 126	1105	3592	0.31	23	M	C	3 yr	7	90	0.76
CS 132	83	254	0.33	38	F	C	15 yr	12	55	0.75
CS 134	78	56	1.39	26	F	C	4 yr	11	15	0.42
CS 135	14	43	0.33	35	M	C	20 yr	20	8	0.75
CS 137	29	43	0.67	19	M	C	1 yr	2	9	0.60
CS 138	< 62.5	67	0.93	21	M	C	2 yr	6	25	> 0.52
CS 139	20	131	0.15	32	M	C	3 yr	5	9	0.87
CS 140	< 12.5	60	0.21	45	M	C	15 yr	9	5	> 0.83
CS 141	< 12.5	59	0.21	27	M	C	2 yr	7	5	> 0.83

Table 3.4: MALDI spectra values of oxidized and reduced peptides in cigarette smokers. CS = cigarette smoker, ND = not detected, Red = reduced, Ox= oxidized, M = male, F = female, A.U = arbitrary units, C = Caucasian, SEA = South East Asian. Daily intake is the number of cigarettes smoked per day. Noise is the background noise of the spectra. % oxidized is the percentage of oxidized peptide.

It is necessary to consider what the effects are of assuming that the amount of any peak that is not detected is equal to 2.5 times the noise level. Again, this is a conservative assumption that has the effect of making the average non smoker seem more oxidized and the average smoker less oxidized. This moves the two averages toward each other, but also tends to make them more normally distributed. If we assume that the peak that is not detected is present in the same amount as the noise level (data not shown), the data are no longer normally distributed, failing the Shapiro-Wilk test. However, using the non-parametric Mann-Whitney Rank Sum test we discover, unsurprisingly, that the difference in the median values of the two groups (Red/Ox median non smoker 2.043; smoker 0.308) is still greater than would be expected by chance ($P=0.002$).

In a few cases a peak was detected which was greater than the noise level but not 2.5 times larger. We examined the effect of excluding these individuals entirely if both peaks were below this cut-off while if just one of the two peaks was detected, but below 2.5, increasing it to 2.5 the noise level. The data passed the Shapiro-Wilk test ($P=0.516$) but failed the equal variance test, presumably because only three values were left for the non smokers. However, the Mann-Whitney test still showed that the difference (Red/Ox median non smoker 2.498; smoker 0.310) between the two groups was quite significant ($P=0.015$). Clearly, there is a statistically significant difference between the two populations.

Possible Future Work

In the future we would like to expand this study to greater numbers of participants to determine how oxidation varies for the many permutations of smoking behavior. We plan to recruit smokers with a wide range of cigarette consumption habits. We expect a relationship between higher levels of smoking and higher oxidation, and our hypothesis would be further supported if we can establish there is a dose dependent curve. While conducting this study, we expect some donors to display variability that would not be consistent with dose dependent curve. Determining the degree to which there is variability which is not explained by the amount of smoking is very important. It is possible that some individuals resist the oxidation of thrombomodulin better than others. This could be due to multitude of factors some of which could be diet, environment, differential expression of repair proteins, and differential expression of thrombomodulin. Identification of such individuals offers extension of this research project. Firstly, we would identify the factors that make them vary from normal smoking behavior. Next steps would be identifying how influential individual factors are by themselves, and eventually their effects in myriad permutations and combinations.

Pipe and cigar smokers typically do not inhale the smoke, and that differentiates them from cigarette smokers. We are interested to see how pipe and cigar smoke affects thrombomodulin. Since these smokers typically do not inhale the smoke this offers a very useful avenue for testing the hypothesis that the bulk of thrombomodulin oxidation in cigarette smokers is localized to the lungs. It would be informative to recruit smokeless tobacco users, and compare the thrombomodulin oxidation levels.

The American Heart Association has concluded that passive smoking is an important risk factor for heart disease in both adults and children [204]. AHA estimates 22,700 to 69,600 annual premature deaths from heart and blood vessel disease are caused by passive smoking [205]. We would like to examine the effects of secondhand smoke on non-smokers, by recruiting donors who live with smokers or donors working in a bar. It is difficult to predict whether we will be able to detect a measurable, and statistically significant oxidation in secondhand smokers, but in light of our research findings, we believe it will be worthwhile to quantify thrombomodulin methionine oxidation in second hand smokers. The degree of cigarette smoke is measured by counting the number of cigarettes smoked in a day, however for the second hand smoke study we need a quantitative test to record cigarette smoke inhaled by the donor. The self reported method by the non smokers are going to be inaccurate due to multiple factors some of which are proximity of nonsmokers to smokers, ventilation of the room, and individual differences in exposure sensitivity. Nicotine would be an ideal biomarker for this study, however it has a half life of 2 hours making it unsuitable [206]. Nicotine from tobacco smoke when taken into the lungs enters into the bloodstream. When nicotine in the blood stream reaches liver it is processed into cotinine, which is the principle metabolite of nicotine from tobacco smoke. Cotinine has been documented to be a sensitive and specific marker for tobacco exposure [207]. It is found in saliva, plasma and urine [208]. Urinary cotinine concentration has been shown to be accurate biomarker for tobacco exposure [208-210]. The degree of cigarette smoke exposure of the passive smoker can be measured using cotinine ELISA. The thrombomodulin methionine 388 oxidation in passive smokers can be studied using our current protocol combined with cotinine as a biomarker.

The MALDI spectra of chymotrypsin digested thrombomodulin from non smokers and smokers gave us insight into methionine 388 oxidation. We have established that methionine 388 of thrombomodulin in smokers is more oxidized, and we propose that the oxidation of methionine 388 in thrombomodulin could be one of the key molecular causes for cardiovascular diseases in cigarette smokers. The immediate extension of this research would be to find treatment that would reduce the oxidized methionine 388 of thrombomodulin. Since cigarettes are the ones imposing oxidative stress the first step towards treatment would be for a smoker to quit smoking cigarettes. We could set up an experiment where a smoker is willing to quit smoking. The urine samples will be collected at frequent intervals before and after the donor quits. The samples would be collected for six months from the date the smoker quits smoking, with cotinine tests to verify abstinence. The MALDI data collected over that span will be analyzed to see if there are any changes to the oxidation levels of methionine. We speculate that the oxidation levels to decrease. Six months ago we found a donor who volunteered to quit smoking cigarettes for this study. Urine samples were collected for a span of 1 month before the donor quit smoking, and samples have been collected at a frequency of 3 weeks interval. The urine samples have undergone reverse phase HPLC step of purification, and are currently stored in -20°C. When the donor completes 6 six months without smoking, the stored samples will be subjected to chymotrypsin digestion and will be sent for MALDI analysis. We are actively looking for donors who plan on quitting smoking. In the past few months we have found three donors who planned on quitting, and two of them did quit, but only for the span of 1-2 weeks. We could approach smoking cessation clinics to recruit smokers trying to quit. The state of Arkansas has chosen to use some of its tobacco settlement money to subsidize the cost of clinically proven programs, which means that there are a number of fairly popular and successful

programs working in the area. Experiments where many permutations of donors who quit smoking could be designed based on age, sex, race and degree of smoking, and compare each factor towards recovery. For comparison, if we could find a donor who intends to pick up smoking would provide us insight on rate at which cigarette smoke oxidizes methionine 388 of thrombomodulin.

Annually 437,900 people are killed by diseases caused by smoking, and 35% of those deaths are cardiovascular related [20]. Cigarette smoke is the major cause of pulmonary emphysema [21], bronchitis, myocardial infarction, and stroke as well as lung cancer. Four million people die every year from tobacco smoking related diseases worldwide. It has been estimated that 2 billion people use tobacco products [22]. Approximately 64.5 million people in the USA are active smokers, between 1995-1999 the estimate of deaths that resulted from smoking and exposure to secondhand smoke was approximately 440,000 annually [23]. Cigarette smoking ranks number one in the list of preventable disease and death worldwide. In 2004, cigarette smoking cost the United States over \$193 billion, this number includes \$97 and \$96 billion from loss in productivity and in direct health care expenditures respectively [211]. The average cost to the government was \$4,260 per adult smoker [211]. As we have mentioned previously, we propose methionine 388 oxidation is one of the key molecular mechanism for development of cardiovascular diseases in smokers. Treatment to mitigate or regress methionine 388 oxidation in smokers has potential to minimize or prevent morbidity and fatality in cigarette smokers. We could design experiments where mice are subjected to cigarette smoke for a period that would induce cardiovascular like symptoms. Following the development of disease, they are treated with antioxidants like apocynin or Tempol [212-214], and the effectiveness of the antioxidants is monitored.

Prolylcarboxypeptidase (PRCP) is an endothelial membrane-bound serine carboxypeptidase responsible for activation of bradykinin and angiotensin [103-105]. Both proteins regulate the vascular NO to provide protection from thrombosis. PRCP is indirectly involved in maintenance of normal blood pressure and reduction of thrombosis risk. PRCP polymorphism has been associated with hypertension and inflammation [106]. PRCP gene-trapped mice are hypertensive and are prone to faster thrombosis [107]. These mice have increased *in vivo* vascular ROS and uncoupled endothelial nitric oxide synthase (eNOS) and reduced expression of vascular thrombomodulin in their aorta [108, 110]. The hypertensive and prothrombotic state in PRCP gene trapped mice was abrogated by antioxidant treatment using mitoTEMPO, apocynin, and Tempol [107]. The paper demonstrates that the hypertensive and prothrombotic state arose from high levels of ROS in PRCP gene trapped mice, and this state was reversed by antioxidant treatment. The potential of antioxidants treatment in cigarette smokers to suppress smoking related cardiovascular disease is worth exploring.

Shifting from urinary thrombomodulin, we are also interested in plasma thrombomodulin. The level of plasma thrombomodulin has been associated as an indicator of endothelial damage in patients with disseminated intravascular coagulation syndrome, pulmonary thromboembolism, adult respiratory distress syndrome, chronic renal failure, acute hepatic failure, atherosclerosis [121, 215-217]. In one study, plasma thrombomodulin concentrations of smokers and non smokers were studied [218]. They found the plasma levels of cigarette smokers was 15% lower than the mean. We would like to see if there is any correlation between the concentration of plasma thrombomodulin and methionine 388 oxidation in cigarette smokers.

Conclusion

We have shown that methionine 388 is significantly more oxidized in smokers than non-smokers. Oxidative damage to the endothelium from the oxidants in cigarette smoke has been suggested to be an important factor for the development of cardiovascular diseases in smokers. Smokers are in a hypercoagulable state, but this is the first evidence for a specific molecular origin of this state. Methionine 388 oxidation has been shown *in vitro* to have large effects on the activity of the thrombomodulin. The mutation of thrombomodulin methionine 388 to any other residues, except leucine, decreases the anticoagulant cofactor activity of thrombomodulin [91]. More importantly, there is 76-90% loss of activity when Met388 is oxidized [92]. We believe oxidation methionine 388 of thrombomodulin is one of the key molecular causes of the prothrombotic state in smokers and thus a critical factor in the development of thrombosis and premature death in smokers. In this chapter we have shown a method allows the semi quantitative analysis of methionine 388 oxidation in the thrombomodulin isolated from urine sample. This method offers a useful way to compare thrombomodulin oxidation in cigarette smokers and non smokers. In this study we report that methionine 388 of thrombomodulin is more oxidized in cigarette smokers than in non smokers, and we propose that one of the molecular causes for prethrombotic state seen in smokers is from oxidation of methionine 388 of thrombomodulin.

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Appendix

Office of Research Compliance
Institutional Review Board

June 8, 2012

MEMORANDUM

TO: Wesley Stites
Samrat Thapa

FROM: Ro Windwalker
IRB Coordinator

RE: PROJECT CONTINUATION

IRB Protocol #: 05-427

Protocol Title: *Study of Thrombomodulin Oxidation*

Review Type: EXEMPT EXPEDITED FULL IRB

Previous Approval Period: Start Date: 06/27/2005 Expiration Date: 06/26/2012

New Expiration Date: 06/26/2013

Your request to extend the referenced protocol has been approved by the IRB. If at the end of this period you wish to continue the project, you must submit a request using the form *Continuing Review for IRB Approved Projects*, prior to the expiration date. Failure to obtain approval for a continuation on or prior to this new expiration date will result in termination of the protocol and you will be required to submit a new protocol to the IRB before continuing the project. Data collected past the protocol expiration date may need to be eliminated from the dataset should you wish to publish. Only data collected under a currently approved protocol can be certified by the IRB for any purpose.

This protocol has been approved for 106 total participants. If you wish to make *any* modifications in the approved protocol, including enrolling more than this number, you must seek approval *prior* to implementing those changes. All modifications should be requested in writing (email is acceptable) and must provide sufficient detail to assess the impact of the change.

If you have questions or need any assistance from the IRB, please contact me at 210 Administration Building, 5-2208, or irb@uark.edu.