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CONFORMATIONAL DYNAMICS LEADING TO ACTIVATION OF THE
TRANSGLUTAMINASE FACTOR XIII

By
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B.S., Biochemistry, College of Charleston, 2000
B.S., Chemistry, College of Charleston, 2001

A Dissertation
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville
In Partial Fulfillment of the Requirements
For the Degree of

Doctor of Philosophy

Department of Chemistry
University of Louisville
Louisville, Ky

December 2011

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Dissertation approved on

December 16, 2011

by the following Dissertation Committee:

Dissertation Director, Muriel C Maurer

DEDICATION

This dissertation is dedicated to all the love and support of my family. First and foremost, graduate school would not have been possible without the loving support of my beautiful wife Molly. I am truly fortunate to have fallen in love with my best friend. Thank you for all of your assistance/love/support throughout this journey.

Denise, I did not know my own capacity to love until you were brought into this world in 2005. You are a mere 6 years old and I admire your wisdom and drive to learn. I look forward to watching you become a “veterinarian Princess.”

Isaac, where do I start? You have brought nothing but smiles since you were born in 2007. You have an amazing ability to make people laugh. You are now 4 years old and your imagination is unstoppable.

Norah, you came into this family ready to play. You joined our family in 2009 and there is nothing that slows you down. Your manners and thoughtfulness are second to none.

I love you guys... You are my Life.

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ABSTRACT

CONFORMATIONAL DYNAMICS LEADING TO ACTIVATION OF THE
TRANSGLUTAMINASE FACTOR XIII

Richard Tatum Woofers II

16 December 2011

One of the last events that occurs during blood coagulation, a process taken for granted on a daily basis, involves Factor XIII (FXIII) cross-linking fibrin monomers to form an insoluble clot. In plasma, FXIII-A₂ is not active and exists as the heterotetramer FXIII-A₂B₂. Through the utilization of hydrogen – deuterium exchange (HDX) coupled with Matrix Assisted Laser Desorption ionization – Time of Flight – Mass Spectrometry (MALDI-TOF-MS), it was determined that FXIII-A₂ becomes nearly uniformly protected when bound to FXIII-B₂ and the FXIII-A₂ β-barrels play a major role in heterotetramer formation.

After dissociation from FXIII-B₂, FXIII-A₂ has the ability to become activated in the presence of Ca²⁺. The regions/residues of FXIII-A₂ Ca²⁺ affects during activation were identified using HDX. It is debated whether FXIII-A₂ undergoes an open conformation during activation. Transglutaminase 2 (TG2) has been observed crystallographically in an open conformation. HDX was utilized to compare the conformational dynamics of Transglutaminase 2 in solution to that of FXIII-A₂. The increase in exposure between the catalytic core and β-barrels of TG2 yields evidence of an open conformation. A structural

comparison of FXIII-A₂ and TG2 identified steric hinderance within the A₂ dimer that could thwart a similar conformational change.

Once activated physiologically, FXIII-A₂ is solely responsible for forming the cross-links between fibrin monomers. The α C (233 – 425) region of fibrin contains three reactive Gln residues and acts as a substrate for FXIII. Fibrin α C (233 – 425) was expressed and its structure investigated via ¹⁵N-HSQC when in solution with FXIII-A₂.

The integral role of FXIII-A₂ in the coagulation cascade leads to a dire need for investigating its conformational dynamics during activation. The research herein provides a stronger knowledge of FXIII-A₂ structural changes during activation and outlines FXIII-A₂ interactions with B₂ and the fibrin α C domain. This progress in understanding FXIII-A₂ dynamics could lead to improved treatments for excessive bleeding and thrombosis.

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CHAPTER I

INTRODUCTION

Blood Coagulation Overview

The highway system that transports nutrients to all cells within the body and aids in eliminating cellular waste is referred to as the circulatory system. The circulatory system involves many organ systems working in harmony with one another. The organ that acts as the carrier for all the nutrients, amino acids, hormones and cellular waste is blood (1). If there is a breach in the vasculature there needs to be processes in place which quickly and efficiently close the breach while not allowing for any disturbance in the flow of blood (nutrients) to cells throughout the body. Haemostasis ('hemo' = blood + 'stasis' = stagnation), the process of stopping blood flow in damaged tissue, starts with vasoconstriction to slow the flow of blood to the damaged area. This physiological process can also be assisted by external pressure on wounded tissue. Following vasoconstriction, platelets are recruited to the damaged tissue to form a plug. As the platelets aggregate, tissue factors signal the coagulation cascade which ultimately concludes in a soluble fibrin clot being crosslinked by factor XIII (FXIII) to form an insoluble clot (1).

Historically it was believed that coagulation was initiated after an injury when blood was exposed to air. It was not until the mid-19th century before it was proposed that a specific component in the blood initiated coagulation (2). Nearly a century later in 1964, the “Cascade” or “Waterfall” model was released by two independent groups (3-4). These models were developed under the premise of defining the

Figure 1: The conventional Y-shaped cascade model of coagulation. This model initiates via the Intrinsic (contact factor) pathway or the extrinsic (tissue factor) pathway. This diagram was adapted from a poster distributed by Enzyme Research Laboratories, South Bend, IN.

identity and function of the procoagulant enzymes. The early models involved the familiar Y-shaped scheme (Figure 1 or for more detail Appendix A) where the “intrinsic” and “extrinsic” pathways converged in a common pathway with the production of Factor Xa. After several decades of discoveries, questions started to arise concerning the independent nature of the intrinsic and extrinsic pathways. One such question being, why do patients with a deficiency of FVIII and FIX (see appendix B for coagulation enzyme abbreviations) in the “intrinsic” pathway contract hemophilia A and B respectively when they still have an intact “extrinsic” pathway(5). Hoffman and Monroe have since worked to develop a model in which coagulation is mediated by the cell surfaces where they occur. They propose a model that incorporates three overlapping stages: 1- initiation,

occurring on tissue factor bearing cells; 2-amplification, occurs when platelets and cofactors become activated prior to thrombin production; and 3- propagation, where large amounts of thrombin are generated on the platelet surface (6).

Initiation

Tissue factor (TF) is an integral membrane protein that plays the role of initiator in coagulation *in vivo*. As a membrane bound protein, TF remains localized to the cell in which it was synthesized and these cells are generally located outside the vasculature (7). Once there is a breach in the vessel, blood will come into contact with the TF-bearing extravascular cells. FVII is easily activated by coagulation and non-coagulation proteases and rapidly binds to cellular TF. The FVIIa-TF complex then goes on to further activate more FVII as well as both FX and FIX. The FX that is activated by the FVIIa-TF complex is quickly degraded if it leaves the cell surface, but when it remains within the confines of the forming thrombus, it can combine with FVa to produce thrombin which will aid in activating platelets and FVIII during amplification (8).

Amplification

The next step is to amplify the coagulation response that was just initiated. The damage to the endothelium that initiated the response also allows platelets to come in contact with TF-bearing cells. The thrombin that was produced during the last stages of initiation is then utilized to enhance platelet adhesion and activation (9). Thrombin also assists in preparing for procoagulant activity by activating FV, FVIII, and FXI (8).

Figure 2: The Cell-Based Model of Hemostasis. In the cell based model, several cell surfaces are utilized in during the series of events that lead to a fibrin clot. (A) During the Initiation phase TF-bearing cells become exposed to plasma after injury and FIXa and IIa are released and diffuse to the platelet surface. (B) During Amplification the IIa released on the TF-bearing surface serves to activate the platelets and FXI as well as release vWF yielding FVIIIa . (C) During Propagation several of the enzymes that were activated earlier assemble on the surface of the activated platelet. $\text{FIXa}/\text{FVIIIa}$ form the tenase which aid in FXa formation. FXa/FVa then form prothrombinase which results in the burst of IIa . IIa then goes on to cleave fibrinogen and activate FXIII which crosslinks the fibrin to form an insoluble clot. Figure adapted from cell-based hemostasis models (2, 6).

Thrombin continues its action by activating platelets through the utilization of their protease-activated receptors (PAR) (10). FV is released from platelets and subsequently activated by thrombin. Some of the thrombin remains on the surface of the platelet and is available for cleaving other coagulation enzymes. von Willebrand factor (vWF)/ FVIII circulate together and when bound to the

platelet surface, thrombin activates the FVIII through the release of vWF (11).

The amplification process has now produced activated platelets with activated cofactors such as FVa, FVIIIa and FXIa on their surfaces; therefore, propagation is ready to commence.

Propagation

The FIXa and FVIIIa that were activated during initiation and propagation respectively are now poised to form the intrinsic tenase complex on the surface of activated platelets. Once FIXa diffuses from the TF-bearing cell to the platelet these complexes begin to form. In addition to FIXa diffusing from the neighboring TF-bearing cell, it can also be activated on the platelet surface by FXIa (12). The tenase complexes then continue the coagulation processes by activating FX on the platelet surface where it is poised to complex with FVa. These FXa/FVa “prothrombinase” complexes convert II to IIa generating a IIa burst (6).

The IIa generated during initiation, amplification and propagation is further utilized in clot stabilization. Fibrinogen is a 340 kDa protein that consists of three chains ($A\alpha$, $B\beta$ and γ) forming the homodimer $(A\alpha B\beta\gamma)_2$. Thrombin converts fibrinogen to fibrin through the cleavage of fibrinopeptides A and B. Fibrin then non-covalently self associates in the formation of a soft clot (13). The soluble fibrin clot is then stabilized through γ -glutamyl- ϵ -lysinyI cross-links formed between the fibrin $\gamma - \gamma$ and $\gamma - \alpha$ chains by IIa-activated FXIII (14). Thrombin activated FXIII further stabilizes and protects the growing fibrin network from lysis

by cross-linking it to thrombin-activatable fibrinolysis inhibitor (TAFI) and α_2 antiplasmin (α_2 AP) (15-17).

The process of haemostasis has been broken down into three overlapping stages: Initiation, Amplification, and Propagation. Of the many enzymes and cofactors involved, the research presented here focuses FXIII. The use of techniques such as HDX coupled with MALDI-TOF-MS aim to shed light into the illusive FXIII conformational changes.

Transglutaminase Factor XIII

FXIII-A₂ has gained much interest over the last couple decades due to its presence in the cytoplasm of several different cell types: macrophages, megakaryocytes, and connective tissue histiocytes to name a few (19-21). Factor XIII plays an

integral role in the coagulation cascade, and there is much need to investigate the conditions that affect activation/activity as well as the structural dynamics of the enzyme during activation. A better understanding of the structural changes around the active site of FXIII during activation, as well as how it interacts with the other enzymes and proteins involved in the coagulation cascade, could lead to more targeted therapeutic agents for treatment of excessive bleeding, thrombosis, and atherosclerosis.

Figure 3: Labeled FXIII-A (1FIE) This figure was made using PyMol(18).

The action of Factor XIII was first reported in the early 1920s when Barkan and Gaspar observed that fibrin clots formed in the presence of Ca^{2+} were insoluble in weak bases (22). Over 25 years later in a letter to Nature, Lorand reported that a protein was responsible for the formation of insoluble clots because the “fibrin stabilizing factor” was thermolabile, nondialyzable and required Ca^{2+} (23). Then after the acknowledgement of fibrin stabilizing factor as a clotting factor in 1963 by the International Committee on Blood Clotting Factors, it became factor XIII (24). It soon became obvious that FXIII was essential for stabilizing the non-covalent soft clots formed in blood coagulation after thrombin cleaves the A and B fibrinopeptides from fibrinogen.

Factor XIII is a protransglutaminase in plasma with a tetrameric structure consisting of two A subunits and two B subunits ($\text{FXIII-A}_2\text{B}_2$). The details of FXIII-B will be further discussed in chapter IV, but briefly, all FXIII-A in plasma is found bound to FXIII-B as a $\text{FXIII-A}_2\text{B}_2$ heterotetramer with an apparent K_d of 25 nM (25). In platelets, FXIII-A_2 is found without the B_2 dimer and is referred to as cellular FXIII (26). The ~83 kDa factor XIII-A has been successfully expressed and the recombinant form, rFXIII, has been subsequently crystallized (27). The crystal structure illustrates the well-defined and sequentially folded domains: the activation peptide (1-37), the β -sandwich (38-184), the catalytic core (185-515), β -barrel 1 (516-628), and β -barrel 2 (629-730) (Figure 3).

Figure 4: Factor XIII activation scheme. **A)** Plasma FXIII activation. Intact FXIII-A₂B₂, thrombin cleaved FXIII-A', Ca²⁺ induced dissociation of FXIII-A₂ and FXIII-B₂ yielding active FXIII-A* (FXIIIa^{IIa}). **B – D)** Cellular FXIII-A₂ activation. **B)** Thrombin proteolytic activated FXIII-A₂, thrombin cleaved FXIII-A' and fully active FXIII-A* (FXIIIa^{IIa}) in the presence of low mM Ca²⁺. **C)** FXIII-A₂ nonproteolytic activation in the presence of high (50 mM) [Ca²⁺] FXIII-A° (FXIIIa^{Ca}) **D)** FXIII-A₂ nonproteolytic activation in the presence of low mM Ca²⁺ and high (> 150 mM) NaCl yielding FXIII-A° (FXIIIa^{Na}).

When FXIII is activated physiologically (Figure 4), thrombin (IIa) cleaves the N-terminal activation peptide (residues 1-37). Following activation peptide cleavage, both low mM Ca²⁺ and the C-terminal portion of the fibrin α -chain assists in the dissociation of the two B subunits (28-32). Once the FXIII-A₂ is free from FXIII-B₂ it is active (FXIIIa^{IIa}). Factor XIII exists as a dimer and the active site consists of a catalytic triad (C³¹⁴, H³⁷³ and D³⁹⁶). The catalytic C314 has an apparent pK_a of 6.0, similar to that of a histidine imidazole (33). The hydroxyl oxygen of Y560 forms a hydrogen bond with C314 (27). This H-bonding interaction is indicative of a thiolate – imidazolium pair (34). The catalytic sulfur anion is stabilized at physiological pH through the H-bonding with Y560 and ion-pair interactions with the H373 imidazolium (27, 34-35).

Dimer formation is important for the stability of FXIII-A. The dimer interface covers 2280 Å² and there is a 10.3 – 14.4 kcal/mol decrease in its free energy upon dimerization (36). When in the presence of a suitable acyl-donor glutamine containing substrate, C314 of FXIIIa forms a thioester bond and ammonia is released. The acyl-donor substrate is then covalently linked to a primary amine (lysine) acyl-acceptor forming γ-glutamyl-ε-lysine cross-links between the α- and γ-chains of fibrin which stabilizes the growing clot. In addition to clot stabilization, FXIII also assists in protecting the clot from plasmin degradation via crosslinks between the Aα-chain of fibrin and α₂ antiplasmin(15, 17, 37).

FXIII can also be non-proteolytically activated (Figure 4 C and D) when the Ca²⁺ concentration is greater than 50 mM (FXIIIa^{Ca}) (38-39). Under these non-physiological conditions the activation peptide is not cleaved and the enzyme shows activity. Another nonproteolytic activation mechanism has been demonstrated that is more physiologically relevant. When the factor XIII-A₂ subunits are in the presence of 150mM Na⁺ with low mM Ca²⁺ (FXIIIa^{Na}), albeit slow, transglutaminase activity is observed (40). When haemostasis concludes, one means that FXIII-A₂ can be rendered inactive is through cleavage at the secondary cleavage site (K513-S514).

Hydrogen Deuterium Exchange (HDX) Coupled with Mass Spectrometry

The major theme underlying this research is establishing the conformational dynamics of FXIII-A₂ in solution. The FXIII-A₂ crystal structures provided to date for zymogen (27), FXIIIa^{IIa} (41), and FXIIIa^{Ca} (42) display no

significant RMSD between the activated and unactivated forms. The active site is occluded in all crystal structures so an active conformation has yet to be observed. In an effort to gain a better understanding of FXIII-A₂ during activation, HDX has been utilized in several studies (43-46). HDX has proved to be a worthy method of isolating structural changes to defined regions of the molecule

Proteins in solution are not static and in fact are naturally moving, flexing, and possibly even opening and closing. These movements termed, conformational dynamics, are difficult to monitor and impossible to see via crystallography. One benefit of HDX in the field of protein dynamics is the needed sample size. A typical HDX sample contains 12 μ l of 30 – 50 μ M protein whereas NMR would require approximately 350 μ l and preferably > 100 μ M protein. At pH 7.0 exposed protein backbone amide hydrogens exchange with D₂O within 1 – 10 seconds (47). Within the interior of the protein, exchange is limited because there is not only restricted access but also nearly all amide hydrogens are involved in hydrogen bonds. The H-bonding protects the hydrogen from exchange (48). When running HDX at physiological pH, base-catalyzed exchange is the dominant mechanism of hydrogen exchange. This occurs in the presence of a catalyst (hydroxide) and a new hydrogen (water) (47).

The rate of hydrogen exchange in an unfolded protein is dependent upon several factors.

Equation 1: Rate Constants for hydrogen-deuterium exchange where F and U represent folded and unfolded protein respectively (47).

In Equation 1, U and F represent

the unfolded and folded protein respectively; therefore, hydrogen-deuterium exchange is not only dependent upon (k_2), but also the k_1 and k_{-1} (the unfolding and folding of the protein). The exchange kinetics are broken down into two pathways, EX1 and EX2 kinetics. When $k_2 \gg k_{-1}$ EX1 kinetics ensue. Under EX1 kinetics, there is an unfolding event and the residues involved are exchanged prior to refolding. This is very rarely seen naturally, without denaturants. Therefore, EX2 kinetics typically prevail where the protein is quickly exchanging between two states and the refolding rate is faster than the exchange rate ($k_{-1} \gg k_2$). Once hydrogen-deuterium exchange occurs, the half-life for amides increases to over an hour by lowering the pH to between 2 and 3 at 0 °C (48). This low pH – low temperature quench allows for protein digestion and mass spectrometric analysis.

Figure 5: Schematic describing the processes involved in hydrogen-deuterium exchange coupled with MALDI-TOF-MS. The details are in-text.

The details of the method used for each HDX experiment are explained in the methods section of each respective chapter. Figure 5 is a pictorial representation to assist in visualizing the process. The starting material is a lyophilized protein containing hydrogens (blue circles) at all amide linkages. The protein is resuspended in D_2O , introducing the protein to a deuterium (red circles) environment. During the 10 minute room temperature incubation, the hydrogens are given time to exchange with the deuterons as described by the kinetic regime presented above. To quench the exchange, samples were placed on ice and trifluoroacetic acid was added dropping the pH to 2.2. The protease utilized was pepsin due to its ability to function at low pH. After 10 min, aliquots of the peptic digestion were quick-frozen in liquid nitrogen and stored cryogenically until analysis.

Matrix was added to the sample spotted on MALDI plates for analysis. The spectra in Figure 5 are a representative FXIII-A₂ pepsin digest. The three spectrum labeled A, B and C represent an undeuterated spectra (A), and two different states (B) and (C) where (C) displays the highest level of deuterium incorporation or exposure.

Hydrogen-deuterium exchange coupled with mass spectrometry provides an extremely powerful tool to analyze the conformational dynamics of proteins in solution. In the research presented HDX has successfully identified regions of FXIII that experience protection or exposure due to physiological Ca²⁺ concentrations, as well as after non-proteolytic activation. To approach the question of whether FXIII-A₂ adopts an open conformation when activated, FXIII-A₂ HDX was compared to transglutaminase 2 (TG2), an enzyme with the same domain structure as FXIII, in both an open and closed conformation. Lastly, the interface between FXIII-A₂ and FXIII-B₂ is not well defined and HDX has uncovered the regions of FXIII-A that become protected when in the heterotetramer conformation.

Research Goals

FXIII-A₂ solution conformational dynamics is the basis of all research presented in this dissertation. The overarching goal is to gain a better understanding of the solution structure of FXIII-A and understand how it interacts with substrate. HDX was the primary method for studying FXIII-A conformational dynamics, but NMR was utilized in analyzing α C (233 – 425) structure in solution with FXIII-A₂ (described in chapter V).

In Chapter II, the FXIII-A₂ conformational dynamics journey starts by investigating the solution structure of FXIII-A₂ in the presence of physiological Ca²⁺ (1 mM)(44). To determine whether the conformational changes seen in the presence of Ca²⁺ were Ca²⁺ specific, Mg²⁺, Ba²⁺ and Cu²⁺ were also investigated. Non-proteolytically Ca²⁺ activated FXIIIa^{Ca} was also analyzed by HDX and compared to FXIIIa^{Na}. These HDX studies led to many new discoveries concerning the Ca²⁺ specific conformational dynamics of FXIII-A₂ both before (1 mM Ca²⁺) and after activation.

After Pinkas *et al.* reported that the transglutaminase TG2 could be trapped in an open conformation (49), HDX was utilized to determine if FXIII assumed a similar open conformation. Chapter III describes the conformational dynamics of TG2 in the activated state and also in the open state after inhibition with TG-DON. HDX has already been used to analyze FXIII-A₂ in the activated

state (Chapter II) and inhibited with the substrate peptide K9-Don (43). TG2 conformational dynamics have yet to be investigated by HDX and these analyses allow for a great comparison for FXIII-A to a known 'open' conformation.

The previous studies focused on the conformational dynamics of FXIII during the last phases of activation. Chapter IV took a step backward in the activation process to investigate the interaction between FXIII-A₂ and FXIIIB₂. The FXIII-A₂B₂ heterotetramer plays a major role physiologically but the interface between the FXIII-A and FXIII-B subunits is not very well understood. HDX once again proved to be an invaluable tool in determining the regions of FXIII-A₂ that become protected when bound to FXIII-B₂.

When studying protein conformational dynamics, it is beneficial to establish an expression system for your protein of interest. The development of an expression system opens the doors to introducing mutations and/or the incorporation of isotopic labels for NMR analysis. The α C domain of fibrin is known to interact with FXIII-A₂B₂ and the α C (242 – 424) region aids in the dissociation of FXIII-A₂B₂ (28). Chapter V describes the expression system established and utilized to express ¹⁵N- α C (233 – 425). The ¹⁵N-labeled α C was then analyzed via ¹⁵N-HSQC to determine if it adopted any compact structure when interacting with FXIII-A₂.

CHAPTER II

ROLE OF CALCIUM IN THE CONFORMATIONAL DYNAMICS OF FACTOR XIII
ACTIVATION EXAMINED BY HYDROGEN/DEUTERIUM EXCHANGE
COUPLED WITH MALDI-TOF MS

Introduction

Due to the critical role of FXIII in coagulation, there is a need to better understand the conditions that influence FXIII activation/activity as well as the structural dynamics of this enzyme system. Recombinant FXIII A₂ has been successfully crystallized (27), and the resultant structure exhibits well-defined and sequentially folded domains that include the activation peptide (1-37), the β sandwich (38-184), the catalytic core (185-515), β -barrel 1 (516-628), and β -barrel 2 (629-730) Figure 6.

When FXIII is activated physiologically, thrombin (IIa) cleaves the N-terminal activation peptide (residues 1-37). The presence of low mM Ca²⁺ assists in the dissociation of the B subunits from plasma FXIII. In addition, Ca²⁺ promotes exposure of the catalytic C314 from both plasma and cellular FXIII resulting in an active A₂ dimer (FXIIIa*) (26, 30, 32). Like other transglutaminases (TGases), the active site of FXIII consists of a thiol-containing

catalytic triad (C314, H373, and D396), but FXIII is unique in the fact that it exists as a dimer.

Figure 6: The FXIII A₂ zymogen 2.1 Å crystal structure 1F13 (50). (A) A cartoon model to illustrate the intimate contact at the dimer interface with the domains being color coded (grey) activation peptide, (white)β-Sandwich, (black) catalytic core, (white) β-barrel 1 and (black) β-barrel 2. (B) Ribbon view of one FXIII-A monomer with the domains represented by the same colors as (A). The regions of interest are labeled: activation peptide (1-37), Gln recognition peptide 4 (72-97), Gln recognition peptide 7 (190-230), catalytic triad (314, 373 and 396), Tyrosine 560, secondary cleavage site (513-514) and the proposed lysine recognition region (646-658). These figures were created using VMD (51).

In addition to IIa proteolytic activation, FXIII A₂ can be non-proteolytically activated when the Ca²⁺ concentration is greater than 50 mM (FXIIIa^o or FXIIIa^{Ca}) (38-39). FXIII A₂ has also been observed in an activated state in the presence of >150 mM NaCl and low mM Ca²⁺ (FXIIIa^{Na}) (40). A related form of nonproteolytic FXIII activation has been documented in platelets (40, 52-53). Thrombin-stimulated platelets may exhibit increased Ca²⁺ levels that promote formation of an active intracellular FXIII species which has not been hydrolyzed at the R37-G38 peptide bond. In plasma, these different forms of nonproteolytic activation are minimized by the presence of the B₂ subunits (54). Regardless of the mode of activation, FXIIIa function can be hindered following hydrolysis at the

secondary cleavage site (K513-S514) by thrombin (55-56). Occupation of a nearby site with Ca^{2+} and other select metals can help protect this secondary hydrolysis from occurring (27, 57).

Even though the catalytic C314 is only alkylated by iodoacetamide (IAA) after activation, (33, 58-59) the crystal structures for FXIIIa^{IIa} and FXIIIa^{Ca} lack any major structural changes when compared to that of zymogen (27, 41-42). The Maurer lab has used MALDI-TOF mass spectrometry approaches coupled with hydrogen-deuterium exchange or chemical modification to probe FXIII conformation changes occurring in solution. During activation, portions of the FXIII catalytic core and the A₂ dimer interface were found to be more accessible to solvent (45, 60). By contrast, the addition of an inhibitory peptide with a glutamine isostere caused selected FXIII regions to become more protected from solvent (43). As expected from the secondary cleavage studies, Ca^{2+} binding hindered solvent exposure in a FXIII segment around 513-514. Inhibition at the active site could lead to further long-range decreases in HDX in this region.

A recent paper by Kristiansen and Andersen questions our use of borate buffer supplemented with CaCl_2 for mass spectrometry based projects. Our HDX studies have not, however, been carried out under deactivated concentrations (0.3 mM CaCl_2) as they presume, but at 1 mM CaCl_2 following either proteolytic or nonproteolytic activation. According to their paper, FXIII A₂ that is activated nonproteolytically at 50 mM CaCl_2 and then buffer exchanged into 1mM CaCl_2 maintains 90% of its enzyme activity (61). Moreover, many of the conformational events reported as being important for FXIII activation have been

confirmed by HDX studies by Andersen and Faber using a buffer system of 200 mM Hepes, 150 mM NaCl, and 50 mM CaCl_2 (46).

The focus of our present HDX work was to further investigate the conformational changes that occur to FXIII due to monovalent and divalent cation binding. FXIII A₂ was monitored in the presence of Ca^{2+} , Mg^{2+} , Ba^{2+} , Cu^{2+} , Na^+ , tetramethylammonium chloride (TMAC^+) or ethylenediamine (EDA^{2+}) with concentrations ranging from physiological Ca^{2+} concentration, 1-2 mM, up to 50-500 mM. On its own, the low mM Ca^{2+} condition is unable to support FXIII activity. The HDX effects observed in this environment thus provide valuable information on early conformational changes needed in preparation for activation. Often these effects further increased as the Ca^{2+} concentration was raised to the levels required for nonproteolytic activation (50-500 mM). Several of the conformational changes observed with physiological 1 mM Ca^{2+} also occurred with the other divalent metals whereas other effects were distinct for a particular metal. In the current investigation, a novel method of nonproteolytic FXIII activation was characterized utilizing high concentrations of organic cations TMAC^+ or EDA^{2+} . Knowledge gained from these different HDX studies will help identify the elusive structural roles of different divalent and monovalent cations. A greater understanding of the conformational dynamics of FXIII will aid in the development of new therapeutic strategies to control excessive bleeding, thrombosis, and/or atherosclerosis.

Materials and Methods

Factor XIII Preparation and Synthetic Peptides

Recombinant human cellular factor XIII A₂ (FXIII A₂) was generously provided by Dr. Paul Bishop (ZymoGenetics, Inc., Seattle, WA). After reconstituting the lyophilized FXIII in 18 MΩ deionized water, FXIII was buffer exchanged into 6.67 mM borate at pH 8.3. The concentration of FXIII was determined on a Cary 100 UV/vis spectrophotometer. The absorbance was monitored at 280 nm and concentration calculated with the FXIII extinction coefficient of 1.49 ml/mg cm. Aliquots (36 µl) of 16.7 µM FXIII in 6.67 mM borate were dried in a SpeedVac (Savant) and stored at -70 °C until future use.

The β casein derived FXIII substrate peptide K9 (Ac-LGPGQSKVIG-OMe) was synthesized by Peptides International (Louisville, KY). K9 was reconstituted in 18 MΩ deionized water and the concentration was confirmed by quantitative amino acid analysis (AAA Service Laboratory, Inc., Boring, OR). Purity was assessed by HPLC and mass spectrometry approaches.

Transglutaminase Activity Assay

Factor XIII activity was determined using a modified version of the Dade-Behring Berichrom Assay (62-63). Briefly, this assay utilizes a coupled reaction involving both FXIII and glutamate dehydrogenase (GDH). FXIIIa reacts with the acyl-donor K9 releasing NH₃ and the transglutaminase reaction concludes when the primary amine acyl-acceptor, glycine ethyl ester, forms an isopeptide bond with the K9 peptide. This transglutaminase activity is monitored via the

conversion of α -ketoglutarate and NH_3 into glutamate in the presence of reducing equivalents of NADH. The oxidation of NADH results in decreased absorbance at 340 nm.

Dry FXIII aliquots were activated nonproteolytically in a total volume of 12 μl dH_2O . The final working concentration was 50 μM FXIII in 20 mM Borate at pH 8.3 with 50 mM CaCl_2 , 50 mM MgCl_2 with 2 mM CaCl_2 , 50mM BaCl_2 with 2 mM CaCl_2 , 50 mM CuCl_2 with 2 mM CaCl_2 , 500 mM ethylenediamine (EDA^{2+}) with 2 mM CaCl_2 , 500 mM NaCl with 2 mM CaCl_2 , and 500 mM tetramethylammonium chloride (TMAC^+) with 2 mM CaCl_2 . FXIII was activated for 10 min at 37 °C before adding 3 μl to the assay. For each assay, the volume of activator reagent (163 μl) and detector reagent (250 μl) as well as the concentration of FXIII (300 nM) and K9 (500 μM) were held constant (64). The assay contents were placed in a Cary 100 UV/vis spectrophotometer for 2 min at 37 °C for equilibration before the K9 substrate peptide was added. After introducing the K9 peptide, the oxidation of NADH was monitored for 25 minutes at 340 nm. Due to the coupled nature of this FXIII activity assay, there is a brief delay before consumption of NADH can be detected. The FXIII transglutaminase activity was later determined by the steepest part of the slope which correlates with enzyme catalyzed velocity ($\Delta\text{abs}/\text{min}$). The final divalent and monovalent cation concentrations in the assays (0.3-3 mM) did not interfere with GDH reactivity. Using the molar absorptivity value for NADH ($6220 \text{ M}^{-1}\text{cm}^{-1}$ at 340nm), the transglutaminase based velocities were converted into ($\mu\text{M}/\text{min}$) and the final velocity values compared. For the studies involving the novel cations EDA^{2+} ,

Na⁺, and TMAC⁺, transglutaminase activity was compared relative to that of FXIII^{Ca} with % FXIII^{Ca} Activity defined as (FXIIIa activity / FXIII^{Ca}) x 100.

Standard Deviations of the mean for three independent trials were calculated.

HDX Experimentation

There were two different groups of HDX samples, one monitoring the effects of low mM concentrations of divalent metals and the other examining non-proteolytic activation. Both groups utilized the dry buffer exchanged FXIII aliquots. To probe the effects of low mM divalent metals, 0.6 µl of 20 mM metal-chloride (CaCl₂, MgCl₂, BaCl₂ or CuCl₂) was added to the dry FXIII aliquot and evaporated to dryness in the SpeedVac and stored at -70 °C. The following HDX protocol was then adapted from methodology established in the Komives laboratory (65-66). The dry FXIII aliquot was allowed to reach room temp before 12 µl of 99.996% D₂O (Cambridge Isotope Laboratories) was added yielding a final working concentration of 50 µM FXIII and 1mM Ca²⁺, Mg²⁺, Ba²⁺ or Cu²⁺ in 20 mM Borate at pH 8.3. The samples were incubated at room temperature for 10 minutes before the HDX was quenched by adding 120 µl of chilled 0.1% TFA at pH 2.5. The quenched reaction was then immediately transferred to a tube containing activated pepsin bound to 6% agarose (Thermo Scientific, Rockford, IL). Pepsin digestion occurred for 10 min on ice. Following digestion, the reaction mix was centrifuged for 30 sec to separate the FXIII digest from the pepsin beads. Three 8.2 µl aliquots were immediately frozen in liquid N₂ and each reaction condition was performed three times.

When investigating nonproteolytically activated FXIII, aliquots were prepared as described previously. Divalent and monovalent cations (< 3 μ l) were added to the dry FXIII aliquot for each of the activation conditions (50 mM Ca^{2+} , 500 mM ethylenediamine (EDA) with 2 mM Ca^{2+} , 500 mM Na^+ with 2 mM Ca^{2+} , and 500 mM tetramethylammonium chloride (TMAC^+) with 2 mM Ca^{2+}) and evaporated to dryness in a SpeedVac and stored at -70°C . After allowing the sample to reach room temperature, 12 μ l 99.996% D_2O was added and the HDX occurred at 37°C for 10 min. The activated FXIII was digested and quenched as stated above.

HDX Analysis

A FXIII(a) HDX aliquot was thawed at room temperature and immediately mixed with an equal volume of 10 mg/ml α -cyano-hydroxycinnamic acid matrix (α -CHCA) (Aldrich) in 1:1:1 ethanol/ CH_3CN /0.1%TFA at pH 2.2, and 0.5 μ l was spotted on a chilled MALDI plate. The sample spot was then quickly dried by placing the MALDI plate into a SpeedVac. The plate was then immediately inserted into the MALDI-TOF-MS (Voyager DE-Pro, Applied Biosystems). By limiting time for this procedure to < 5 min, HDX back exchange is kept at a minimum. Spectra were collected in reflector mode with 256 shots/spectrum. All peptides in the peptic digest were previously identified by Brian T. Turner, Jr. (45) and/or confirmed by MS/MS analysis on an Applied Biosystems 4700. The MS/MS peptide identifications were performed using Applied Biosystems GPS software which utilized the Mascot database. Peptide identification was confirmed through observing the same peptide sequence in multiple independent

digests, with an ion score preferably above 20 (peptic peptides Appendix C).

After digestion by pepsin, the FXIII A₂ peptides identified represent 40 %

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1  SETSRTAFGG RRVPPNNSN AAEDDLPTVE LOGVVPRGVN LQEFLNVTSV HLFKERWDTN 60
61 KVDHHTDKYE NNKLIVRRGO SFYVQIDFSR PYDPRRDLFR VEYVIGRYPQ ENKGTIIPVP 120
121 IVSELQSGKW GAKIVMREDR SVRLSIQSSP KCIVGKFRMY VAVWTPYGVL RTSRNPETDT 180
181 YILFNPWCED DAVYLDNEKE REEYVLNDIG VIFYGEVNDI KTRSWSYGQF EDGILDTCLY 240
241 VMDRAQMDLS GRGNPIKVS VGSAMVNAKD DEGLVGSWD NIYAYGVPPS AWTGSVDILL 300
301 EYRSSENFVR YGQCWVFAGV FNTFLRCLGI PARIVTNYFS AHDNDANLQM DIFLEEDGNV 360
361 NSKLTKDSVW NYHCWNEAWM TRPDLPVGF GWQAVDSTPQ ENSDGMYRCG PASVQAIKHG 420
421 HVCFQFDAPF VFAEVNSDLI YITAKKDGTH VVENVDATHI GKLIVTKQIG GDGMMDITDT 480
481 YKFQEGQEEE RLALETALMY GAKKPLNTEG VMKSRSNVDM DFEVENAVLG KDFKLSITFR 540
541 NNSHNRYTIT AYLSANITFY TGVPKAEFFK ETFDVTLEPL SFKKEAVLIQ AGEYMGQLE 600
601 QASLHFFVTA RINETRDVLA KQKSTVLTIP EIIIKVRGTQ VVGSDMTVTV QFTNPLKETL 660
661 RNVVHLDGP GVTRPMKKMF REIRPNSTVQ WEEVCRPWVS GHRKLIASMS SDSLRHVYGE 720
721 LDVQIQRRPS M

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Figure 7: FXIII peptides observed by MALDI-MS after 10 minute pepsin digestion are underlined. The underlined peptides represent 40 % coverage.

coverage (Figure 7). These peptides focus on key FXIII regions found within the β -sandwich, the catalytic core, and the β -barrel 1 region. Additional studies with acid dependent type XIII protease did not significantly improve sequence coverage. All the MALDI spectra derived from pepsin digests were analyzed using Data Explorer (Applied Biosystems) and calibrated using two singly protonated reference peptides; monoisotopic mass 850.4787 Da (residues 535-541) and quadraisotopic mass of 1375.7097 Da (residues 220-230).

Deuterium incorporation for each isotopic cluster was quantified as described by Sabo *et al.* (67). To determine the change in deuteration for FXIII under different conditions the percent deuteration for each peptide was calculated using equation 1:

$$\%difference = \frac{(D - D_{FXIII})}{D_{max}} \times 100 \quad (1)$$

where D_{FXIII} is the amount of deuterium incorporated in FXIII zymogen, D is the amount of deuterium incorporation in FXIII under different conditions and D_{max} is the theoretical maximum amount of deuterium incorporation for the given peptide under these conditions. The theoretical maximum depends on the final percentage of D_2O under quench conditions (4.5 %) and accounts for all exchangeable backbone amide protons as well as a fraction of N-terminal, C-terminal and side chain exchangeable protons. In accordance with previous HDX data analysis, percent differences greater than 4.5 % are considered significant (45, 67-69), 3 – 4.5 % is moderate and < 3 % is modest.

Results

1 mM Divalent Metals and FXIII – HDX-MS

Human plasma contains 1-2 mM calcium, therefore the solvent accessibility of FXIII zymogen (without metals) was compared to that of FXIII in the presence of 1 mM Ca^{2+} , Mg^{2+} , Ba^{2+} or Cu^{2+} . All studies would thus be carried out in the presence of a physiological cation concentration environment. Prior knowledge about these divalent cations includes the following derived from studies carried out at concentrations from 0.1 to 10mM. Mg^{2+} is smaller in size than Ca^{2+} and does not promote the conformation-based fluorescence changes that are observed upon Ca^{2+} binding to FXIII (70). Mg^{2+} also does not support

Figure 8: The number of deuterons incorporated at 10 min for FXIII under the following conditions zymogenic (no metal) FXIII (black), 1 mM Ca^{2+} (up diagonal), 1 mM Mg^{2+} (grey), 1 mM Ba^{2+} (down diagonal) and 1 mM Cu^{2+} (white). The top of the graph illustrates regions of interest in FXIII. Error bars represent the standard deviation of the mean for 3 independent trials.

FXIIIa^{IIa} activity but does protect against thrombin-catalyzed proteolysis at the secondary cleavage site (57, 70). Larger in size than Ca²⁺, Ba²⁺ exhibits no protection against secondary cleavage and modest ability to support FXIIIa^{IIa} activity (33, 57). Cu²⁺ is a transition metal that is closer in size to Ca²⁺ and exerts no protection of the K513-S514 site but can support some FXIIIa^{IIa} activity (33, 57).

For the current conformational studies on FXIII A₂, Table 1 displays changes in deuterium incorporation of FXIII zymogen compared to FXIII-(Ca, Mg,

Table 1: Changes in Percent Deuteration: FXIII with 1 mM Metal Relative to FXIII Zymogen^a.

Residues	Theo D _{max} ^b	1 mM Ca ²⁺	1 mM Mg ²⁺	1 mM Ba ²⁺	1 mM Cu ²⁺
1-23	23.3	-19.1^c	-14.6	-22.9	-20.7
32-40	8.9	-12.7	-6.6	-10.9	-8.5
83-99	23.2	-0.4	-5.6	-6.3	-6.5
88-98	10.9	9.2	7.3	4.6	3.5
100-111	11.8	-4.1	-3.4	-5.0	-3.6
214-230	18.8	8.8	n/a ^d	6.2	2.5
220-230	12.3	5.6	0.7	4.3	3.1
240-247	7.5	5.3	2.2	6.3	8.7
248-264	15.9	2.9	3.3	3.6	4.0
248-265	18.8	2.1	0.3	1.4	2.8
300-314	15.8	8.7	0.8	3.0	-7.4
364-372	8.4	3.5	-1.0	-2.9	-2.3
407-424	18.8	3.6	4.0	9.7	-2.8
513-522	9.7	-9.0	-9.7	-11.3	-11.6
533-550	20.1	0.5	-6.0	-6.4	-6.8
632-646	14.7	3.2	-2.8	-1.9	-1.1

^a The % change for a particular peptide was calculated by the following equation: % difference = ((D - D_{FXIII})/D_{max}) × 100 %, where D is the amount of deuterium incorporated in FXIII with metal, D_{FXIII} is the amount of deuterium incorporated in the zymogenic state (no metal), and D_{max} is the theoretical maximum number of exchangeable protons within the indicated peptide. ^b The maximum number of exchangeable protons within the indicated peptide, assuming 100 % deuteration. This value accounts for all exchangeable backbone amide protons and a slight fraction of N-terminal, C-terminal, and side chain exchangeable protons, which are dependent on the final percentage of D₂O in solution under quench conditions (~4.5 %). A fully deuterated peptide would theoretically have acquired this amount of deuterons. ^c The values in bold represent significant changes in deuteration greater than ~4.5 %. ^d n/a refers to a peptide that was either not observed in the peptic digest or was not of sufficient intensity to quantify.

Ba and Cu). The total deuterium incorporated for each pepsin-derived peptide is displayed in Figure 8. Note that the top of the chart highlights the different FXIII regions where the peptides reside.

Within the β sandwich domain, the two main regions of interest are the activation peptide (1-37) and the putative Gln substrate recognition site (72-97), also known as peptide 4. After 10 minutes of HDX, the activation peptide became more protected from solvent relative to its values in the zymogen state of FXIII. Such protection was observed for all the metals tested and ranged from -6.6 % for FXIII-Mg to -22.9 % for FXIII-Ba. The peptide 4 segment (71), is represented by the pepsin derived peptides 83-99 and 88-98. For peptide 83-99, changes in deuteration ranged from -0.4 % for FXIII-Ca to -6.5 % for FXIII-Cu. By contrast, the region 88-98 became more exposed in the presence of all metals ranging from 3.5 % for FXIII-Cu to 9.2 % for FXIII-Ca. The different responses for 83-99 versus 88-98 suggest that the amide protons from residues 83-87 and/or 99 may be responsible for the HDX protection observed for 83-99 in the presence of divalent metals (Figure 8). Further support for the importance of residue 99 comes from the observation that the next FXIII segment 100-111 exhibits solvent protection in the presence of all the cations tested.

The catalytic core contains several regions of interest. Peptide 7 (190-230), another putative glutamine-substrate region (71), displayed increased deuterium incorporation for metals when compared to zymogen. FXIII-Ca was the most exposed for peptides 214-230 and 220-230 followed by FXIII-Ba, FXIII-Cu, and finally FXIII-Mg. The dimer interface between the two FXIII-A monomers

(residues 240-265) was another region where increases in deuterium incorporation for all the metals was observed. Exposure within the dimer interface (240-247) for FXIII-(Ca, Mg, Ba and Cu) when compared to zymogen was 5.3, 2.2, 6.3, and 8.7 % respectively. As with the peptide 7 region, FXIII-Mg exhibited the smallest effect. Interestingly, protection from solvent (-3.4 to -5.0%) was observed for the residues 100-111. The C-terminal portion of this segment is located at the dimer interface; however, the more N-terminal portion is found in a β -sandwich region.

The catalytic C314 is found within segment 300-314 and the catalytic H373 is found one residue outside 364-372. For the 364-372 segment containing the H373, 1 mM Ca^{2+} showed a moderate 3.5 % increase in HDX exposure whereas the other metals all showed a modest protection. By contrast, FXIII-Ca displayed considerable exposure (8.7 %) for 300-314 when compared to FXIII-(Mg and Ba) with 0.8 % and 3.0 % exposure, respectively. FXIII-Cu was the only condition found to be protected in this region (-7.4 %). FXIII-Cu also became modestly protected in the region of 407-424 which is N-terminal to the FXIII Ca^{2+} binding site (-2.84 %) and makes contacts across the dimer interface. FXIII-(Ca, Mg, and Ba), however, displayed increases in deuterium incorporation for this segment. The 407-424 segment may be influenced by a nearby non-prolyl cis peptide bond that is proposed to play a role in FXIII activation (50).

The β -barrels contain the secondary cleavage site (513-514) and the lysine recognition site (646-658) (72-73). FXIII-(Ca, Mg, Ba and Cu) all displayed protection within 513-522 when compared to zymogen (-9.0 to -11.6%). The

nearby FXIII 533-550 is not influenced by 1 mM Ca whereas it is significantly solvent protected in FXIII-(Mg, Ba, and Cu) at levels of -6.0 to -6.8%. Peptide 632-646 is on the N-terminal end of the proposed lysine recognition site and is moderately exposed in FXIII-Ca (3.6 %) (73). All other conditions showed little if any protection in this region of β -barrel 2.

TGase Activity of Nonproteolytically Activated FXIII

FXIII was nonproteolytically activated under a series of conditions and TGase activity monitored using a modified version of the Dade-Behring

Figure 9: TGase activity of nonproteolytically activated FXIII. Samples were incubated at 37 °C for 10 minutes then added to the assay components. At 2 minutes (12 min total) the K9 substrate was added to the assay and the oxidation of NADH is depicted as a function of time. The experimental groups are as follows: 50 mM Cu²⁺ with 2 mM Ca²⁺ (0.0 μM/min), 50 mM Ba²⁺ with 2 mM Ca²⁺ (1.98 μM/min), 50 mM Mg²⁺ with 2 mM Ca²⁺ (9.5 μM/min) and 50 mM Ca²⁺ (10.7 μM/min).

Berichrom assay (64). When incubated in the presence of several different divalent metals including 50 mM Ca²⁺, Mg²⁺, Ba²⁺ or Cu²⁺, only Ca²⁺ displayed activity (data not shown). After changing the activation conditions to include 2 mM Ca²⁺ with 50 mM Mg²⁺, Ba²⁺ or Cu²⁺, minor activity was observed in Mg²⁺ and Ba²⁺ but not Cu²⁺ (Figure 9). Factor XIII activity was then monitored using a series of other nonproteolytic techniques. FXIIIa^{Ca} was compared to FXIII activated in the presence of 500 mM EDA²⁺ (FXIIIa^{EDA}) with 2 mM Ca²⁺, 500 mM

Na⁺ with 2 mM Ca²⁺ (FXIIIa^{Na}) and 500 mM TMAC⁺ with 2 mM Ca²⁺ (FXIIIa^{TMAC}). Using the slope (μM NADH consumed / min) as an indicator of activity, EDA²⁺, Na⁺ and TMAC⁺ displayed an increase in FXIII activity of 291 ± 27.7 %, 156.7 ± 16.3 % and 194.8 ± 5.5 %, respectively when compared to FXIIIa^{Ca}.

Nonproteolytically activated FXIIIa – HDX-MS

The conformational dynamics of nonproteolytically activated FXIII were also probed using HDX-MS. Due to peak suppression in the MALDI, it was not possible to quantify the deuterium incorporated into FXIIIa^{EDA} and FXIIIa^{TMAC}. Table 2 thus displays changes in deuterium incorporation of FXIII zymogen compared to FXIII in 2 mM Ca²⁺, FXIIIa^{Ca} and FXIIIa^{Na}. The total deuterium incorporated in each peptide is displayed in Figure 10.

Table 2: Changes in Percent Deuteration: Nonproteolytically Activated FXIII Relative to FXIII Zymogen^a.

Peptide	Theo D _{max} ^b	2 mM Ca ²⁺	50 mM Ca ²⁺	50 mM Na ⁺ with 2 mM Ca ²⁺
1-23	23.3	-16.3^c	-8.3	n/a
32-40	8.9	-12.1	-12.7	-12.7
88-98	10.9	-0.9	-1.9	-1.3
100-111	11.8	-4.5	-6.2	-5.9
220-230	12.3	9.3	13.2	17.1
240-247	7.5	7.2	18.2	19.8
248-264	15.9	1.4	n/a ^d	2.5
248-265	18.8	4.7	2.2	4.5
300-314	15.8	7.1	7.7	11.7
407-424	18.8	0.2	-0.1	-0.8
513-522	9.7	0.0	1.7	1.9
533-550	20.1	2.6	16.8	n/a

^a The % change for a particular peptide was calculated by the following equation: % difference = ((D - D_{FXIII})/D_{max}) × 100 %, where D is the amount of deuterium incorporated in FXIII with metal, D_{FXIII} is the amount of deuterium incorporated in the zymogenic state (no metal), and D_{max} is the theoretical maximum number of exchangeable protons within the indicated peptide. ^b The maximum number of exchangeable protons within the indicated peptide, assuming 100 % deuteration. This value accounts for all exchangeable backbone amide protons and a slight fraction of N-terminal, C-terminal, and side chain exchangeable protons, which are dependent on the final percentage of D₂O in solution under quench conditions (~4.5 %). A fully deuterated peptide would theoretically have acquired this amount of deuterons. ^c The values in bold represent significant changes in deuteration greater than ~4.5 %. ^d n/a refers to a peptide that was either not observed in the peptic digest or was not of sufficient intensity to quantify.

To ensure activity was achieved during HDX, the same activation conditions used to confirm nonproteolytic activity were utilized for HDX analysis, except D₂O was substituted for H₂O. The extent of deuterium incorporation may vary relative to previous work since this HDX project was conducted at 37°C. When activated in the presence of 50 mM Ca²⁺ or in 500 mM Na⁺ with 2 mM Ca²⁺, the β sandwich activation peptide of FXIIIa^{Ca} and FXIIIa^{Na} was more protected than in FXIII zymogen. This effect was seen in the -12.7 % change found for residues 32-40 in both FXIIIa^{Ca} and FXIIIa^{Na}. Residues 100-111 fall within 4 Å of the dimer interface and they too displayed protection of -6.2 % and -5.9 % for FXIIIa^{Ca} and FXIIIa^{Na}, respectively when compared to zymogen.

Peptide 7 within the catalytic core became notably exposed when nonproteolytically activated leading to a 13.2 % increase in deuterium incorporation for FXIIIa^{Ca} and a 17.1 % increase for FXIIIa^{Na} at residues 220-230. The FXIII dimer interface (240-247) within the catalytic core experienced an 18.2 % (FXIIIa^{Ca}) and 19.8 % (FXIIIa^{Na}) increase in solvent accessibility when activated. Increased accessibility was also evident within the catalytic cysteine containing segment (300-314). This region changed 7.7 % and 11.7 % for FXIIIa^{Ca} and FXIIIa^{Na} respectively when compared to zymogen (Table 2). These various changes in solvent exposure were all greater than what could be observed under a more physiological 1-2 mM Ca²⁺ concentration. Such results suggest that these two FXIII regions (peptide 7 and dimer interface) become more solvent accessible as the cation concentration is increased (Figure 5). In a Na⁺-containing environment, low mM calcium is required for activation.

Events occurring within the β barrels are also important to consider.

Unlike the studies done in the presence of 1 mM Ca^{2+} , no substantial changes in solvent exposure were observed for β -barrel 1 residues 513-522 under the nonproteolytic activation conditions at 37 °C. By contrast, accessibility to solvent increased steadily from 2 to 50 mM Ca^{2+} for the neighboring segment 533-550 (2.6 to 16.8%). Within β -barrel 2, peptide 632-646 is located N-terminal to the lysine recognition region (646-658) and exhibited an increased deuterium incorporation of 6.2 % with $\text{FXIIIa}^{\text{Na}}$.

Figure 10: The number of deuterons incorporated after 10 min at 37 °C for nonproteolytically activated FXIII. Deuterium exchange was monitored under the following conditions zymogenic (no metal) FXIII (black), 2 mM Ca^{2+} (up diagonal), $\text{FXIIIa}^{\text{Ca}}$ 50 mM Ca^{2+} (grey), $\text{FXIIIa}^{\text{Na}}$ 500 mM Na^+ with 2 mM Ca^{2+} (whites). The top of the graph illustrates regions of interest in FXIII. Error bars represent the standard deviation of the mean for 3 independent trials.

Discussion

Calcium plays a vital role in the activation and regulation of cellular (A_2) and plasma (A_2B_2) FXIII (33, 57-58, 74). HDX provides an effective solution-based approach for dissecting out how Ca^{2+} and other divalent and monovalent species (Mg^{2+} , Ba^{2+} , Cu^{2+} , Na^+ , EDA^{2+} , $TMAC^+$) participate in the conformational dynamics of this transglutaminase.

Impact on Ca^{2+} binding site and surrounding regions

The FXIII ion binding helix (485-501) is located within 10 Å of β -barrel 1 and also makes contact with the other FXIII-A monomer across the dimer interface (42, 55, 75). See golden helix in Figure 11. The primary calcium binding site involves FXIII residues Asn436, Asp438, Ala457, Glu485 and Glu490 (Figure 11 pink sticks) and site-directed mutagenesis studies have demonstrated that when the glutamate residues are removed, FXIII sensitivity to Ca^{2+} decreases (27, 55-56). According to Lewis *et al.*, when Ca^{2+} concentrations are < 2.5 mM, there is one Ca^{2+} bound per FXIII-A, but there are up to 8 low affinity sites when the concentration is raised above that threshold (70). The presence of strong and weak Ca^{2+} binding sites has also been confirmed by ^{43}Ca NMR studies (76). Our studies to determine the conformational dynamics of FXIII A_2 in 1 mM metal were performed to mimic physiological Ca^{2+} concentrations and to monitor events where a single Ca^{2+} should target the higher affinity metal site on FXIII. The other divalent metals were examined under these same 1 mM concentration values.

Although sequence coverage was not observed for the FXIII calcium binding site, 40 % of the enzyme including a segment containing (513-514), a secondary cleavage site that is protected from hydrolysis in the presence of calcium was observed (57). This site of proteolytic degradation (red sticks) is located just 12 residues down from the ion binding helix (485-510). See Figure 11. All metals tested at 1 mM concentration (Ca^{2+} , Mg^{2+} , Ba^{2+} , and Cu^{2+}) demonstrated solvent protection around peptide 513-522. This finding was surprising considering that Mary *et al.* reported Ba^{2+} and Cu^{2+} provided no protection against 2° cleavage (57).

Unlike the 513-522 region, 1 mM Ca^{2+} did not show any deuterium protection for the nearby 533-550 segment (Figure 11 purple) which is part of an anti-parallel β -sheet that runs adjacent to 513-522. By contrast, the other divalent metals Mg^{2+} , Ba^{2+} and Cu^{2+} all showed substantial protection within 533-550 when compared to FXIII in 1 mM Ca^{2+} . The HDX results suggest that Ba^{2+} and Cu^{2+} exert a greater influence on 533-550 with additional effects on 513-522; however, these events are not sufficient to protect FXIII from proteolysis. 1 mM Ca^{2+} may exert a more direct influence on the secondary cleavage site 513-514.

The ability of 1 mM Mg^{2+} , Ba^{2+} and Cu^{2+} to influence FXIII dynamics brings up the question of whether such divalent metals could support nonproteolytically-derived TGase activity as already observed with Ca^{2+} . Solutions containing 50 mM Mg^{2+} or Ba^{2+} , however, all required the added presence of 2 mM Ca^{2+} to produce FXIII activity following 10 minutes of nonproteolytic activation at 37°C. Furthermore, their levels of enzymatic activity were all less than those for 50 mM

Ca^{2+} . By contrast, Factor XIII in 50 mM Cu^{2+} , even in the presence of 2 mM Ca^{2+} , was not able to yield TGase activity. From these studies, it is evident that a series of divalent metals can support FXIII activation and TGase activity but low mM Ca^{2+} is still needed to occupy what is likely the high affinity ion binding site on FXIII.

Figure 11: Illustration of the regions within the catalytic core and the Ca^{2+} binding site mentioned in the conformational dynamics of FXIII A₂ (1F13). FXIII A₂ (inset) has each domain labeled: β -sandwich (blue), catalytic core (red), β -barrel 1 (green) and β -barrel 2 (yellow) with the box representing the region of interest. The Ca^{2+} binding site (pink) and the ion binding helix (gold) are connected to the secondary cleavage site 513-514 (red sticks) within peptide 513 to 522 (dark blue) through the (green) connector. The influence on the catalytic triad (light blue sticks) is seen through the (green β -sheet) connector between peptide 533-550 (purple) and Tyr560 (yellow). FXIII monomer A is (black) and monomer B is (silver). Image constructed utilizing VMD (51).

As mentioned previously, the FXIII segment 513-522 exhibits a decrease in deuteration in the presence of 1 mM Ca^{2+} and no change is observed for the segment 533-550. These results led to an interest in monitoring FXIII conformational dynamics after incubation in an environment of 2-50 mM Ca^{2+} at physiological 37°C. Curiously, the protective effect on 513-522 was lost and replaced with a steady increase in the exposure of 533-550. In the X-ray crystals, the FXIII active site is occluded by Y560 (Figure 11 yellow sticks) which resides only 10 residues from 533-550. Steady increases in Ca^{2+} concentration

might promote movement of Y560 thus assisting C314 exposure to substrate. X-ray crystallographers have previously predicted that β -barrel 1 must move somewhat away from the catalytic core during the activation process (42). Moreover, Y560 will need to be displaced from its H-bonding interaction with catalytic C314. Since the active site region becomes more exposed at higher Ca^{2+} levels, it is possible the FXIII 533-550 segment contributes a line of communication to this process. In further support of this proposal, Andersen and Faber observed their own increases in HDX based solvent exposure for FXIII peptides spanning 533-551, 556-559, and 560-573 upon formation of FXIII^{Ca} (46).

Metal Influence on Dimer Interface

The ion binding helix has direct interactions with the FXIII A_2 dimer interface. According to surface calculations, the dimer interface covers $2280 \text{ \AA}^2$ and there is a 10.3 – 14.4 kcal/mol decrease in FXIII-A free energy upon

Figure 12: An illustration of all peptic peptides which yield sequence coverage along the dimer interface (100-111, 240-265 and 407-424) are labeled (red) for FXIII monomer A and (gold) for monomer B. Image constructed utilizing VMD (51).

dimerization (36, 42). Key interactions between the two FXIII-A monomers are found between K113-D367', K257-E401' and R260-D404'. See Figure 12 for HDX coverage of the dimer interface. The exposure reported previously (43) around the dimer interface for peptide 240-247 is also seen in the presence of just 1 mM Ca^{2+} . This dimer interface exposure is further supported by increases in deuterium incorporation now recorded for 248-264 and 248-265. Solvent exposure around the dimer interface is also observed with 1 mM Ba^{2+} and Cu^{2+} but to a lesser extent by Mg^{2+} . Although 100-111 is also positioned along the dimer interface, this peptide segment exhibits protection from deuterium exchange. Only the most C-terminal portion is actually along the dimer interface. The remaining segment is directed into the β -sandwich region which exhibits protection. These results indicate that a series of different divalent ions can disturb interactions between the two FXIII-A monomers. Such events occur even at Ca^{2+} levels insufficient to support FXIII activation.

Further increases in solvent accessibility along the A_2 dimer interface (aa 240-264) could be observed when the FXIII was nonproteolytically activated at 37°C with 50 mM Ca^{2+} (FXIII^{Ca}) or 500mM Na^+ / 2mM Ca^{2+} (FXIII^{Na}). Under different HDX conditions, this same region was seen to increase in accessibility when activated by IIa as well as when inhibited by IAA and K9 DON (43). The disruption along the dimer interface due to increasing levels of cations may also be due to the noncanonical Ca^{2+} binding site reported for TG2 (S1 226-233) which is homologous to 264-271 found on the dimer interface of FXIII (77). Interestingly, a Yb binding site (Figure 13 green) has been shown by X-ray

crystallography to be in the vicinity of FXIII residue 271 (42). Yee et al. proposed that an opening in the FXIII A₂ dimer interface may allow for the lysine acyl-acceptor to access the catalytic core (27).

Catalytic C314 Region and Substrate Recognition

The exposure of the catalytic C314 is imperative for FXIII activity. Of the metals tested at 1 mM, Ca²⁺ promoted the largest increase in solvent accessibility for the FXIII 300-314 region. See orange peptide in Figure 13. Ba²⁺ exerted a mild increase on this region and Mg²⁺ remained essentially the same as zymogen. Cu²⁺ was unique in that it demonstrated substantial protection of 300-314 when compared to zymogen. These HDX results substantiate the lack of TGase activity observed in the presence of Cu²⁺ and suggest that the observed solvent protection might thwart substrate interactions around the catalytic cysteine.

Figure 13: Illustration of the substrate recognition regions and dimer interface mentioned in the conformational dynamics of FXIII A₂ (1F13). FXIII A₂ (inset) has each domain labeled: β -sandwich (blue), catalytic core (red), β -barrel 1 (green) and β -barrel 2 (yellow) with the box representing the region of interest. The sequence coverage around the peptide 7 (cyan) the catalytic triad (red) and their close proximity to the dimer interface and the Yb binding site 270-272 (green). Pepsin peptide 300-314 is (orange) and 407-424 is (magenta). FXIII monomer A is (black) and monomer B is (silver). Image constructed utilizing VMD (51).

Additional HDX results at physiological temperature revealed that 2 mM Ca^{2+} had nearly the same effect on exposure of the 300-314 region as activating FXIII with 50 mM Ca^{2+} (FXIIIa^{Ca}). Such results suggest that low mM Ca^{2+} contribute to initial exposure of C314. Further conformational changes likely located within the catalytic core, the dimer interface, and/or β -barrel 1 must provide the vital link to achieve full FXIII activation and thus ability to target actions at C314.

Interestingly, segment 407-424 exhibits increased deuteration at 1mM Ca^{2+} but loses this solvent exposure when the temperature is raised to 37°C and then 2-50 mM Ca^{2+} is employed. The 407-424 segment may be influenced by a nearby non-prolyl cis peptide bond Q425-F426 that is in proximity to the dimer interface. Earlier work by our group (60) demonstrated that that FXIII C409 could be alkylated with NEM when the enzyme was nonproteolytically activated with 50 mM Ca^{2+} and then re-equilibrated back to 1 mM. This ability was, however, lost when the exposed FXIII active site was blocked with an inhibitory Q-containing peptide (43). Weiss and coworkers proposed that conversion of a FXIII non-prolyl cis peptide bond to the more energetically favored trans could help drive larger scale conformational changes to fully expose the FXIII active site region (50). The current increases in Ca^{2+} concentration and temperature may have aided in establishing an environment that helps promote the next alterations in FXIII conformation. Such an environment may contain a more solvent protected FXIII 407-424 segment.

Peptide 4 (72-97) in the β -sandwich and peptide 7 (190-230) in the catalytic core are proposed glutamine substrate recognition regions (71). Peptide 7 is represented by the cyan peptide in Figure 13. FXIII A₂ in 1 mM Ca²⁺ displayed enhanced solvent accessibility relative to zymogen for both 88-98 and 214-230. This FXIII exposure around the glutamine recognition region also appears to be Ca²⁺ specific as it induces the greatest effect compared to the other metals. By contrast, Cu²⁺ exhibited the smallest amount of exposure and thus appears to be least likely to allow for glutamine recognition. Within peptide 4, there are differences in solvent exposure depending on the metal tested. In the peptide segment 83-99, the percent incorporation with 1 mM Ca²⁺ is essentially the same as with zymogen whereas Mg²⁺, Ba²⁺, and Cu²⁺ show protection. Within the peptide segment 88-98, Ca²⁺ promoted more exposure than Mg²⁺ and Cu²⁺. Another region of interest is the Lysine recognition site 646-658 in β -barrel 2 (72). A peptide located N-terminally to this site, 632-646, is only exposed in the presence of 1 mM Ca²⁺, whereas Mg²⁺, Ba²⁺ and Cu²⁺ all display protection in this region.

The proposed glutamine substrate binding site, peptide 7 (190-230), is located in close proximity to the Ca²⁺ binding site and comes within 3 Å of a loop which connects D438 to E485 and E490 within the Ca²⁺ binding pocket. For the nonproteolytically activated forms FXIIIa^{Ca} and FXIIIa^{Na}, the residues 220-230 exhibited further increases in deuterium incorporation in comparison to the 1-2 mM Ca²⁺ conditions. The peptide 7 substrate recognition sequence is another example of a FXIII region that undergoes initial conformational changes at

physiological Ca^{2+} levels and then exhibits greater exposure at higher ion concentrations.

Low mM Metals: Activation Peptide

The activation peptide (1-37) is cleaved by IIa during physiological activation. Residues 1-23 became protected in the presence of 1 mM Ca^{2+} as well as 1 mM Mg^{2+} , Ba^{2+} and Cu^{2+} . The activation peptide was also protected after nonproteolytic activation with 50 mM Ca^{2+} at 37°C, albeit somewhat less than 1 mM Ca^{2+} at room temperature. Both the FXIII 1-23 and 32-40 segments exhibited protection in the HDX studies.

Figure 14: Illustration of the activation peptide and its intimate contact with the opposing FXIII-A monomer (1F13). FXIII A₂ (inset) has each domain labeled: β -sandwich (blue), catalytic core (red), β -barrel 1 (green) and β -barrel 2 (yellow) with the box representing the region of interest. Depiction of the N-terminal activation peptide of FXIII monomer B (silver surface) labeled according to residue type (green = polar, white = nonpolar, red = acidic and blue = basic) and Arg11 and Arg12 are shown as (blue) sticks. The AP crosses the dimer interface and Asp373' from FXIII monomer A is hydrogen bonded to Arg11 in close proximity to the catalytic triad (yellow) and Tyr560 (orange). Image constructed utilizing VMD (51).

It was reported recently that the FXIII AP is free in solution after IIa cleavage and available for binding to monoclonal antibodies specific to FXIII-AP (78). Our HDX results suggest that in the presence of Ca^{2+} the unhydrolyzed AP remains tightly associated with FXIII A₂. Figure 14 shows how the activation

peptide segment straddles across both monomer units and that this segment contains a combination of polar and nonpolar residues. Moreover, there are acidic and basic side chains that can participate in stabilizing interactions with the FXIII surface. Interestingly, R11 (Figure 14 blue sticks) from the activation peptide segment is hydrogen bonded to D373' on the opposite unit of the dimer. This R11 – D373' interaction is also in close proximity to the FXIII catalytic triad and the vital Y560. Other hydrogen bonds also seem to anchor the FXIII AP above the catalytic core.

Earlier studies by Lewis *et al* reported that an additional 8 weak metal sites could be found on FXIII at higher Ca^{2+} concentrations. The AP region and/or a complementary segment on the β -sandwich domain may accommodate some of these divalent ions (70). Further studies will be needed to assess whether the AP segment may aid in channeling Gln substrates towards the catalytic core. An alternative possibility is that the binding of large physiological substrates will later assist in displacing this cleaved portion of FXIII from the vicinity of the transglutaminase active site.

Further Characterizing Nonproteolytic Activation

Polgar *et al.* demonstrated that FXIII A₂ can be activated albeit slowly by 150 mM NaCl and 2 mM Ca^{2+} (40). More efficient activation occurs when NaCl is raised to the 500 – 1000mM level. Low calcium, however, is still necessary. In the current work, the organic mono and divalent cations, TMAC⁺ and EDA²⁺, were investigated for the first time. The pH of the TGase activity assay is 7.4 and EDA²⁺ pK_{a1} is 7.56 and pK_{a2} is 10.71 ensuring a mostly divalent (60 %) EDA²⁺

ion. As observed with Na^+ , TMAC^+ and EDA^{2+} each required some calcium to elicit FXIII transglutaminase activity. Curiously, TGase activity for the TMAC^+ , Na^+ , and EDA^{2+} conditions (all containing 2 mM Ca^{2+}) were greater than that of 50 mM Ca^{2+} , our standard nonproteolytic activation method. Such results further emphasize the important role of Ca^{2+} . Moreover, the novel use of organic cations EDA^{2+} and TMAC^+ suggests that activation is not only driven by metal ion binding interactions. Unfortunately, ionization issues in the MALDI hindered ability to carry out HDX projects with $\text{FXIIIa}^{\text{EDA}}$ and $\text{FXIIIa}^{\text{TMAC}}$.

Overview of Results:

In the current project, the FXIII A_2 was monitored in the presence of Ca^{2+} , Mg^{2+} , Ba^{2+} , Cu^{2+} , Na^+ , EDA^{2+} , and TMAC^+ with concentrations ranging from 1-2 mM up to 50-500 mM for nonproteolytic activation. Several regions were found where physiological Ca^{2+} concentrations initiate the changes needed for activity. As expected, 1 mM Ca^{2+} does lead to solvent protection for the FXIII region containing the 513-514 secondary cleavage site. Surprisingly, Ba^{2+} and Cu^{2+} could protect FXIII 513-522 and 533-550 from solvent even though these two divalent cations do not shield against secondary cleavage (57). By contrast, increasing concentrations of Ca^{2+} promoted exposure of 533-550. Such Ca^{2+} induced effects may play a role in exposing the FXIII reactive thiol group by helping to disrupt the H-bond between C314 and Y560. When considering other cations, Mg^{2+} and Ba^{2+} could both promote nonproteolytic activation in the presence of low mM Ca^{2+} whereas Cu^{2+} could not. The presence of Cu^{2+} seems to hinder exposure of the active site C314 as well as the Gln substrate

recognition region. Our HDX studies also revealed that 1 mM Ca^{2+} increased deuterium incorporation at the dimer interface, around the lysine recognition region (632-646), and at two glutamine substrate recognition regions, peptide 4 (83-99) and peptide 7 (214-230). These trends continued in nonproteolytically activated FXIII.

There has been much speculation about whether FXIII A₂ can adopt an open conformation similar to that reported by Pinkas et al. for TG2 (49). Conformational changes to activated FXIII A₂ can clearly be observed by mass spectrometry approaches. The gross increases in solvent accessibility that would be expected if the β -barrels had pulled fully away from contacting the catalytic core surface, however, are not observed. The fact that FXIII functions as a dimer may be a contributing element to the difference in behavior of TG2 and FXIII-A₂. The dynamic nature of the FXIII in solution is also important to consider.

Calcium appears to play an intriguing regulatory role in FXIII (33, 56, 58, 79). Low mM levels already begin to initiate important conformational changes needed to expose the active site and make the enzyme ready to accommodate incoming substrates. Often as divalent cation concentration increases, the effects are also enhanced. Physiologically, FXIII is poised to respond to transient influxes of Ca^{2+} in the presence of a Na^+ containing environment (40, 52-53). Platelet FXIII A₂ is quite sensitive to such changes in solution environment and can be more readily activated nonproteolytically (40, 52-53). The initial conformation events that have been observed in the current study help set the

stage for the larger conformational changes that are anticipated in the presence of a physiological substrate or inhibitor.

CHAPTER III

COMPARING THE SOLVENT ACCESSIBILITY OF FXIII-A₂ TO THE OPEN AND CLOSED FORM OF TRANSGLUTAMINASE 2.

Introduction

Very few structural changes have been observed in FXIII when comparing the crystal structures of proteolytically activated and nonproteolytically activated FXIII to zymogen. TG2 on the other hand was recently crystallized bound to an inhibitory peptide substrate in an 'open' conformation (49, 51). Comparing these two enzymes in solution will allow for a much better judgment as to whether the

Figure 15: Structure of GDP-Bound and Inhibitor-Bound TG2 and FXIII-A₂ Dimer. The N-terminal β -sandwich is in blue, catalytic core in green, and the C-terminal β -barrel 1 and β -barrel 2 in yellow and red respectively. A- GDP-bound (closed) TG2 (1KV3). B- TG2 inhibited (open) with substrate peptide inhibitor Ac-P(DON)LPF-NH₂ (2Q3Z). C- Factor XIII-A₂ dimer (1F13).

FXIII-A₂ dimer also goes through an 'open' conformation like the TG2 monomer when activated.

Comparing the conformational dynamics of FXIII to that of TG2 offers a unique opportunity. Although TG2 is physiologically a monomer, it contains the same 4 domains (β -sandwich, catalytic core, β -barrel 1 and β -barrel 2) as well as the same general shape as a FXIII-A monomer. As with FXIII, TG2 is proposed to go through a major conformational change during activation or substrate binding, but unlike FXIII, TG2 bound to substrate has now been crystallized in an open conformation (Figure 15 (49, 80)). TG2 is activated in the presence of millimolar Ca²⁺ but unlike FXIII it is inhibited by guanine nucleotides. The guanine nucleotide binding site is located between the catalytic core and β -barrel 1. When GTP is bound, the active site is blocked by two loops and the catalytic cysteine (C277) is H-bonded to Y516 a neighboring tyrosine (81). In the absence of Ca²⁺, the GTPase activity of TG2 utilizes several G protein-coupled receptors to mediate intracellular signaling (21, 25).

The first step in comparing the solvent accessibility of FXIII to that of TG2 is identifying the peptides generated during TG2 pepsin digestion. Pepsin is a non-specific specific enzyme; therefore, it is difficult to predict cleavage products but their cleavage pattern is very reproducible when conducted under the same conditions (pH, Temp., etc.). Once the peptic peptides were identified and sequence coverage determined, HDX was used to determine the conformational dynamics of TG2 while unactivated (zymogen), activated in the presence of Ca²⁺ and substrate inhibited.

Although Hausch was the first to inhibit transglutaminase activity by replacing the substrate reactive glutamine with 6-diazo-5-oxo-L-norleucine (DON), Sabo *et al.* was the first to utilize DON to monitor the conformational dynamics of a transglutaminase with substrate bound (43, 82). DON is an electrophilic amino acid which acts as an inhibitor by covalently binding to the catalytic cysteine via a thioether linkage (Figure 16). The substrate peptide K9 (Ac-LGPG(DON)SLVIG-OMe) was synthesized and utilized to characterize the

Figure 16: K9 DON Inhibition of FXIIIa. **A.** The isosteres glutamine and 6-diazo-5-oxonorleucine. **B.** The proposed mechanism of inhibition proceeds through tetrahedral intermediate formation resulting from cysteine attack at the carbonyl group. The methylene carbon then abstracts a proton from the active site H373. Finally, a thioether is formed resulting in the release of N₂ and irreversible inhibition of FXIIIa(43, 80).

conformational dynamics of FXIII-A₂ when activated and bound to inhibitor (43). HDX was utilized in the current study to monitor TG2 conformational dynamics during activation as a function of calcium concentration and when bound to TG-DON, a gluten peptide (Ac-P(DON)LPF-NH₂) inhibitor substrate. Surprisingly there was not a gross increase in deuterium incorporation across TG2. Within the catalytic core, several regions, 451-461 and 369-377, became protected when activated and inhibited. The regions 259-269 and 475-487 became more exposed only when TG2 was in an open conformation bound to inhibitor substrate. There are antibodies already available that are specific for the open conformation of TG2 and the open conformation is being targeted for pharmaceutical design. The knowledge gained in understanding the regions of TG2 that become more accessible to solvent when in the open conformation will assist in determining whether FXIII-A₂ undergoes a similar open conformation that has been reported for TG2 (49).

Materials and Methods

Transglutaminase 2 and TG-DON Preparation

Transglutaminase 2 was purchased from Zedira (Darmstadt, Germany). Lyophilized recombinant human TG2 (1 mg) was reconstituted in 18 M Ω deionized water, then buffer exchanged into 25 mM Tris-base at pH 7.4. The concentration of TG2 was determined on a Cary 100 UV/vis spectrophotometer. The absorbance was monitored at 280 nm and concentration calculated with the TG2 extinction coefficient of 106,020 M⁻¹cm⁻¹. The extinction coefficient was determined using Expasy ProtParam tool (<http://web.expasy.org/protparam/>). Aliquots (12 μ l) of 50 μ M TG2 in 25 mM Tris-base were stored at -70 °C until future use.

The gluten derived TG2 inhibitory peptide TG-DON (Ac-P(DON)LPF-NH₂) was purchased from Zedira (Darmstadt, Germany). The lyophilized powder (10 mg) was solubilized in 1.5 ml DMSO and a sample was sent to AAA Service Laboratories (Damascus, OR) for analysis. The TG-DON concentration was determined to be 8.5 mM and stored at 4°C. Purity was assessed by HPLC and mass spectrometry approaches.

The α_2 antiplasmin (α_2 AP-Q4N) derived substrate peptide (¹NQENVSP¹⁵LTLLKLG¹⁵N) was synthesized by SynPep Corp (Dublin, CA). The α_2 AP peptide was reconstituted in 18 M Ω deionized water and the concentration (6.8 mM) was confirmed by quantitative amino acid analysis (AAA Service Laboratory, Inc., Boring, OR). Purity was assessed by HPLC and mass spectrometry approaches.

TG2 Transglutaminase Activity Assay

TG2 activity was determined using a modified version of the Dade-Behring Berichrom Assay (62-63). Briefly, this assay utilizes a coupled reaction involving both TG2 and glutamate dehydrogenase (GDH). Activated TG2 reacts with the acyl-donor α_2 AP-Q4N releasing NH_3 and the transglutaminase reaction concludes when the primary amine acyl-acceptor, glycine ethyl ester, forms an isopeptide bond with the α_2 AP-Q4N peptide. This transglutaminase activity is monitored via the conversion of α -ketoglutarate and NH_3 into glutamate in the presence of reducing equivalents of NADH. The oxidation of NADH results in decreased absorbance at 340 nm.

TG2 aliquots were diluted to 10 μM in 25 mM Tris. For each assay, TG2 (200 nM) was in solution with activator reagent (163 μl), detector reagent (250 μl) and CaCl_2 (10 mM) (64). For monitoring TG-DON inhibition, TG-DON was also added (0.01 – 1000 nM). The assay contents were placed in a Cary 100 UV/vis spectrophotometer and incubated for 8 min at 37 °C for before the α_2 AP-Q4N substrate peptide was added. Oxidation of NADH was monitored for 30 minutes at 340 nm and the activity was determined by the steepest part of the slope. The slope represents the velocity of the reaction as $\Delta\text{abs}/\text{min}$. TG-DON is solubilized in DMSO, therefore a control experiment was run to confirm that 2 % DMSO did not affect activity of TG2 (data not shown). In previous studies, Factor XIII activity also was not affected with 2 % DMSO (43).

HDX Experimentation

Hydrogen deuterium exchange was utilized to elucidate regions of TG2 that become more accessible to solvent when activated and inhibited. A 12 μl aliquot of 50 μM TG2 was allowed to come to room temperature. Due to TG-DON being solubilized in DMSO, all samples were adjusted to the same concentration of DMSO. TG2 zymogen was prepared by adding 1.0 μl dH_2O and 0.3 μl DMSO to a 12 μl aliquot of 50 μM TG2 for an assay concentration of 45 μM TG-2 in 2 % DMSO. For Ca^{2+} activated TG2, 1.0 μl 26.6 mM CaCl_2 and 0.3 μl DMSO were added to a 12 μl aliquot of 50 μM TG2 for an assay concentration of 45 μM TG2 in 2 mM Ca^{2+} and 2% DMSO. Lastly, the activated and inhibited TG2 was prepared by adding 1.0 μl 26.6 mM CaCl_2 and 0.3 μl 8.5 mM TG-DON to a 12 μl aliquot of 50 μM TG2 for an assay concentration of 45 μM TG2 in 2 mM Ca^{2+} , 191 μM TG-DON and 2% DMSO. Each sample was incubated at 37 $^\circ\text{C}$ for 10 min. then evaporated to dryness in a CentriVap Centrifugal Vacuum Concentrator (Labconco, Kansas City, MO) and placed at -70 $^\circ\text{C}$ until use.

The following HDX protocol was adapted from methodology established in the Komives laboratory (65-66). The dry FXIII aliquot was allowed to reach room temp before 12 μl of 99.996% D_2O (Cambridge Isotope Laboratories) was added yielding a final working concentration of 50 μM TG2, 2.2 μM Ca^{2+} and 213 μM TG-DON. The samples were incubated at room temperature for 10 minutes before the HDX was quenched by adding 120 μl of chilled 0.1% TFA at pH 2.5. The quenched reaction was then immediately transferred to a tube containing activated pepsin bound to 6% agarose (Thermo Scientific, Rockford, IL). Pepsin digestion occurred for 10 min on ice. Following digestion, the reaction mix was

centrifuged for 30 sec to separate the TG2 digest from the pepsin beads. Three 8.2 μ l aliquots were immediately frozen in liquid N₂ and each reaction condition was performed three times.

In addition to investigating TG2 while inhibited with TG-DON, TG2 was also activated under different concentrations of Ca²⁺ (2, 5, and 10 mM) in the absence of DMSO, and it was determined that DMSO did not play a role in the conformational changes reported (data not shown).

HDX Analysis

A FXIII(a) HDX aliquot was thawed at room temperature and immediately mixed with an equal volume of 10 mg/ml α -cyano-hydroxycinnamic acid matrix (α -CHCA) (Aldrich) in 1:1:1 ethanol/CH₃CN/0.1%TFA at pH 2.2, and 0.5 μ l was spotted on a chilled MALDI plate. The sample spot was then quickly dried by placing the MALDI plate into a SpeedVac. The plate was then immediately inserted into the MALDI-TOF-MS (Voyager DE-Pro, Applied Biosystems).

Spectra were collected in reflector mode with 256 shots/spectrum. All peptides in the peptic digest were identified using GPS Explorer™ after MS/MS analysis on an Applied Biosystems 4700 in the laboratory of M. Merchant at University of Louisville. After digestion by pepsin, the TG2 peptides identified represent 37 % coverage (Figure 17). Peptide identification was confirmed through observing the same peptide sequence in multiple independent digests, with an ion score preferably above 20 (peptic peptides Appendix D). These peptides focus on key TG2 regions found within the β -sandwich, the catalytic core, and the β -barrels. Additional studies with acid dependent type XIII protease did not significantly

1	MAEELVLERCDLELETNGRDHHTADLCREKLVVRRGQPFWLTLHFEGRNYEASVDSLTF	60
61	VVTGPAPSQEAGTKARFPLRDAVEEGDWTATVVDQQDCTLSQLTTPANAPIGLYRLSLE	120
121	ASTGYQGSSFVLGHFILLFNAWCPADAVYLDSEEEROEYVITQQGFIYQGSAKFIKNIPW	180
181	NFGQFEDGILDICLILLDVNPKFLKNAGRDCSRSSPVYVGRVVSVMVNCNDDQGVLLGR	240
241	WDNNYGDGVSPMSWIGSVDILRRWKNHGCQRVKYGQCWVFVAAVACTVLRCLGIPTRVVTN	300
301	YNSAHDQNSNLLIEYFRNEFGIEIQGDKSEMIWNFHCWVESWMTRPDLQPGYEGWQALDPT	360
361	PQEKSEGTYCCGPVPVRAIKEGDLSTKYDAPFVFAEVNADVVDWIQQDDGSVHKSINRSL	420
421	IVGLKISTKSVGRDEREDITHTYKYPEGSSEEREAFTTRANHLNKLAEKEETGMAMRIRVG	480
481	QSMNMGSDFDVFAHITNNTAEEYVCRLLLCARTVSYNGILGPECCKYLLNLNLEPFSEK	540
541	SVPLCILYEKYRDCITESNLIKVRALLVEPVINSYLLAERDLYLENPEIKIRILGEPKQK	540
601	RKLVAEVSILQNPLPVALEGCTFTVEGAGLTEEQKTVEIPDPVEAGEEVKVRMDLLPLHMG	660
661	LHKLVVNEESDKLKA VKGFRNVIIGPA	687

Figure 17: TG2 peptides observed by MALDI-TOF-MS after 10 minute pepsin digestion. The magenta area represent peptides observed for TG2 (37 % coverage).

improve sequence coverage. All the MALDI spectra derived from pepsin digests were analyzed using Data Explorer version 4.8(Applied Biosystems) and calibrated using two singly protonated reference peptides; monoisotopic mass 901.44 Da (residues 369-377) and 2042.18 Da + 11 deuterons at mass 2053.29 Da (residues 668-686).

Deuterium incorporation for each isotopic cluster was quantified as described by Sabo *et al.* (67). To determine the change in deuteration for TG2 under different conditions the percent deuteration for each peptide was calculated using equation 1 (page 23). In accordance with previous HDX data analysis, percent differences greater than 4.5 % are considered significant (45, 67-69), 3-4.5 % is moderate and < 3 % is modest.

Results

TG2 Activity and Inhibition with TG-DON

The kinetics of α_2 AP as FXIII-A substrate are well established (83). It has also been demonstrated through a Q2N mutation that FXIII will not utilize the Gln in position 4 on the α_2 AP peptide (83). To determine if TG2 also recognizes only the Gln in position 2, TG2 activity was monitored with α_2 AP-Q2N as substrate. Like FXIII, TG2 was not catalytically active in the presence of α_2 AP-Q2N (data not shown). The activity of TG2 was confirmed using α_2 AP-Q4N as substrate. Following an 8 minute incubation in the presence of 10 mM Ca^{2+} at 37 °C the substrate peptide α_2 AP-Q4N was added to the cuvette. The inhibitory action of TG-DON on TG2 activity was determined by adding TG-DON to the cuvette during the 8 minute incubation prior to the addition of substrate. The TG2

Figure 18: A plot of % inhibition vs the log of TG-DON concentration. Seven separate trials were performed at varying inhibitor concentrations: TG2 in the absence of TG-DON; 15 nM TG-DON; 30 nM TG-DON; 50 nM TG-DON; 90 nM K9 DON; 180 nM K9 DON; and 1000 nM K9 DON. The assay components, TG2, and the indicated amount of TG-DON were incubated for 8 min at 37 °C, at which point the substrate α_2 AP-Q4N was introduced to the assay. All points on the curve are marked with red squares and the linear portion used for determining $\text{IC}_{50} = 55$ nM is blue diamonds inside red squares.

concentration during activation was 200 nM and TG-DON was monitored over a concentration range of 0 – 1000 nM. By plotting the % TGase inhibition versus log TG-DON concentration, a sigmoidal dose response curve is observed (Figure 18). With a TG2 concentration of 200 nM, the IC₅₀ was calculated to be 55 nM TG-DON.

MALDI-TOF-TOF for TG2 Pepsin Peptide Identification

The first step in comparing the solvent accessibility of FXIII to that of TG2 was identifying the peptic peptides for TG2. Pepsin is a non-specific specific protease; therefore, it is difficult to predict cleavage products but the cleavage pattern is very reproducible when conducted under the same conditions (pH, Temperature, etc.). Previously in our lab, the peptic peptides of FXIII were determined on an Applied Biosystems DE-PRO using Post-Source Decay (PSD) (45). MALDI-TOF-TOF MS allows for MS/MS capabilities. An Applied Biosystems 4700 was used for acquiring the MS and MS/MS spectra of TG2 digest products. The mass list was subsequently uploaded into GPS Explorer™ software available through Applied Biosystems. Using GPS Explorer™ to search the MASCOT™ database 16 peptides were identified representing 37 % of TG2 (Figure 17 in Material and Methods).

HDX Analysis of TG2

Once TG-DON binding and inhibition were confirmed HDX was utilized to monitor the conformational dynamics of TG2 during activation and in the open-inhibited conformation. TG2 has been crystallized in an open conformation leading to the hypothesis that a gross increase in solvent accessibility would be

Table 3: Changes in Percent Deuteration^a: Active TG2 (2 mM Ca²⁺) and Inhibited TG2 (2 mM Ca²⁺ with TG-DON) relative to TG2 zymogen^a.

	Residues	Theo. D _{max} ^b	2mM Ca ²⁺	2mM Ca ²⁺ TG-DON
β-Sandwich	13-26	15.8	-0.3	-0.4
	27-40	15.2	-0.7	-0.6
	32-40	9.4	-0.8	-0.4
Cat. Core	155-161	8.2	-0.4	-0.7
	238-254	18.0	-0.4	0.2
	260-270	13.0	1.1	4.8
	316-330	17.0	0.0	-0.2
	342-354	12.5	3.3	3.6
	370-378	8.0	-4.2	-4.0
	452-462	12.7	-22.6	-23.4
	453-462	11.6	-23.2	-23.7
β-Barrel 1	476-488	14.9	0.2	5.3
	503-509	8.0	0.4	-0.1
	548-555	9.4	2.0	2.3
	561-569	10.2	0.5	1.1
β-Barrel 2	669-687	20.3	6.0	8.2

^a The % change for a particular peptide was calculated by the following equation: % difference = $((D - D_{FXIII})/D_{max}) \times 100 \%$, where D is the amount of deuterium incorporated in FXIII with metal, D_{FXIII} is the amount of deuterium incorporated in the zymogenic state (no metal), and D_{max} is the theoretical maximum number of exchangeable protons within the indicated peptide. ^b The maximum number of exchangeable protons within the indicated peptide, assuming 100 % deuteration. This value accounts for all exchangeable backbone amide protons and a slight fraction of N-terminal, C-terminal, and side chain exchangeable protons, which are dependent on the final percentage of D₂O in solution under quench conditions (~4.5 %). A fully deuterated peptide would theoretically have acquired this amount of deuterons. ^c The values in bold represent significant changes in deuteration greater than ~4.5 %

observed by HDX around the catalytic core and β-barrel interface. HDX allows for monitoring TG2 conformational changes in solution, eliminating any possible artifacts of the crystallization process. There were four peptides monitored within the N-terminal β-sandwich region, none of which displayed any significant changes in solvent accessibility. The catalytic core was better represented and several peptides displayed not only increases in solvent accessibility, but also protection (Table 3 and Figure 19).

There were 8 peptides analyzed representing 41 % of the TG2 catalytic core (139 – 471). The majority of the catalytic core did not show any large changes in solvent accessibility. Starting at the N-terminal end of the catalytic

core, peptide 260 - 270 is the first to show a significant change in deuteration. When compared to zymogen, TG2 activated with 2 mM Ca^{2+} only displayed a 1.1 % increase in solvent accessibility whereas the TG-DON inhibited form increased 4.8 %. Continuing toward the C-terminus, the next notable region was 342 - 354, where both the Ca^{2+} activated and the TG-DON inhibited conformations had a moderate increase in solvent accessibility, 3.3 and 3.6 % respectively. The next few regions all experienced protection. The first of which is 370 - 378 which experienced a -4.2 and -4.0 % protection for Ca^{2+} activated and TG-DON inhibited respectively. There were two peptides spanning from 452 - 462 and they both became highly protected when both became protected 22.6 – 23.7 %

Figure 19: The number of deuterons incorporated at 10 min for TG2 under the following conditions zymogenic (no metal) TG2 (Blue), 2 mM Ca^{2+} (Red), 2 mM Ca^{2+} with TG-DON (Green). Error bars represent the standard deviation of the mean for 3 independent trials.

when activated or inhibited.

Lastly there are the two β -barrels (β -barrel 1 472 – 584 and β -barrel 2 585 – 686). The 4 peptides that were analyzed in β -barrel 1 represented 65 % of the 113 amino acids. Only one peptide, 476 - 488 had a significant change in solvent accessibility. Like the 260 - 270 region, there was not any notable change in solvent accessibility when activated with 2 mM Ca^{2+} , but there was an increase was observed when inhibited with TG-DON. When inhibited, there was a 5.3 % increase in dueteration, whereas Ca^{2+} activated TG2 only saw a 0.2% increase. There was only one peptide (669 - 687) representing 19 % of β -barrel 2 available for analysis. Peptide 669 - 687 also represented the 19 C-terminal amino acids of TG2. This region saw increases in solvent availability in both the Ca^{2+} activated and TG-DON inhibited TG2. The Ca^{2+} activated increased 6.0 % and the TG-DON inhibited increased 8.2 % when compared to zymogen.

Discussion

Lorand and Graham coined transglutaminase 2 “the bête noir” of the transglutaminase family because it was the first of the TGase family discovered and its physiological role remains elusive (84). Progress in understanding the physiological role of TG2 in apoptosis, cell-matrix interactions and human diseases like Central Nervous System (CNS) neurodegenerative diseases, celiac disease and cancer has been nicely reviewed (84-86). Understanding the function and physiological role of TG2 has also been assisted with a greater understanding of its conformational dynamics (49, 87-89).

Two recent studies have utilized mass spectrometry to monitor the conformational dynamics of TG2 (87, 90). Greenberg, *et al.* monitored TG2 activity after lysine modification and determined that acetylation of Lys 444, 468 and 663 were essential for inhibition (87). The other study identified a redox-sensitive cysteine triad (Cys 230, 370 and 371) which need to be fully reduced for TGase activity (90). Pinkas *et al.* has crystallized TG2 trapped in an open conformation (49). In the current work, the conformational dynamics of TG2 were monitored in solution, for the first time, through the utilization of HDX coupled with MALDI-TOF-MS.

TG2 HDX

Crystallography has been utilized to investigate the structure of TG2 both in the inhibited (open) and GDP-bound (closed) conformations (49, 91). It was apparent that within the β -sandwich domain there was very little structural variability (Figure 20). This lack of conformational dynamics was supported by

HDX where there were no regions within the β -sandwich that exhibited significant changes in solvent accessibility.

Catalytic Core

The catalytic core proved to be more flexible during activation and inhibition. In addition to the catalytic triad (C277, H335 and D358), it also contains the putative

Figure 20: Overlay comparison of the TG2 β -Sandwich domain in the open and closed conformations. The green represents GDP-bound TG2 in the closed conformation and the grey is the TG-DON inhibited form of TG2. The overlay was performed using the MultiSeq tool in VMD(51).

Ca^{2+} binding sites, the redox-sensitive cysteine triad (C230, 370 and 371) and two of the three lysines (448 and 468) that are essential for inhibition after acetylation (77, 87, 90). The exposure experienced from 260 - 270 falls N-terminally of the catalytic C277. During Ca^{2+} activation the 260 - 270 region experienced a modest (1.1 %) increase in exposure whereas when bound to TG-DON, there was a 4.8 % increase in exposure. This region is represented by the magenta helix (Figure 21) and is separated from C277 by a short β -strand. The 260 - 270 helix does not change orientation in the GDP-bound versus TG-DON inhibited crystal structures; therefore, the differences observed in deuterium incorporation for activated versus TG-DON inhibited TG2 is due to the dislocation of the β -barrels. The residues of 260 - 270 are $< 10 \text{ \AA}$ from residues 631-635 of β -barrel 2 in the closed conformation. The movement of the two β -barrel

domains during inhibition allows for the 4.8 % increase in deuterium incorporation.

Figure 21: Overlay comparison of TG2 in the open and closed conformations. (A) The green represents GDP-bound TG2 in the closed conformation and the grey is the TG-DON inhibited form of TG2. Regions of interest are labeled according to protection or exposure to solvent after activation and inhibition. Peptides 452-462 and 370-378 (Blue) experienced solvent protection. Peptide 342-354 (pink) experienced moderate exposure. Peptides 260-270 and 476-488 (magenta) experienced exposure only after inhibition. Peptide 669-687 (red) experienced exposure. (B) GDP-bound TG2 in the closed conformation with the same labeling as (A). (C) Inhibited TG2 in the open conformation with the same labeling as (A).

In addition to C277, the catalytic triad also consists of H335 and D358. Peptide 342 – 354 falls between these two residues and 370 – 378 falls 12 residues C-terminal of D358. Residues 342 – 354 experienced moderate exposure when activated and when inhibited (Figure 21 and Figure 22 pink). This peptide's conformation did not change in the GDP-bound structure when compared to the TG-DON inhibited crystal structure. These observations indicate that 342 – 354 may become more exposed if the solvent accessibility of GDP-bound TG2 was compared to zymogen. The similar structure of 342 – 354 in GDP-bound and TG-DON inhibited TG2 could be due to the importance of

maintaining structural integrity between two of the three catalytic residues.

Unlike 342 – 354 which became more exposed and displayed a similar conformation in the two TG2 crystal structures, 370 – 378 experienced moderate protection and a conformational

change when inhibited. A closer look at this region demonstrated the intimate contact between 370 - 378 and the catalytic D358. In Figure

22, 370 – 378 (ball and stick, red = closed and blue = open), the region observed in HDX

was viewed with an extension, 355 – 369 (new cartoon), which contains D358. The beta-hairpin motif on the N-terminus of 370 – 378 in the

GDP-bound TG2 lost its character and became random coil in the TG-DON inhibited TG2 crystal structure. During HDX, the solvent exposure of 370 – 378 decreases 4.2 and 4.0 % for Ca^{2+} activated and TG-DON inhibited TG2 respectively when compared to zymogen. In the inhibited form of TG2 the N-terminus of 370 – 378 underwent a conformational change and lay closer to the surface thus supporting the protection observed by HDX (Figure 22).

There is a disulfide bond reported between C370 and C371 in the open conformation of TG2 (49, 90). Stamnaes *et al.* monitored TG2 under different

Figure 22: Illustration of the intimate connection between the catalytic triad and peptides 342-354 and 370-378. The catalytic triad (C277, H335 and D358) are green ball and stick. Peptide 342-354 (pink) is connected to 370-378 (ball and stick) via a linker containing D358. Peptide 342-354 and the linker are labeled according to conformation (red = closed and blue = open).

oxidizing (GSH/GSSG) conditions and determined that the vicinal C370 – C371 disulfide was prevalent in the open and oxidized form (90). The solvent protection and observed conformational change at the N-terminus of 370 – 378 help to substantiate the evidence of vicinal disulfide formation in the open conformation.

The Ca^{2+} binding site on TG2 has yet to be observed in crystal structures and has only been identified through modeling and mutations of noncanonical Ca^{2+} binding regions (77, 88). One such proposed Ca^{2+} binding site (S2, residues 445 – 455) was identified through sequence homology with the FXIII-A Ca^{2+} binding site (77). The HDX of this proposed Ca^{2+} binding region is

Figure 23; Overlay of TG2 and Factor XIII-A₂. (A) The overlay of FXIII-A₂ and the closed conformation of TG2. One monomer of the FXIII-A₂ dimer is shaded while the other is (blue). The GDP-bound (closed) TG2 is (red) and the catalytic triad is (yellow sticks). (B) The overlay of FXIII-A₂ and the open conformation of TG2. One monomer of FXIII-A₂ is shaded while the other is (blue). The TG-DON inhibited conformation of TG2 is overlaid in (red) and the catalytic triad is (yellow sticks). The overlay was performed using the MultiSeq tool in VMD(51).

represented by 452 – 462, an alpha-helix that lay in a flexible region between the catalytic core and β -barrel 1. The > 20 % protection observed in for 452 - 462 supports the evidence of a noncanonical Ca^{2+} binding site in this region.

In addition to the noncanonical Ca^{2+} binding site Lai *et al.* reported that K468 is one of three lysine residues that, when acetylated, inhibit TG2 activity (87). The decrease in TG2 activity after acetylation was attributed to the inability of this flexible region to change conformation and allow for the open conformation. The α -helix character of 452 - 462 in TG-DON inhibited TG2 extends C-terminally when compared to GDP-bound TG2. This extension of α -helix character would allow for more intra-helix H-bonding thus decreasing the exchangeable protons. The 452 – 462 region was represented by two peptides that shared 462 as their C-terminus. Both peptides experienced the same amount protection, further supporting the evidence that the C-

Figure 24: Overlay of FXIII-A₂ and the open conformation of TG2. One FXIII-A₂ monomer (blue) was aligned with TG-DON (yellow) inhibited TG2 (red). The other FXIII-A₂ monomer displays a surface projection emphasizing the interference between the FXIII-A β -sandwich (surface) and the β -barrel 1 of TG2 in the open conformation. The overlay was performed using the MultiSeq tool in VMD(51).

terminus is important for the observed protection. This helix lies on the backside of the 'hinge' during inhibition (opening) of TG2. Due to this placement it is not surprising that it would become more protected as the TG2 molecule opens.

The conformational dynamics of Factor XIII has been well characterized by HDX and chemical modification techniques (43-44, 46, 60, 92). TG2 and the FXIII-A monomer share a high degree of sequence and structural homology (Figure 23). Due to differences in sequence coverage a direct comparison between TG2 and FXIII HDX was not possible. One region of FXIII which has received lots of attention is the exposure of the dimer interface during activation and inhibition (43-44). The equivalent residues on TG2 were not observed. There has been debate as to whether FXIII-A2 undergoes a large conformational change when inhibited like that reported for TG (46, 49). The increase of exposure around the FXIII-A2 dimer interface would seem to support this hypothesis; but, if it is assumed that FXIII-A2 opens in a manner similar to TG2, it would have to overcome substantial steric hinderance between the β -sandwich region of monomer 1 and the β -barrel 1 of monomer 2. When the FXIII-A2 dimer is overlaid on the open conformation of TG2 this blockage between the opposing monomers of FXIII become evident (Figure 24). Komáromi, *et al.* have attempted to model FXIII-A2 undergoing an open conformation and reported that they had to "eliminate two conformational bumpings by making cuts in the structure and manually deriving new positions for them" (93). Although the data provided to date would not suggest a major conformational change in FXIII-A2 during activation, more work needs to be done to place any certainty on this

hypothesis. Since 2007 when TG2 was captured in the open conformation there have already been antibodies designed for the detection of the open conformation (Zedira, Darmstadt, Germany). The possibility of trapping FXIII-A2 in a particular conformation via specific antibodies or pharmaceuticals would open new avenues for dealing with aberrant coagulation.

CHAPTER IV

UTILIZATION OF HDX TECHNOLOGY TO ELUCIDATE THE FXIII A RESIDUES CRUCIAL FOR HETEROTETRAMER FORMATION WITH FXIII B.

Introduction

In plasma, factor XIII is a protransglutaminase with a tetrameric structure consisting of two A subunits and two B subunits (FXIII-A₂B₂), where the B subunits act as a carrier for the catalytically active A subunits. All FXIII-A in plasma is found in the complexed A₂B₂ form, but the B₂ subunits are in excess and about 50% are in the uncomplexed B₂ form (94). To date there is not a crystal structure available for FXIII-B, but by electron microscopy they appear as thin flexible and kinked fibers (95). Factor XIII-B is a glycoprotein which contains 8.5% carbohydrate and has a molecular mass of ~80 kDa. Structurally, the B subunit contains 10 “sushi-domain” repeats (Figure 25), each of which consist of 60 amino acids and 2 disulfide bridges (96-97).

Figure 25: Primary sequence of FXIII B₂ illustrating the 10 sushi-domain repeats each containing 60 AA and 2 disulfide bonds. This research was originally published in The Journal of Biological Chemistry. Ichinose, A., Bottenus, R.E., Davie, E.W. Structure of Transglutaminases. Journal of Biological Chemistry. 1990; 265:23 13411 - 13414. © the American Society for Biochemistry and Molecular Biology (96).

Factor XIII-A exists as the ~320 kDa A₂B₂ heterotetramer with a concentration of 14-28 µg/ml in plasma. The apparent binding constant for the association of A₂ and B₂ subunits was determined to be $4 \times 10^7 \text{ M}^{-1}$ (K_d = 0.025 µM)(25). Souri recently utilized several truncated forms of FXIII-B which contained different regions of the molecule (eg. sushi domains 1-9, 1-5, 1-4, 5-10, etc.) to identify the sushi domains responsible for dimer (B₂) formation and heterotetramer (A₂B₂) formation. The fourth and ninth domains are crucial for dimer formation and the first sushi domain on the N-terminus is needed for heterotetramer formation (Figure 23) (98). The exact residues on FXIII-B that are important to the B₂-dimer and A₂B₂-tetramer formation have not been

Figure 26: Cartoon model of FXIII B homodimer illustrating sushi-domains 4 and 9 (yellow) which are crucial for FXIII B₂ dimer formation and domain 1 (red) which is utilized in heterotetramer formation with FXIII A₂. Figure used from (98).

determined. When complexed with FXIII-B, FXIII-A is stabilized in plasma increasing its half-life. The concentration of FXIII-A decreased 40% after 2 hr incubation at 37 °C. Further evidence of the stabilization of FXIII-A is the resistance to proteolytic attack by trypsin and thrombin observed when FXIII-A is bound to FXIII-B (99). When bound, the protective effects of the B-subunit are well documented, but the FXIII-A₂ residues required for tetramer formation have yet to be elucidated. It is hypothesized that the beta-barrels play a major role in the tetramer formation because when truncated the tetramer is not observed by co-immunoprecipitation with an anti-XIII-B antibody (99).

In the current study hydrogen-deuterium exchange coupled with MALDI-TOF-MS was utilized to unveil the residues on FXIII-A that become less accessible to solvent when it associates with the B₂ subunit forming the A₂B₂ heterotetramer. Monitoring FXIII-A solvent protection by HDX aided in painting a more complete picture of how FXIII-A and FXIII-B interact in solution. These experiments demonstrated that although FXIII-B₂ incurs FXIII-A₂ proteolytic

resistance 14 % sequence coverage was still observed. Even with decreased sequence coverage it was demonstrated that much of the FXIII-A molecule becomes protected in the presence of FXIII-B, but significant protection is observed around the dimer interface and both of the beta-barrels. This evidence supports earlier reports of β -barrel involvement in heterotetramer formation (98-99).

Materials and Methods

Factor XIII A₂ and Factor XIII B₂ Preparation

Recombinant human cellular FXIII A₂ was generously provided by Dr. Paul Bishop (ZymoGenetics, Inc., Seattle, WA). After reconstituting the lyophilized FXIII in 18 MΩ deionized water, FXIII was buffer exchanged into 25 mM Tris-base at pH 7.4. The concentration of FXIII was determined on a Cary 100 UV/vis spectrophotometer. The absorbance was monitored at 280 nm and concentration calculated with the FXIII extinction coefficient of 1.49 ml/mg cm. Aliquots (12 μl) of 34 μM FXIII in 25 mM Tris-base were stored at -70 °C until future use.

A 200 μg ampoule of recombinant human FXIII B₂ (Zedira, Darmstadt, Germany), lyophilized in the presence of 25 mM Tris, was resuspended with 18 MΩ deionized water to a concentration of 24 μM. Aliquots of 10 μl were prepared and stored at -20 °C until use.

HDX Experimentation

Hydrogen deuterium exchange was utilized to elucidate points of contact between the FXIII A₂ and FXIII B₂. An aliquot of 24 μM FXIII B₂ and 34 μM FXIII A₂ were allowed to come to room temperature. For analysis of FXIII A₂ in solution, 6 μl FXIII was added to 10 μl 25 mM Tris for a final concentration of 13 μM FXIII-A. To allow for FXIII A₂ and FXIII B₂ binding before HDX, 6 μl 34 μM FXIII A₂ was added to 10 μl 24 μM FXIII B₂ yielding 13 μM FXIII A and 15 μM FXIII B in solution. All assays were then allowed to incubate for 20 minutes on ice. Following incubation, the assay tubes were then evaporated to dryness in

the SpeedVac and then HDX was performed on the dry samples. The following HDX protocol was then adapted from methodology established in the Komives laboratory (65-66). The dry FXIII aliquot was allowed to reach room temp before 12 μ l of 99.996% D₂O (Cambridge Isotope Laboratories) was added yielding a final working concentration of 17 μ M FXIII A and 20 μ M FXIII B. The samples were incubated at room temperature for 10 minutes before the HDX was quenched by adding 120 μ l of chilled 0.1% TFA at pH 2.5. The quenched reaction was then immediately transferred to a tube containing activated pepsin bound to 6% agarose (Thermo Scientific, Rockford, IL). Pepsin digestion occurred for 10 min on ice. Following digestion, the reaction mix was centrifuged for 30 sec to separate the FXIII digest from the pepsin beads. Three 8.2 μ l aliquots were immediately frozen in liquid N₂ and each reaction condition was performed three times.

HDX Analysis

A FXIII(a) HDX aliquot was thawed at room temperature and immediately mixed with an equal volume of 10 mg/ml α -cyano-hydroxycinnamic acid matrix (α -CHCA) (Aldrich) in 1:1:1 ethanol/CH₃CN/0.1%TFA at pH 2.2, and 0.5 μ l was spotted on a chilled MALDI plate. The sample spot was then quickly dried by placing the MALDI plate into a SpeedVac. The plate was then immediately inserted into the MALDI-TOF-MS (Voyager DE-Pro, Applied Biosystems). Spectra were collected in reflector mode with 256 shots/spectrum. All peptides in the peptic digest were previously identified by Brian T. Turner, Jr. (45) and/or

confirmed by MS/MS analysis on an Applied Biosystems 4700. Peptide identification was confirmed through observing the same peptide sequence in multiple independent digests, with an ion score preferably above 20 (peptic peptides Appendix C). After digestion by pepsin, the FXIII A₂ peptides identified represent 40 % coverage. These peptides focus on key FXIII regions found within the β -sandwich, the catalytic core, and the β -barrel 1 region. Additional studies with acid dependent type XIII protease did not significantly improve sequence coverage. All the MALDI spectra derived from pepsin digests were analyzed using Data Explorer (Applied Biosystems) and calibrated using two singly protonated reference peptides; monoisotopic mass 850.4787 Da (residues 535-541) and quadraisotopic mass of 1375.7097 Da (residues 220-230).

Deuterium incorporation for each isotopic cluster was quantified as described by Sabo *et al.* (67). To determine the change in deuteration for FXIII under different conditions the percent deuteration for each peptide was calculated using equation 1 (page 23). In accordance with previous HDX data analysis, percent differences greater than 4.5 % are considered significant (45, 67-69), 3-4.5 % is moderate and < 3 % is modest.

Native Gel

To mimic the HDX conditions, prior to running on the gel, 3 μ l 34 μ M FXIII-A was added to 5 μ l 24 μ M FXIII-B. A 13 μ M FXIII-A and 15 μ M FXIII-B aliquot was also prepared and all samples incubated for 20 minutes on ice. Each sample was then added to 16 μ l sample buffer (63 mM Tris pH6.8, 10 % glycerol and 0.05 % bromophenol blue). The samples were electrophoresed using a 4.0

% polyacrylamide stacking gel (125 mM Tris-base, pH = 6.8) on a 7.5 % polyacrylamide resolving gel (375 mM Tris-base, pH=8.8) and a running buffer of 5 mM Tris Base and 38.4 mM glycine pH = 8.3. The gels subsequently were stained with a 0.1 % Coomassie blue solution containing 40 % Methanol and 10 % Acetic acid. A Gel Doc™ XR+ System (Bio-Rad, Hercules, CA) was then utilized for gel imaging.

Results

FXIII-B induced proteolytic resistance

All HDX that have been run in our lab to this point have included proteins with a molecular weight of no more than ~160 kDa (FXIII-A = ~80 kDa). The FXIII-A₂B₂ heterotetramer has a molecular weight of ~320 kDa (4 × ~80 kDa). This increase in total protein mass along with the known proteolytic resistance of FXIII-A in the presence of FXIII-B (98-99) led to an exploratory experiment to determine the feasibility of performing HDX on this system. Following a 10 minute pepsin digest mimicking HDX conditions, peptides produced were

Figure 27: Representative spectra for FXIII-A₂ (A), FXIII-B₂ (B) and FXIII-A₂B₂ (C) following pepsin digestion.

monitored for FXIII-A, FXIII-B and FXIII-A₂B₂ on the MALDI-TOF-MS (Figure 27A-C). At 13 μ M, FXIII-A produced pepsin digest with many clear and identifiable peaks (Figure 27A). FXIII-B contains 2 disulfide bonds per sushi domain which confers an innate proteolytic resistance. Due to the proteolytic resistance, only two major peaks (m/z = 1350.65 and 2039.95) are found in the FXIII-B pepsin digest (Figure 27B). FXIII-B did elicit proteolytic protection upon FXIII-A when in the heterotetramer form as seen in Figure 27C. The intensity of the most abundant ion (m/z = 1216.70) in Figure 27A is 5 fold higher than that for the spectra representing 13 μ M FXIII-A in the presence of 15 μ M FXIII-B.

Suppression within the FXIII-A₂B₂ pepsin digest resulted in a decrease in sequence coverage for the HDX. After digestion by pepsin, the FXIII-A₂ peptides identified represent 40 % coverage. These peptides focus on key FXIII regions

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1  SETSRTAFGG RRAVPNNNSN AAEDDLPTVE LQGVVPRGVN LQEFNLVTSV HLFKERWDTN 60
61  KVDHHTDKYE NNKLIVRRGQ SFYVQIDFSR PYDFPRDLER VEYVIGRYPQ ENKGTYPVP 120
121 IVSELQSGKW GAKIVM FDR SVRLSIQSSP KCIVGKPRMY VAVWTPYGLV RISHNPETDT 180
181 YILFNPWCED DAVYLDNEKE REEYVLNDIG VIFVGEVNDI KTRSWSYGQF EDGILDTCL 240
241 VMDRAQMDLS GRGNPIKVSF VGSAMVNAKD DEGLVVGSD NIYAYGVPPS AWGSDVILL 300
301 EYRSSENPVR YGQCWVFAGV FNTFLRCLGI PARIVTNVFS AHDNDANLQM DIFLEEDGNV 360
361 NSKLTKDSVW NYHCWNEAWM TRPDLPVGE GWQAVDSTPQ ENSDGMVRCG PASVQAIRHG 420
421 HVCFQFDAPF VFAEVNSDLI YITAKKDGTH VVENVDATHI GKLIIVTKQIG GDGMMDITDT 480
481 YKFPQEGQEEE RLALETALMY GAKKPLNTEG VMKSRSHVDM DEEVENAVLG KD FHLSTTFR 540
541 NNSHNRYTIT AYLSANITFY TGVPKAEFKK ETPDVTLEPL SFKKEAVLIQ AGEYMGQLE 600
601 QASLHFF VTA RINETRDVLA KQKSTVLTIP ELIIKVRGTQ VVGSDMTVTV QFTNPLKETL 660
661 RNVVHLDGP GVTRPMKKMF REIRPNSTVQ WEEVCRPWVS GHRKLIASMS SDSLRHVYGE 720
721 LDVQIQRRPS M
FXIII-A, Pepsin Coverage = 40%
FXIII-A2B2, Pepsin Coverage = 14%

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Figure 28: FXIII peptides observed by MALDI-TOF-MS after 10 minute pepsin digestion. The magenta and green represent peptides observed for FXIII-A₂ (40 % coverage). The green peptides represent those observed in FXIII-A₂B₂ digests (14 %).

found within the β -sandwich, the catalytic core, and the β -barrel 1 region. Although the peaks from the FXIII-A₂ digest are observed in the FXIII-A₂B₂ digest, there is a large decrease in sequence

coverage due to lower sensitivity as well as peak interference (overlap of unidentified peptides). Following pepsin digestion, FXIII-A₂ sequence coverage reduces to 14 % when bound to FXIII-B₂ (Figure 25).

Native Gel – Heterotetramer confirmation

FXIII-A₂ has a high affinity for FXIII-B₂ ($K_d = 25$ nM) and is found almost exclusively as a FXIII-A₂B₂ heterotetramer in plasma. To confirm FXIII-A₂B₂ formation for HDX analysis native (no SDS) PAGE was utilized. By mirroring the solution conditions for HDX, native PAGE identified FXIII-A₂ and FXIII-B₂ dimers as well as FXIII-A₂B₂ heterotetramer formation in solution (Figure 26). FXIII-A₂ migrated the furthest and FXIII-B₂ having a similar molecular weight migrated in almost as far as FXIII-A₂. The FXIII-A₂B₂ complex was confirmed in the higher molecular weight (slower migrating band).

Figure 29: Native PAGE illustrating the formation of FXIII-A₂B₂. Molecular weight standards are marked (97.4 kDa).

After confirming FXIII-A₂B₂ formation, HDX analysis was performed to monitor the regions of FXIII-A₂ which become more protected in the presence of FXIII-B₂. Due to the interactions between FXIII-A₂ and FXIII-B₂ during heterotetramer formation, there was a substantial

decrease in sequence coverage. Even with this decrease in coverage, regions of FXIII-A₂ important for heterotetramer formation were monitored, for the first time, using HDX coupled with MALDI-TOF MS.

FXIII-A₂B₂ HDX Analysis

To date FXIII-A₂B₂ has yet to be crystallized; therefore, HDX was utilized in an effort to ascertain the regions of FXIII-A₂ that are involved in heterotetramer formation with FXIII-B₂. Since Ca²⁺ is a FXIII-A₂ cofactor that aids in FXIII-A₂B₂ separation and FXIII-A₂ activation, all studies were run in the absence of divalent metal cations(39, 58). For the current conformational studies on FXIII A₂B₂ Table 4 displays changes in deuterium incorporation of FXIII-A₂ zymogen compared to FXIII-A₂B₂. The total deuterium incorporated for each pepsin-derived peptide is displayed in Figure 30.

The β -sandwich region of FXIII-A₂ contains the activation peptide as well as peptide 4, a Gln

substrate recognition

region. Due to the

decrease in FXIII-A₂

sequence coverage, there

were not any quantifiable

peptic peptides within the β -

sandwich region. The

catalytic core contains

several regions of interest.

Another important putative

Gln substrate recognition,

peptide 7 (190-230), is

Table 4: Changes in Percent Deuteration: FXIII-A₂B₂ relative to FXIII-A₂ zymogen^a

Residues	Theo. D _{max} ^b	FXIII-A ₂ B ₂
137-144	7.90	-0.8
145-159	15.58	-1.6
220-230	12.31	-5.8^d
240-247	7.50	-16.7
298-305	7.50	-6.4
325-338	14.40	0.9
328-338	9.5	-2.4
535-541	6.5	-1.2
607-619	12.80	-14.5
632-646	14.70	-10.6

^a The % change for a particular peptide was calculated by the following equation: % difference = ((D - D_{FXIII})/D_{max}) × 100 %, where *D* is the amount of deuterium incorporated in FXIII with metal, D_{FXIII} is the amount of deuterium incorporated in the zymogenic state (no metal), and D_{max} is the theoretical maximum number of exchangeable protons within the indicated peptide. ^b The maximum number of exchangeable protons within the indicated peptide, assuming 100 % deuteration. This value accounts for all exchangeable backbone amide protons and a slight fraction of N-terminal, C-terminal, and side chain exchangeable protons, which are dependent on the final percentage of D₂O in solution under quench conditions (~4.5 %). A fully deuterated peptide would theoretically have acquired this amount of deuterons. ^c The values in bold represent significant changes in deuteration greater than ~4.5 %

Figure 30: The number of deuterons incorporated after 10 min at 37 °C for FXIII-A₂ (Blue) and FXIII-A₂B₂ (Red) Error bars represent the standard deviation of the mean for 3 independent trials.

found in the catalytic core(71). In the peptide 7 region, 220-230, FXIII-A experienced significant 5.8 % protection from solvent when bound to FXIII-B. One of the regions which displayed the most protection when bound to FXIII-B was the dimer interface. FXIII-A (240-247), underwent a 16.7 % decrease in solvent accessibility. There were two other regions of the catalytic core represented (298-305) which showed a 6.4 % decrease in solvent accessibility and (325-338) which did not change significantly. FXIII-A peptide 325-338 was the only peptide quantified that displayed an increase (0.9 %) in HDX exposure. Peptide 328-338 found within 325-338 experienced 2.3 % protection, thus residues 325, 326 and/or 327 must be exposed to explain the 0.9 % exposure of 325-338.

The β -barrels have always been poorly represented during HDX analysis (43-44, 60), but they appear to be quite important for FXIII-A₂B₂ heterotetramer

formation. Protection within the FXIII-A₂ β -barrel 1 is evident in the 14.5 % protection at peptide 607-619. This large increase in protection also carries over into β -barrel 2 where peptide 632-646, found on the N-terminal end of a proposed Lys recognition region (646-658) (73), is protected 10.6 % when bound to FXIII-B₂.

Discussion

Factor XIII has been studied extensively over the past 30+ years and the knowledge gained is extensive. Ironically very little has been done to determine how FXIII-A and FXIII-B interact. The recent work of Souri *et al.* has shed new light on the sushi domains that are essential for FXIII-B₂ dimer formation as well as FXIII-A₂B₂ heterotetramer formation (98). The FXIII-A regions necessary for heterotetramer formation have also been investigated and it was determined that the β -barrels play an integral role (99). These studies have aided in generating a better understanding of FXIII- A₂B₂ formation but there still needs to be more work done to identify the binding epitopes involved on both FXIII-A and FXIII-B.

The preliminary experiments to determine feasibility yielded evidence that FXIII-B is inherently resistant to pepsin proteolysis. After 10 min digestion on ice, FXIII-B only produced two quantifiable peptides (m/z = 1350.65 and 2039.95). This was promising considering that they did not overlap with any of our known peptic peptides produced in FXIII-A. Although the digest containing only FXIII-B did not produce any peptides that overlapped with FXIII-A digests, there were some peaks lost in the FXIII- A₂B₂ digest due to suppression and/or overlap of unidentified peptides. The protective effect of FXIII-B on the proteolytic action of pepsin on FXIII-A was observed in all FXIII-A₂B₂ digests. FXIII-B has been shown to stabilize FXIII-A *in vitro* (98-99). FXIII-B protected wild type and, to a lesser extent, Y283C FXIII-A from trypsin digestion, but this protection was not seen in a I464stop mutant which does not contain the β -barrels (99). A decrease in

FXIII-A proteolytic degradation due to heterotetramer formation was not only observed with trypsin, but also with the more unspecific proteinase K (98). The intensity of FXIII- A₂B₂ pepsin digests decreases nearly 5 fold in comparison to FXIII-A₂. This signal suppression can be attributed in part to the proteolytic resistance of FXIII- A₂ when associated with FXIII-B₂. The protective effect of FXIII-B induced a decrease in digestion efficiency leading to a decrease in FXIII-A sequence coverage from 40 % to 14 % (Figure 31).

Once it was determined that HDX was possible with the FXIII- A₂B₂ complex, a native gel was utilized to confirm heterotetramer formation. FXIII-B₂ was the smallest of the components and thus migrated the furthest. FXIII-A₂ produced a nice band just above that of FXIII-B₂ and heterotetramer formation is confirmed in the slower migrating FXIII-A₂B₂ band (Figure 26). A

Figure 31: Illustration of FXIII-A₂B₂ HDX sequence coverage. FXIII-A₂ (1F13) is represented by the ribbons. The cartoon peptides represent 14 % of the molecule. Protection is noted in (blue), significant protection (purple) and exposure (red). Image was constructed utilizing VMD (51)

1:1 ratio of FXIII-A₂ to FXIII-B₂ has been confirmed in previous studies (98-99). Therefore, a faint FXIII-B₂ band is observed in the FXIII-A₂B₂ lane due to a small excess of FXIII-B₂ (15 µM) versus FXIII-A₂ (13 µM).

The utilization of HDX to monitor the conformational dynamics of FXIII-A₂ when associated with FXIII-B₂ allows for identifying regions that become protected due to the heterotetramer formation. It has been reported that the β -barrels play a crucial role in FXIII-

Figure 32: Representative HDX Spectra. The three representative FXIII-A₂ spectra illustrate the isotopic clusters of peptide 240-247 after 10 min. HDX. The FXIII state is listed in the figure. This peak cluster represents a peptide found on the FXIII-A₂ dimer interface. Protection due to heterotetramer formation was observed for FXIII-A₂B₂.

A₂B₂ formation (99), but it is unknown as to how the rest of the FXIII-A₂ molecule is affected. The sequence coverage of FXIII-A₂ can be seen in Figure 31. Nearly the whole molecule becomes protected when interacting with FXIII-B₂.

Within the catalytic core there were three peptides which underwent significant (>4.5 %) protection. The first regions of importance was the putative Gln binding site, peptide 7 (190-230), represented by peptide 220-230. This region has been shown to display exposure when activated proteolytically and non-proteolytically, in the presence of physiological concentrations of Ca²⁺, and when inhibited (43-44). It is thought that FXIII-A₂B₂ is associated with fibrinogen in plasma. By limiting substrate recognition at peptide 7, FXIII-B could be protecting against FXIII-A₂ utilizing the fibrinogen as substrate. One peptide falls along the dimer interface (240-247) and it becomes substantially protected in the

heterotetramer form as seen by the isotopic cluster shift to a lighter mass (Figure 32). The dimer interface becomes more exposed as FXIII-A₂ becomes activated so therefore, by remaining protected, FXIII-B₂ is limiting the ability of FXIII to aberrantly become activated in plasma. The catalytic cysteine was not represented, but peptide 298-305 resides 9 residues N-terminally were protected 6.4 % yet again illustrating the protective nature of FXIII-B₂ on FXIII-A₂.

Prior to the elucidation of the FXIII-A₂ crystal structure, Lai *et al.* utilized several different C-terminally truncated FXIII-A mutants to define the minimum structure required for transglutaminase activity (56). When truncated at K513 and A502, FXIII-A affinity for small Gln containing substrates only decreases 2 fold, but the turnover rate experiences a 4 fold decrease. This decrease in turnover when the β -barrels are truncated and complete abolishment of activity when truncated at the Ca²⁺ binding site demonstrates not only the importance the β -barrels play in primary amine recognition/turnover, but also the necessity of an intact Ca²⁺ binding site within the catalytic core (56). Souri *et al.* has nicely demonstrated the importance of the β -barrels in heterotetramer formation (98-99). FXIII-A I464stop as well as two mutants that excluded either β -barrel 1 or β -barrel 2 were all unable to form FXIII-A₂B₂ heterotetramers. In the present study 607-619 in β -barrel 1 and 632-646 in β -barrel 2 experienced 14.5 and 10.6 % protection from solvent when bound to FXIII-B. These results further substantiate the hypothesis that the β -barrels are essential for heterotetramer formation and give specific residues involved in the FXIII-A₂ – FXIII-B₂ interaction.

It is known that in plasma FXIII-A₂B₂ is largely associated with fibrinogen (100). The binding site of fibrinogen on FXIII-A and/or FXIII-B has yet to be identified. Lai *et al.* demonstrated that even when truncated at Y481, FXIII-A still binds to fibrinogen even though it has lost > 99 % of FXIIIa activity (56). It was proposed that the FXIII-A fibrinogen binding site was independent of the substrate recognition site (71). The protection seen in the β -barrels would allow for FXIII-A₂ – FXIII-B₂ interactions while still bound to fibrinogen.

Although FXIII-B has not been crystallized, β 2-glycoprotein I (β 2-GPI) contains similar short consensus repeats also referred to as GP-I's or sushi domains.

Topological comparisons confirmed structural similarity between the 4 sushi domains of β 2-GPI and the sushi domains in 3 other proteins (r.m.s.d

< 2.5 Å) (101). Using the sushi domains of β 2-GPI as a model, it was

determined that a typical sushi domain is 38 Å × 20 Å × 17 Å (Figure 30), therefore, an elongated FXIII-B would be ~380 Å in length (101). The FXIII-A₂

Figure 33: β 2-glycoprotein I – Representative sushi domain. The two representative sushi domains are illustrated as (cyan) cartoon ribbons with the four cysteines involved in disulfide bond denoted as (red) sticks. Each sushi domain is ~ 38 × 17 × 20 Å so therefore, the 10 FXIII-B sushi domains would span ~380 Å(101).

dimer has a length of ~ 102 Å and each β -barrel is approximately the same size as a sushi domain (41-42). If FXIII-B₂ took the orientation proposed by Souri *et al.* (Figure 30) where the first sushi domain of each FXIII-B was interacting with the β -barrels and domains 4 and 9 were involved in FXIII-B₂ dimer interactions, FXIII-B₂ would stretch > 380 Å. Since the FXIII-A₂ dimer is only 102 Å, the FXIII-B₂ dimer would create a D-shaped loop tethered by the first sushi domain to opposing FXIII-A₂ β -barrels. Judging by the HDX results, the FXIII-B loop elicits protection across FXIII-A₂, but solvent protection is primarily observed at the β -barrels (the putative FXIII-B₂ binding site) and the dimer interface.

HDX has allowed for the first glimpse of the regions of FXIII-A₂ that play an integral role in FXIII-A₂B₂ heterotetramer formation. It has long been known that when bound to FXIII-B₂, FXIII-A₂ is protected from proteolytic degradation. The near global HDX protection observed in FXIII-A₂ in the FXIII-A₂B₂ heterotetramer, brings forth new evidence of the protection bestowed upon FXIII-A₂. The data presented here further emphasizes the importance of the FXIII-A₂ β -barrels in the interaction between FXIII-A₂ and FXIII-B₂. These studies aid in presenting a clearer picture of FXIII-A₂B₂ heterotetramer formation. A better understanding of the residues involved in this interaction could potentially be used to promote either the FXIII-A₂B₂ formation for prevention of aberrant clotting or the release of FXIII-A₂ for pro-coagulant activity.

Chapter V

DEVELOPMENT OF A FIBRINOGEN ALPHA-C EXPRESSION SYSTEM UTILIZING MINIMAL MEDIA AND SUBSEQUENT ALPHA-C STRUCTURAL ANALYSIS VIA NMR

Introduction

Factor XIII works in concert with thrombin (IIa) and fibrinogen during the last steps of the blood coagulation cascade. Fibrinogen (Figure 34) a 340 kDa

Figure 34: Schematic of fibrinogen and the dissociation of the α C domain when converted to fibrin(102). A. A Fibrinogen molecule with the D-E-D nodules linked by the coiled coil connectors and the α C domains interacting with each other and the central E domain via the B peptides. Residue 374 has been labeled to give a point of reference. B. Diagram of a fibrin protofibril where the α C domains have dissociated after Fbg peptides A and B (marked with * in A) were cleaved by IIa.

protein consists of three chains ($A\alpha$, $B\beta$ and γ) forming the homodimer $(A\alpha B\beta\gamma)_2$. The rod-like dimer of trimers has a central E-region which contains the N-termini of the fibrinogen chains. The two $A\alpha$, $B\beta$ and γ chains extend in opposing directions from the E-region in a coiled coil and terminate in two globular D-regions. Thrombin is responsible for cleaving fibrinopeptides A and

B (marked with * in Figure 34 A) from the N-termini of the $A\alpha$ and $B\beta$ chains to form fibrin I and fibrin II respectively (103). The resultant fibrin monomers can align E and D-regions to form a soft clot. Thrombin, as mentioned previously, is

also responsible for activating FXIII by cleaving the activation peptide at R37-G38. Fibrin acts as a cofactor for the activation of FXIII by decreasing the concentration of Ca^{2+} needed for FXIII-A₂ to dissociate from FXIII-B₂ and also accelerating the activation peptide cleavage by IIa (29, 31, 74).

The C-terminal portion of the A α chain extends beyond the D-region to form the α C-domain (104). The α C-domain (221-610) interacts intramolecularly with each other and the central E-region of fibrinogen until fibrin assembly. During assembly, they dissociate from the E-region and interact intermolecularly promoting lateral aggregation of protofibrils in a process catalyzed by FXIII (105-107). The process of aggregation begins with γ -chain dimerization of fibrin and is followed by α -chain polymerization (108). The sites of intramolecular cross-linking on the α C-domain are: Gln-A α -328, Gln-A α -366, Lys-A α -508, Lys-A α -556 and Lys-A α -562 (109). There are several crystal structures of fibrinogen, but the α C-domain has been elusive in structural characterization (110-111). The α C-domain, primarily bovine 374 – 538 (human equivalent 392 – 610) has been studied extensively by NMR. These studies have led to the discovery of a β -hairpin which is held together by a disulfide linkage C423 – C453 at its base (102, 112-113). The crosslinking of fibrin monomers by FXIII-A₂ is critical to the formation of stable insoluble clots, yet to date there have not been any studies exploring the structure of the α C-domain in the presence of FXIII-A₂.

Fibrinogen α C-domain (233-425) expression

In order to monitor the conformational dynamics of the fibrinogen α C-domain in the presence of FXIII-A₂, α C-domain (233-425) was expressed in *E. coli*. The gene coding for α C (233-425) was cloned into the pGEX-6P-1 vector which has a glutathione-S-transferase (GST) tag as well as ampicillin resistance. There were no mutations made to the primary sequence, so the purified DNA was transformed into DH5 α *E. coli* to create DNA stocks. After collecting DNA via mini prep, the DNA was subsequently transformed into BL21 Gold (DE3) cells for protein expression. The BL21 Gold (DE3) competent cells are the suggested host for pGEX-6P-1 plasmids.

Once α C expression and purification was confirmed (Figure 35) using conventional methods (LB broth), a method for protein expression was developed utilizing minimal media. Minimal media allows for growth with only the essential nutrients. Providing only the essential nutrients allows for maximizing the incorporation of isotopic labels not found in standard media. To enable future studies using NMR, it was imperative to develop a method of expressing the α C (233-425) domain in the presence of isotopically labeled media (^{15}N , ^{13}C and/or ^2H). Due to the high cost of isotopically labeled nutrient sources, there have been several methods designed to maximize growth with limited nutrients (114-116). The α C domain (233-425) was successfully expressed in BL21-Gold

Figure 35: α C Domain (233-425) Purification Confirmation. This is a representative PAGE gel confirming the presence of α C after expression in BL21 Gold cells and purification using GST affinity columns on an AKTA prime system.

cells using a method adapted from Marley *et. al* and Paliy, *et. al* with minimal media containing $^{15}\text{NH}_4\text{Cl}$ (114-115).

NMR methods for analyzing unstructured proteins

Over the past few decades there have been tremendous advancements in NMR technology allowing for protein analysis. NMR has situated itself at the forefront as a method of elucidating protein structure, monitoring molecular dynamics, ligand-receptor interactions, and kinetics (117). To date over 8300 NMR-derived protein structures have been submitted to the Protein Data Bank (PDB). One benefit to NMR analysis of proteins is the ability to gather structural information that is not available through contemporary crystallization techniques. Proteins that do not crystallize well such as membrane bound proteins or proteins that contain flexible regions (118) that are not visible using X-ray technology are of particular interest.

Of particular importance in the initiation of protein structural analysis is the 1D ^1H NMR and the ^{15}N -HSQC (Heteronuclear Single-Quantum Correlation) spectrum (119). The 1D ^1H NMR simply displays signals for each proton and the 2D ^{15}N -HSQC shows a unique signal for each proton bound to a ^{15}N . In a 2D ^{15}N -HSQC experiment, the applied magnetization is transferred from any ^1H attached to a ^{15}N nuclei, to the ^{15}N through scalar coupling (*J-coupling*). The chemical shift then evolves and is detected after it is transferred back to the ^1H (Figure 36). The resulting 2D spectrum contains a ^{15}N axis and a ^1H axis, and the ^1H chemical shifts depend upon the chemical environment (electron density) surrounding it. Therefore each backbone amide (except proline) gives rise to a

Figure 36: Representative tripeptide displaying the magnetization transfer during a ^{15}N -HSQC. The amide N and H are red to illustrate the atoms involved in the transfer of magnetization. The arrow indicates the travel of energy from the ^1H to the ^{15}N then back to the ^1H for detection. The 'C_R' represents the amino acid side chains.

unique resonance at the ^{15}N and ^1H frequencies for that individual amide group.

The more shielded the amide is the more its signal will shift upfield and vice versa. By observing the dispersion of the ^{15}N -HSQC chemical shifts, one can estimate whether a protein is unstructured or contains any defined structure.

The αC domain (233-425) contains 2 glutamine residues Gln-A α -328 and Gln-A α -366 that are known substrates for FXIII-A₂ during blood coagulation. Prior to transglutmainase activity, the αC domain of fibrin is also known to associate with FXIII-A2B2. The αC domain is believed to be unstructured in solution therefore eluding crystallography (40-41). The minimal binding domain of hypoxia-inducible transcription factor HIF-1 α is an example of an unstructured protein in solution that adopts a structure after it binds, with nM affinity, to CBP (CREB-binding protein) (118, 120). This conformational change in HIF-1 α is easily observed through the dispersion of the ^{15}N -HSQC chemical shifts after addition of CBP to ^{15}N labeled HIF-1 α . Using these same techniques the αC domain (233-425) of fibrin was expressed in minimal media to produce ^{15}N -

labeled protein. ^{15}N -HSQC was then utilized to monitor peak dispersion (structure formation) when FXIII-A₂ was added to the solution.

Materials and Methods

Preparation of α C domain (233-425) DNA stocks

The α C-domain (233-425) pGEX-6P-1 plasmid (GE Healthcare, Piscataway, NJ) DNA was a generous gift from Helen Philippou, Robert Ariens and Kerrie Smith at the University of Leeds, Leeds Institute of Genetics Health and Therapeutics (121). It should be noted that all materials used during transformation, expression and purification were sterile. DH5 α *E. coli* cells (Invitrogen, Carlsbad, CA) were transformed to build an α C (233-425) DNA stocks. The first step in transforming the DH5 α cells was transferring ~5 ng α C (233-425) DNA into a 50 μ l aliquot of DH5 α competent cells in a 1.5 ml tube. This DNA/*E. coli* mix was placed on ice and incubated for 30 min. Following 30 min on ice the cells were heat shocked for 20 seconds in a 42 °C water bath and then returned to the ice for an additional 2 min. The transformation was then brought to temperature with the addition of 950 μ l 37°C LB media (Appendix C). The cell mix was then incubated for 1 hr at 37 °C while shaking at 225 rpm. After incubation, if transformation efficiency is low, the cells could be concentrated by spinning for 2 sec at 10,000 rpm and removing 500 μ l supernatant prior to resuspending cells. In most cases this step was unnecessary and 75 μ l of the transformed cells were plated on LB agar + Ampicillin plates (Appendix C). The plates incubated for 16 hr at 37 °C. Following transformation, a single intact colony was utilized to inoculate 4 ml LB agar containing 100 μ g/ml Ampicillin. The 4 ml culture was incubated at 37 °C with shaking at 225 rpm for 16 hr. The

DNA was then purified using the QIAprep Miniprep kit (Qiagen Inc., Valencia , CA). Following purification, the DNA concentration was determined using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). A typical DNA yield for an α C-DNA Miniprep was 60-80 ng/ml. Purified DNA was also sent for sequencing using pGEX-6P-1 3' and 5' primers (GE Healthcare, Piscataway, NJ).

Transformation of α C domain (233-425) in BL21 Gold (DE3) cells

Once DNA stocks were established, BL21 Gold (DE3) cells were utilized to express the α C-domain (233-425) for purification. A 1 – 2 μ l aliquot totaling 10 – 25 ng DNA was added to a 14 ml round bottom tube with 100 μ l BL21-Gold (DE3) *E. coli* cells (Agilent Technologies, La Jolla, Ca). The BL21 cells were incubated on ice for 30 min then heat shocked in a water bath for 20 sec at 42°C. Following the 20 sec heat shock, the cells were placed on ice for additional 2 min. The cells were then brought to temperature through the addition of 1 ml prewarmed (42 °C) SOC media (Appendix C). After addition of SOC media, the cells (150 μ l) were then plated on LB agar + Ampicillin and incubated overnight at 37 °C.

Following incubation, a colony of cells was transferred to 5 ml LB broth containing 100 μ g/ml Ampicillin. The liquid culture was placed in the incubator where the cells were incubated at 37 °C for 16-20 hr and then utilized to establish BL21-Gold cell stocks. Stocks were made by adding 5 ml 50 % glycerol to the cells then aliquoting and storing them at -70 °C for later use.

Expressing α C (233-425) in BL21 Gold (DE3) cells

The expression of α C (233-425) in transformed BL21 stocks can take place in any number of volumes. For example sake the steps used for a 2 L culture are described. First a 200 ml starter (or seed) culture was grown overnight after inoculation with the frozen BL21 stocks. The starter culture consisted of 180 ml LB broth, 20 ml phosphate buffer and 200 μ l 100mg/ml Ampicillin (Appendix C). The following day prior to addition of cells, 1800 ml Terrific Broth, 200 ml phosphate buffer and 2 ml 100mg/ml Ampicillin was prepared in a 4 L flask. An aliquot was taken and analyzed on the Cary 50 UV/VIS to establish a baseline ($A_{600} = 0$). Once a baseline was established for $T = 0$, the 200 ml starter culture was added to the flask.

The culture (2 L) was shaken (225 rpm) at 37 °C until the absorbance (cell growth) $A_{600} = 0.8 - 1.0$. At which time 2.2 ml of 1 M Isopropyl β -D-1-thiogalactopyranoside (IPTG), a molecular mimic of a lactose metabolite, was added to the culture. The addition of IPTG triggers *lac* operon transcription on the pGEX-6P-1 plasmid leading to the over expression of α C (233-425). After addition of IPTG, the culture was shaken (180 rpm) at 30°C for 16 hours. The cells were then transferred to centrifuge bottles and harvested during sequential 10 min spins at 5000 g and 4 °C. The harvested cell pellets were resuspended in 180 ml ice cold *E. coli* wash buffer (Appendix C) by stirring (800 rpm) for ~40 min. Lastly, the cell suspension was transferred to 50 ml conical tubes and centrifuged in a swinging bucket rotor 45 min at 5000 g and 4 °C. The

supernatant was discarded and the cell pellets were placed at -20 °C until purification.

Expressing ^{15}N - αC (233-425) in BL21 Gold (DE3) cells with Minimal Media

For isotopically labeling αC (233-425) the transformed BL21 cells were grown in minimal media. The recipes used for M9 salts and M9 media were adapted from Marley *et al.* and Paliy, *et al* (114-115). The seed culture for minimal media expression was grown in standard LB broth. For a 2 L culture, two sterile 50 ml conical tubes containing 36 ml LB broth were inoculated with the frozen stocks of transformed BL21 cells. The starter culture was grown overnight at 37 °C while shaking at 225 rpm. The following morning the two starter cultures were spun at 5000 g for 10 min and the LB broth was discarded. Being careful not to disturb the

pellet, the pellet was

washed 2× with 5 ml

1X M9 salts (Table

5). The pellet was

then resuspended in 2 ml 1× M9 salts and transferred to 2 L of M9 media (Table 5 and Table 6). Prior to addition of cells to the M9 media, an aliquot was taken and used to establish an A_{600} baseline. Cell growth was monitored and the cells were induced with the addition of 2 ml 1 M IPTG. The cells were then incubated overnight at 30°C while shaking at 180 rpm. The 2 L culture was then spun down, washed and spun down again before freezing in the same manner previously described for growth in LB broth.

Table 5: Components of 1 L 5× M9 salts and 1× concentration. The mass needed for 1 L 5× M9 salts and the final salt concentration after dilution to 1×.

	Mass (g)	1× (mM)
KH_2PO_4	30	44
Na_2HPO_4	108	96
NaCl	2.5	9

Table 6: Recipe for M9 minimal media. This table outlines all the components added to 1 L of M9 growth media. For each component, the volume added as well as the final concentration are given.

Volume (ml)	M9 Media component	Final Concentration
200	5× M9 salts	1×
1.00	1 M MgSO ₄	1.0 mM
1.00	200 mM CaCl ₂	0.2 mM
4.00	^a 0.25 g/ml NH ₄ Cl	1.0 g/l
0.05	20 % Thiamine HCl	0.001 %
1.00	100 mg/ml Ampicillin	0.1 mg/ml
40.0	20 % Dextrose	0.8 %
753	Sterile dH ₂ O	

^a For expressing isotopically labeled proteins, ¹⁵NH₄Cl was utilized as opposed to NH₄Cl.

Purification of αC (233-425)

The αC (233-425) produced during growth in LB media or M9 media was purified using the same process. Hot water was run over tubes and a spatula utilized to help transfer the frozen pellets to a clean 500 ml beaker. The tubes were rinsed with and resuspended in ice cold PBS (4 ml PBS/100 ml culture – 88 ml PBS for 2L). The cells were stirred gently at room temperature to resuspend. Once resuspended, 888 µl 100 mg/ml Lysozyme [1 mg/ml] (the [] indicate assay concentration) and 88.8 µl 1 M DTT [1 mM] was added to the cell suspension and incubated at room temperature, stirring gently, for 30 min. The protease inhibitors listed in Table 7 were added to the cell suspension and incubated for 30 min at 4 °C stirring gently. Following incubation with protease inhibitors, 222 µl 100 mM phenylmethylsulfonyl fluoride [0.5 mM] and 449 µl 10% Sodium Deoxycholate were added and the cell mix was stirred for 30 more minutes. The addition of 20% Triton X100 [1%] was done forcefully then 47.3 µl 10mg/ml DNase 1 [5 µg/ml] was introduced with 472.5 µl 1 M MgCl₂ [5 mM]. This was

Table 7: The protease inhibitors utilized in during purification of recombinant protein.

Added to 2 L Culture	Protease Inhibitor	Assay Concentration
88.9 μ l	2 mg/ml Aprotinin	[2 μ g/ml]
88.9 μ l	1 mM Pepstatin A	[1 μ M]
222 μ l	4 M Leupeptin	[10 μ M]
0.069 g	Benzamidine	[4 mM]

incubated for 10 min at 4°C with stirring then quenched with the addition of 1.15 ml 0.5 M EDTA [6 mM]. The cell suspension was then transferred to centrifuge tubes and spun for 20 minutes at 22,000 g. The supernatant was filtered using 0.22 μ m Supor machV 150 ml filter-flasks (Nalgene, Rochester, NY).

The purification process takes advantage of GST that is co-expressed with the α C (233 – 425). Utilizing an AKTA prime system, with GST specific GSTrap™ HP Columns (GE Healthcare, Piscataway, NJ) the GST- α C was trapped on the column (Figure 37). After overnight digestion with PreScission Protease, 1 unit per 100 μ g protein, (GE Healthcare, Piscataway, NJ) the free α C (233 – 425) was eluted from the column. To ensure a clean sample for future NMR studies the unlabeled α C (233 – 425) product was cleaned using a Detergent Removal Spin Column (Pierce, Rockford, IL). The 15 N α C (233 – 425) was dialyzed into 1× PBS and all recombinant α C was concentrated using an Amicon Ultra centrifugal concentration devices MWCO = 10,000 (Millipore, Billerica, MA). The concentration was established using an extinction coefficient of 41,480 M⁻¹ cm⁻¹ calculated using ExPASy ProtParam (<http://web.expasy.org/protparam/>).

Figure 37: Visualization of αC (233-425) purification following expression in BL21 Gold *E. coli* cells.

- A)** There are two different schemes shown; one for eluting αC (233-425) still bound to the GST linker (steps 2 – 4), and the other describes an on column digest to elute just αC (233-425) from the GSTrap column (steps 5 – 9). Step (1) is the addition of cell lysate to the GSTrap (GST affinity column). For elution of the GST- αC conjugate, the column was rinsed (2), and reduced glutathione is pumped across the column (3) releasing the GST-αC from the glutathione-sepharose column material. The GST-αC (233-425) fraction is collected and stored at 4 °C until further analysis (4). For on-column digests, after the addition of cell lysate the column was rinsed (5). PreScission protease was then added to the column (6) and allowed to incubate at 4 °C overnight (7). The following morning an additional GSTrap column was added to capture any possible GST that may be released (8) and the recombinant αC (233-425) was eluted and stored at 4 °C until further analysis (9).
- B)** Representative SDS-PAGE gel and Western Blot image. In the SDS-PAGE gel the green band represents αC (233-425) and the pink band GST- αC (233-425). The GST specific Western Blot is representative of a blot containing un-cleaved GST- αC (233-425).

Adapted from GST Gene Fusion System Handbook (122)

SDS-PAGE and Western Blot

All expression products were confirmed using SDS-PAGE. The 15 % resolving gel was prepared by combining 2.4 ml dH₂O, 2.5 ml 1.5 M Tris (pH = 8.8), 5.0 ml 30 % acrylamide and 0.1 ml 10 % SDS. The acrylamide polymerization was initiated with the addition of 50 µl Ammonium Persulfate (APS) and 5.0 µl Tetramethylethylenediamine (TEMED). The 4.0 % stacking gel which was poured on top of the resolving gel consisted of: 6.1 ml dH₂O, 2.5 ml 0.5 M Tris (pH = 6.8), 1.3 ml 30 % acrylamide and 0.1 ml 10 % SDS. Once again, the polymerization was initiated with 50 µl APS and 10 µl TEMED. Samples were mixed with non-reducing sample buffer. The Bio-Rad MiniGels (Hercules, CA) were run at 200 V for 1 hr. The PAGE gel was then either subjected to Western Blot or Coomassie Blue (0.1 % Coomassie Blue, 40 % MeOH, 10 % Acetic Acid and 50 % dH₂O) staining. For staining, gels were placed in Coomassie Blue stain overnight then destained for approximately 3 hours in Destain (40 % MeOH, 10 % Acetic Acid and 50 % dH₂O). Gels were then imaged using a Gel Doc™ XR+ System (Bio-Rad, Hercules, CA).

After SDS-PAGE, western blots were utilized to confirm the presence of GST – αC (233 – 245) or determine whether all GST was removed after on-column PreScission protease digestion. The gel, fiber pad, filter paper, and nitrocellulose membrane (NitroBind, Osmonics Inc.) were equilibrated by soaking them in the Western Blot Transfer Buffer (25 mM Tris, 192 mM Glycine and 20 % MeOH pH=8.3) for 15-20 min at room temperature. The Mini Trans-Blot (Bio-

Rad Hercules, CA) was loaded as per instructions and run at 100 V for 1 Hr at 4 °C. To decrease non-specific binding, the nitrocellulose membrane was placed in a 3 % BSA Blocking Solution in PBST with stirring overnight at room temperature. The following morning, the membrane was washed in PBST then placed in a solution containing 1:5000 α -GST-HRP (horseradish peroxidase) (GE Healthcare, Piscataway, NJ). After a series of 5 rinses in 1 $\times$ PBST, the membrane was developed using 1-Step TMB-Blotting solution, a blue TMB (3,3',5,5'-tetramethylbenzidine) peroxidase which is an HRP substrate (Pierce, Rockford, IL). Membranes were then dried and visualized on the Gel Doc™ XR+ System (Bio-Rad, Hercules, CA).

¹⁵N-¹H HSQC

After the ¹⁵N labeled and unlabeled α C domain (233 – 425) were dialyzed and concentrated, they were loaded into NMR Shigemi tubes. The concentration in the NMR ranged from < 100 μ M for the unlabeled α C domain (233 – 425) to > 200 μ M for the ¹⁵N labeled material and all samples were run in the presence of 10 % D₂O. The pH of NMR samples was not adjusted below 6.6 due to a theoretical pI of 6.28 (<http://web.expasy.org/protparam/>). Two NMR experiments were run to establish the feasibility of determining α C domain (233 – 425) structure. α C domain (233 – 425) was first analyzed with a Watergate ¹H-TOSCY and then the ¹⁵N- α C domain (233 – 425) was utilized in ¹⁵N-¹H HSQC. To determine if the α C domain (233 – 425) adopts a defined compact structure in the presence of FXIII-A₂ a ¹⁵N-¹H HSQC was performed. For this analysis, 350 μ l of 210 μ M ¹⁵N α C (233 – 425) was used to solubilize 3.2 mg dialyzed and lyophilized FXIII-A₂. The final concentrations in the NMR tube were 110 μ M

FXIII-A₂ and 210 μM ^{15}N αC (233 – 425). All NMR experiments were performed on a 16.4 Tesla 700 Varian Inova MHz NMR or an 18.8 Tesla 800 MHz Varian Inova NMR.

The NMR data shown in Chapter V were collected on the Varian Inova 800 MHz spectrometer which is equipped with a triple resonance probe and pulsed-field triple axis gradients. ^1H – ^{15}N HSQC experiments were run on the ^{15}N αC (233-425) in the absence and presence of unlabeled FXIII A₂. For both systems, the ^1H frequency was 799.708 Hz and the sweep width was set at 12000 whereas the ^{15}N frequency was 81.043 Hz and the sweep width was set at 2300. Spectra were collected with 1800 complex points in the ^1H dimension and 100 increments in the ^{15}N dimension. The number of transients was 8. All HSQC experiments were run at 20°C and water was used as a reference. The NMR data were processed using NMRPipe and NMRDraw version 6.5 (123) and the results visualized using Sparky version 3.1(124). The ppm reference for the center of both 2D spectra was 4.821 ppm and 119.990 ppm. Sparky was also used to overlay the HSQC spectrum of ^{15}N αC (233-425) on to the spectrum of the protein-protein complex involving ^{15}N αC (233-425) and FXIII A₂. The Overlay Views dialog box was used. Both spectra had been subjected to the same experimental parameters, referencing, and data processing strategies.

Results

α C (233 – 425) Expression in LB Broth

To establish an expression system in the lab two systems were chosen for method development, FXIII-A₂ and α C (233 – 425). The α C (233-425) gene was cloned into pGEX-6P-1 vectors (Figure 38). The location of the α C gene insert was ideal for PreScission protease cleavage and GST cleavage during purification.

The first objective was to transform the cloned pGEX-6P-1 vector into

Figure 38: Map of pGEX-6P-1 vector. Elements of interest are labeled. The elements to note are the tac_promoter (Orange), GST-tag which contains the pGEX-5' primer (Blue), alpha_C gene (Red), pGEX_3_primer and the Ampicillin gene (Purple). Image created using DNA 2.0 Gene Designer 2.0 software.

DH5 α cells in order to build a series of DNA stocks. Using the transformation methods set forth by Smith *et. al.*, the DNA yields from a standard MiniPrep were consistently 60-80 ng/ml (121). This allowed for the storage of several DNA aliquots to be used in future studies. To confirm that the purified DNA contained the α C (233 – 425) gene, it was sequenced using the pGEX-6P-1 3' and 5'

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Query 1   ACAGACATGCCGAGATGAGAATGGAGTTAGAGAGACCTGGTGGAAATGAGATTACTCGA 60
          |||
Sbjct 634 ACAGACATGCCGAGATGAGAATGGAGTTAGAGAGACCTGGTGGAAATGAGATTACTCGA 575

Query 61  GGAGGCTCCACCTCTTATGGAACCGGATCAGAGACGAAAGCCCCAGGAACCTAGCAGT 120
          |||
Sbjct 574 GGAGGCTCCACCTCTTATGGAACCGGATCAGAGACGAAAGCCCCAGGAACCTAGCAGT 515

Query 121 GCTGGAAGCTGGAACCTCTGGGAGCTCTGGACCTGGAAGTACTGGAACCGAAACCTGGG 180
          |||
Sbjct 514 GCTGGAAGCTGGAACCTCTGGGAGCTCTGGACCTGGAAGTACTGGAACCGAAACCTGGG 455

Query 181 AGCTCTGGGACTGGAGGGACTGCAACCTGGAACCTGGGAGCTCTGGACCTGGAAGTGCT 240
          |||
Sbjct 454 AGCTCTGGGACTGGAGGGACTGCAACCTGGAACCTGGGAGCTCTGGACCTGGAAGTGCT 395

Query 241 GGAAGCTGGAACCTCTGGGAGCTCTGGAACCTGGAAGTACTGGAACCGAAACCTGGAAGT 300
          |||
Sbjct 394 GGAAGCTGGAACCTCTGGGAGCTCTGGAACCTGGAAGTACTGGAACCGAAACCTGGAAGT 335

Query 301 CCTAGACCTGGTAGTACCGGAACCTGGAATCCTGGCAGCTCTGAACGCGGAAGTGCTGGG 360
          |||
Sbjct 334 CCTAGACCTGGTAGTACCGGAACCTGGAATCCTGGCAGCTCTGAACGCGGAAGTGCTGGG 275

Query 361 CACTGGACCTCTGAGAGCTCTGTATCTGGTAGTACTGGACAATGGCACTCTGAATCTGGA 420
          |||
Sbjct 274 CACTGGACCTCTGAGAGCTCTGTATCTGGTAGTACTGGACAATGGCACTCTGAATCTGGA 215

Query 421 AGTTTTAGGCCAGATAGCCAGGCTCTGGGAACGCGAGGCCTAACAACCCAGACTGGGGC 480
          |||
Sbjct 214 AGTTTTAGGCCAGATAGCCAGGCTCTGGGAACGCGAGGCCTAACAACCCAGACTGGGGC 155

Query 481 ACATTTGAAGAGGTGTCAGGAAATGTAAGTCCAGGGACAAGGAGAGAGTACCACACAGAA 540
          |||
Sbjct 154 ACATTTGAAGAGGTGTCAGGAAATGTAAGTCCAGGGACAAGGAGAGAGTACCACACAGAA 95

Query 541 AACTGGTCACTTCTAAAGGAGATAAAGAGCTCAGGACT 579
          |||
Sbjct 94  AACTGGTCACTTCTAAAGGAGATAAAGAGCTCAGGACT 56

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Figure 39: Alignment of 3' primer sequence data (top) and α C (233 – 425) gene sequence (bottom). primers (3' CCGGGAGCTGCATGTGTCAGAGG and 5'

GGGCTGGCAAGCCACGTTTGGTG) (Figure 38). Both the 5' and 3' primers produced sequence data that confirmed the entire α C (233 – 245) construct sequence (Figure 39). The two mis-hits (nucleotide 297 and 300) in Figure 39

were both mutations that did not lead to a change in amino acid. The G to A (GG(A)) mutation at nucleotide 297 remains a glycine and the C to T (AG(T)) mutation at nucleotide 300 remains a serine.

Initially, protein expression took place in standard LB media. The DNA collected from DH5 α cells was successfully transformed into BL21 Gold (DE3) cells. During the first attempts at collecting purified protein from a 2 L culture, the decision was made to elute the uncleaved GST- α C (233 – 425) from the GSTrap column. The next step was developing a method to release the GST from the α C (233 – 425). This was done via an on-column digest (Figure 37) which utilized PreScission protease. PreScission protease selectively cleaves between Gln and Gly in a sequence (Leu Glu Val Leu Phe Gln | Gly Pro) found N-terminally to α C (233 – 425). Therefore, after

transformation and purification the cell extract was loaded on the GSTrap column and then the column was removed from the AKTAprime system. At which time PreScission protease was added and incubated on-column overnight at 4 °C to allow for cleavage between α C (233 – 425) MW = 19582 g/mol and GST MW = 27,000 g/mol. The GST and GST-tagged PreScission protease remain on the column and the cleaved α C (233 – 425)

Figure 40: Representative GST specific Western Blot. Lane (1) was collected from the waste line while loading the column with cell extract. (2) was the cell extract. (3) was the concentrated α C product with GST contaminant. (4) was the ladder. (5 and 6) were collected during GST elution.

was eluted the following morning. SDS-PAGE and a GST-specific western blot were run to evaluate the expression and purification process (Figure 40). The GST-specific western blot exposed that GST was eluting with the α C (233 – 425); therefore, in future protein elutions an additional GSTrap column was added post on-column digestion to sequester any GST that may be co-eluting with α C (233 – 425).

Figure 41: Representative SDS-PAGE and western blot confirming proper α C (233 – 425) purification and GST cleavage. (A) The first three wells in the SDS-PAGE illustrate the α C (233 – 425) was sufficiently cleaved and eluted. The GST elution illustrated in wells 5 – 7 demonstrated minimal residual α C (233 – 425). This carryover was not affected by increasing the column wash-time/volume between α C (233 – 425) and GST elution. (B) Wells 1 – 3 of the GST-specific western blot demonstrated a clean α C (233 – 425) elution free from GST. Wells 5 – 7 contain the cleaved GST fraction.

Expresssion in minimal media

Expression in minimal media proved to be just as successful as α C (233 – 425) expression in under standard nutrient conditions. The true yields of both LB and minimal media expressions are difficult to report due to loss of material during storage, dialysis, and surfactant removal but both methods were successful in producing enough protein for NMR analysis. During minimal media expression and purification, the GST that had eluted with purified α C (233 – 425) previously was eliminated (Figure 41B). One issue that did occur in all

expressions was mild carryover after α C (233 – 425) elution. The α C (233 – 425) that eluted with the GST was seen as a faint band around 20 kDa in the GST fractions (Figure 41A). This band occurred even after increasing the column wash time following α C (233 – 425) elution. Another possible issue is the band in the western blot around 45 kDa. This could possibly be conjugated GST- α C (mw = 46,582 kDa). If that were the case, additional PreScission protease should yield more α C (233 – 425) and decrease the intensity of the band at 45 kDa. This was attempted and the 45 kDa band was still quite prominent; therefore, the large band around 45 kDa may be due to non-specific binding. The α C (233 – 425) expression system proved to be quite versatile and allowed for protein production under standard nutrient conditions and under minimal media with ^{15}N - NH_4Cl .

α C (233 – 425) ^{15}N -HSQC

Although the ultimate goal of expressing α C (233 – 425) in ^{15}N -labeled minimal media was to enable NMR observations, the first NMR experiments utilized unlabeled α C (233 – 425). A valuable lesson was learned during our first TOSCY analysis of unlabeled α C (233 – 425). There was substantial contamination from a small molecule in our sample. After reviewing the purification procedures, the problem was identified as Triton X-100 which is added to help solubilize the protein before centrifugation. The Triton X-100 concentration is 1 % and the critical micelle concentration is 0.1 %. The Triton X-100 micelles contain 628 Triton units/micelle therefore has an apparent molecular weight of ~90 kDa. This apparent molecular weight caused the Triton

Figure 42: ^{15}N -HSQC spectra of ^{15}N - αC (233-425) conducted on an 800 MHz (18 T) Varian Inova NMR at 20 °C. The assignments can be viewed in appendix F.

X-100 to concentrate with our protein of interest. In order to thwart this problem in the future two different techniques were utilized, 10,000 MWCO dialysis into 1 × PBS and/or Detergent Removal Spin Columns.

The first ^{15}N -HSQC spectra for ^{15}N - αC (233 – 425) were attained on a 16.4 T Varian NMR equipped with a triple resonance cryoprobe and confirmed on the 18 T Varian NMR (Figure 42). The concentration of αC (233 – 425) was 210 μM and pH = 6.6. As this was an aqueous sample, Watergate was utilized for water suppression. These experiments prove that even at sub mM concentrations of αC (233 – 425) one can still observe the ^1H - ^{15}N -HSQC transitions. αC (233 – 425) contains 193 residues, 17 of which are proline, so therefore there are 176 amide hydrogens. In addition to the amide backbone,

there are 13 asparagines, 3 glutamines (each with two proton signals for the same nitrogen) and 7 tryptophans. Therefore, there should be a total of 199 observable ^{15}N - ^1H signals. The αC (233 – 425) ^{15}N -HSQC successfully identified approximately 184 of the 199 possible signals (Appendix F). Of the 7 Trp all but one was visible and 13 of the 19 Asn and Gln were visible.

Once an HSQC for αC (233 – 425) was established, the next goal was to observe αC (233 – 425) in the presence of FXIII-A₂ (Figure 43). The same 210 μM αC (233 – 425) sample used previously was utilized to solubilize 3.2 mg FXIII-A₂. The sample precipitated out of solution and sonication for 5 min at

Figure 43: ^{15}N -HSQC spectra of ^{15}N - αC (233-425) in the presence of FXIII-A₂ conducted at 20 °C on an 800 MHz (18 T) Varian Inova NMR. The assignments can be viewed in Appendix G.

room temperature did not increase solubility. The pH of the α C (233 – 425) – FXIII-A₂ solution was confirmed to be 6.6 and was spun at 14,000 rpm. The supernatant was then transferred to a Shigemi tube for NMR analysis. Due to precipitation and analysis of the supernatant the final concentration of α C and FXIII-A₂ was unknown, but assumed to be less than the starting concentrations (210 μ M and 110 μ M respectively). The structure of α C (233 – 425) in the presence of FXIII-A₂ was also investigated using an 18 T Varian NMR. When aligned with the ^{15}N - α C (233 – 425) ^1H - ^{15}N -HSQC, ^{15}N - α C (233 – 425) with FXIII-A₂ did not exhibit any major peak shifts but several new peaks appeared. In the HSQC for α C (233 – 425) in the presence of FXIII-A₂, approximately 191 of the 199 possible N-H stretches were observed (Appendix G). This was an increase of 7 over the 184 signals observed for α C (233 – 425). The most notable new peaks are at (N-120.7 ppm, H-8.68 ppm) and (N-121.8 ppm, H-8.36 ppm) as well as the Trp residue (N-129.7 ppm, H-10.15 ppm). It is quite promising to see that even under conditions where some material may have been lost during precipitation, α C (233 – 425) still appears to gain some structure when in the presence of FXIII-A₂.

Discussion

Many kinetic and conformational dynamic studies require specific mutations. Thus it is imperative to establish and maintain functional expression systems. The α C (233 – 425) DNA that was given to our lab has now been successfully transformed into both DH5 α and BL21 DE3 (gold) *E. coli* cells. The transformed DNA was then utilized to express and purify α C (233 – 425) for ^{15}N -HSQC NMR analysis.

α C (233 – 425) Expression

The DNA utilized had the α C (233 – 425) gene cloned into a pGEX-6P-1 vector. The pGEX-6P-1 vector has several benefits. Expression of α C (233 – 425) in the pGEX-6P-1 vector is induced with IPTG. α C (233 – 425) expression is under the control of the *tac* promoter which is induced by IPTG, a lactose mimic. The pGEX-6P-1 plasmid also contains a *lacI^f* gene which produces a repressor for the *tac* gene inhibiting expression until IPTG is added. To ease in protein purification following expression, the protein of interest is co-expressed with a GST tag. The GST tag is then subsequently utilized in affinity chromatography to capture the GST- α C product. Another benefit of the pGEX-6P-1 is that it contains an Ampicillin resistance gene to assist in confirming transformation in your cells of interest.

Two different cell lines were chosen for α C expression, the first of which was DH5 α . The DH5 α cells chosen were the Subcloning Efficiency Competent

Cells which allowed for an efficient transformation and subsequent production of purified DNA for future transformations. One lesson learned during this process was that if the transformation does not produce nice round colonies and the plates are covered with a lawn of *E. coli*, the Ampicillin may have expired. According to Ryan, *et al.* Ampicillin starts to lose activity after 30 days at 4 °C (125). Following expression in DH5 α cells the DNA that had been successfully purified was transformed into BL21 cells for α C (233 – 425) expression and purification.

The *E. coli* strain BL21 gold (DE3) is frequently used as a host for protein expression studies. Bhandari and Gowrishankar reported on the robustness of the BL21 gold expression options (126). BL21 cells also limit the degradation of expressed proteins because they naturally lack genes for the proteases *lon* and *ompT* (127). Although transformation efficiency was never calculated, a nice selection of colonies was always observed. The reported efficiency for pUC18 DNA is $\geq 1 \times 10^8$ cfu (colony forming units)/ μ g of DNA (Stratagene, La Jolla, CA).

Once transformed into the BL21 gold cells, selected colonies were then grown in liquid LB broth to create glycerol stocks of cells ready for α C (233 – 425) expression. The glycerol stocks were stored at -70 °C and a sterile loop was utilized to seed cultures for expression. After an overnight incubation, the starter culture was utilized to seed a larger (1-2 L) culture. There are several variables which are critical to maximizing the amount of α C (233 – 425) expressed, one of which is the proper cell density for induction. During log phase, the doubling time of *E.coli* is typically between 20 – 30 min in nutrient rich

media. It is during this time that cells are in the optimal condition for expressing

Figure 44: Theoretical *E. coli* growth curve. Lag phase displays the slow growth after inoculation. During Log Phase, the cell doubling time is around 20 – 30 min. This is the optimal time for induction of protein expression. During stationary phase, nutrients are limited, cells become stressed and protein degradation can occur. During death phase cells begin to die due to lack of nutrients and cell density decreased over time.

protein (Figure 41). During LB broth, nutrient rich, expressions the cells were induced at $A^{600} = 1.0$ and 0.6 for the minimal media expressions. The slope during log phase decreased from $0.54 A^{600}/\text{hour}$ for growth in nutrient rich media to $0.20 A^{600}/\text{hour}$ in minimal media. This > 2.5 fold decrease in slope led to much longer incubation times prior to induction for the minimal media expressions.

To ensure induction during log phase, the induction for minimal media took place at $A^{600} = 0.6$ since nutrient rich growth appears to be approaching stationary phase when $A^{600} = 1.0$ (Figure 42). After IPTG addition both nutrient rich and minimal media cultures were allowed to incubate overnight at 30°C . This is another variable which could possibly be optimized. By decreasing the rpm of the shaker from 225 to 180 rpm and decreasing the temperature from 37

°C to 30 °C, it slows the growth. It may be worth investigating shorter induction periods at 37 °C and 225 rpm. The last issue concerning expression which was

Figure 45: Representative growth curves for BL21 gold (DE3) cells grown in LB broth and minimal media. LB Broth is represented by the (Red Squares) and the time points utilized for calculating the slope of the Log Phase are marked with a black (X). The minimal media growth curve is represented by (blue diamonds) and the time points utilized for calculating the slope of the Log Phase are marked with a black (X).

overcome involved the use of Triton-100 during the purification process. When viewing the first α C (233 – 425) NMR Triton X100 was the only molecule visible. The Triton X100 was concentrated when using 10,000 MWCO centrifuge concentrators to purify the α C (233 – 425). To thwart this in future α C (233 – 425) expressions, both detergent removal spin columns and dialysis were utilized successfully. Further experimentation could be performed to determine which process is the most effective.

α C (233 – 425) ¹⁵N-HSQC

It was first reported in 1981 that the 242-424 region of the fibrinogen α -chain was involved in reducing the concentration of Ca^{2+} needed for FXIII-A₂ to dissociate from FXIII-B₂ (74). Until recently very little research has been dedicated to further investigating this interaction between the α C-domain (C-terminal portion of the fibrin α -chain) and FXIII-A₂. There have been a series of manuscripts which have focused on determining whether bovine and/or human α C-domain contains compact structure (102, 113, 128). Burton *et al.* utilized NMR to identify and characterize a β -hairpin with a disulfide (C423-C453) restricting the base in bovine α C (374 – 538) (102). The β -hairpin was then further characterized with a shorter α C fragment (406 – 483) focusing in on the compact structure (113). During this study α C (406 – 483) was fully characterized by NMR which allowed for the identification of a second loose β -hairpin that is formed by residues 459 – 476. It was also determined by size exclusion chromatography and analytical ultracentrifugation that the α C (406 – 483) fragment formed oligomers in a concentration dependant manner, starting at concentrations as low as 3 mg/ml (113).

More recently, the knowledge gained from investigating the structure of bovine α C has been utilized to better understand human α C structure. It was determined that human α C (425 – 503) and (392 – 610) form oligomers as a function of concentration like their bovine counterparts (128). The NMR analysis of human α C (425 – 503), equivalent to bovine (406 – 483), was limited due to the oligomerization. Since bovine α C (406 – 483) had been fully characterized, the human α C ¹⁵N-HSQC was compared to the bovine. This comparison

revealed that human α C (425 – 503) contained several of the same peaks that were essential for β -hairpin formation in bovine α C (113, 128).

These human α C structural studies utilizing NMR have yet to describe how α C interacts with FXIII. Smith *et al.* has extensively studied the human α C (233 – 425) fragment and how it interacts with unactivated FXIII-A₂, activated FXIII-A₂ and FXIII-A₂B₂ (121). Surface plasmon resonance (SPR) was used to study a series of truncated α C fragments and it was determined that activated

Figure 46: ^{15}N -HSQC spectra of ^{15}N - α C (233-425) overlaid on the spectra for ^{15}N - α C (233-425) in the presence of FXIII-A₂. The ^{15}N -HSQC spectrum for α C (233-425) is displayed in (blue) and α C (233-425) in the presence of FXIII-A₂ in (pink). The three peaks noted are only visible in the ^{15}N -HSQC spectra for α C (233-425) with FXIII-A₂. The image was produced using the overlay command within Sparky 3 (124).

FXIII-A₂ had low μM affinity for α C and it was localized to residues 389 – 403.

FXIII-A₂B₂ was found to have low nM affinity and surprisingly, FXIII-A₂ zymogen did not bind to any of the α C fragments (121). The ^{15}N -HSQC data reported here investigates α C (233 – 425) both in the presence and absence of FXIII-A₂ zymogen. After the addition of α C to the lyophilized FXIII-A₂ there was a

substantial amount of precipitate formed. It is unknown whether the precipitate correlates to α C oligomerization or possible α C-FXIII-A₂ interactions. The sample for α C with FXIII-A₂ ¹⁵N-HSQC analysis had to be spun down and only the supernatant was analyzed. The increase in total peaks identified for the α C (233 – 425) with FXIII-A₂, as seen in the overlay of the two spectra, alludes to an interaction that induces structure in α C (Figure 46). Our lab has now established an α C (233 – 425) expression system in standard media as well as minimal media capable of producing enough protein for NMR analysis. These NMR studies have now opened the door to further investigation of α C (233 – 425) in the presence of FXIII-A₂.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Factor XIII is one of the last enzymes involved in the blood coagulation cascade and is responsible for cross-linking fibrin monomers to form an insoluble clot. Through the utilization of HDX coupled with MALDI-TOF MS, great strides have been made in clarifying FXIII-A₂ conformational dynamics during activation. The importance of FXIII during haemostasis has led to increased interest in acquiring a greater understanding of how this enzyme interacts with its substrates and cofactors in solution. Therefore, in addition to studying FXIII, NMR was utilized to investigate the FXIII-A₂ substrate fibrin α C (233-425).

The conformational dynamics of Factor XIII-A₂ (zymogen, FXIIIa^{Ia}, FXIIIa^{Ca}, and inhibited with K9-Don) in solution has been extensively studied using HDX (43, 45-46). One area that lacked understanding was how the FXIII-A₂ structure was dependent upon physiological Ca²⁺ concentrations. Chapter II describes the first objective of this project; to investigate the conformational dynamics of FXIII-A₂ under physiological Ca²⁺ concentrations and compare to other metals of interest (Mg²⁺, Ba²⁺, and Cu²⁺). HDX analysis identified regions of FXIII-A₂ where Ca²⁺ elicited unique conformational differences when compared to the other metals studied. There were also several regions of FXIII-A₂ where all metals examined yielded similar conformational changes. Due to the similarities

observed for these metals, they were then tested to determine whether they could promote non –proteolytic FXIII-A₂ activity.

In addition to studying FXIII-A₂ non-proteolytic activation in the presence of 50 mM Ca²⁺, Mg²⁺, Ba²⁺ and Cu²⁺, we also investigated 500 mM Na⁺ and two organic cations EDA²⁺ and TMAC⁺. When investigating each cation individually only 50 mM Ca²⁺ could promote FXIII-A₂ activity. The cations Mg²⁺, Ba²⁺, Na⁺, EDA²⁺ and TMAC⁺ all induced FXIII-A₂ activity when in the presence of 2 mM Ca²⁺. Low mM Ca²⁺ was essential for non-proteolytic activation. The activity observed using organic cations (EDA²⁺ and TMAC⁺) revealed that non-proteolytic activation is not unique to metal ions (44). HDX analysis was then utilized to study the conformational dynamics of non-proteolytically activated FXIII-A₂. These analyses revealed that exposure around the active site and substrate recognition regions was initiated by physiological Ca²⁺ concentrations and set the stage for the larger conformational changes observed after activation.

While working on the FXIII-A₂ conformational dynamics presented in Chapter II, Pinkas *et al.* reported that TG2 is trapped in an open conformation when bound to a substrate-based inhibitor (49). It has been debated whether FXIII-A₂ undergoes a similar conformational change; therefore, in Chapter III HDX was utilized to compare the solvent accessibility of TG2 zymogen to that of activated TG2 and also inhibited TG2. Evidence of the open conformation was observed as increased exposure on the β -barrels and regions of the catalytic core. When comparing FXIII-A₂ to the open conformation of TG2 it becomes obvious that in order to exist in an open conformation, FXIII-A₂ must overcome

substantial steric strain between the β -sandwich of one FXIII-A₂ monomer and β -barrel 2 of the opposing monomer. If FXIII-A₂ does adopt an open conformation, it could lead to further developments in pharmaceuticals and antibodies specific for the open conformation which could be utilized in correcting aberrant FXIII-A during coagulation.

In regards to comparing TG2 and FXIII-A₂ conformational dynamics, future work could take a few directions. Y238C FXIII, a physiological mutant, is a monomer. Now that our lab has established expression systems, it may be possible to express FXIII-A Y283C and compare FXIII-A in the monomer form to TG2. The Y283C monomer would be utilized because, as mentioned in Chapter III, the 'open' conformation seen in TG2 may be sterically impossible for the FXIII-A₂ dimer. Although the Y283C mutant is physiological, it is not stable in whole plasma, having a shorter $t_{1/2}$ than wild type FXIII (99). If the Y283C mutant shows promise, mutation of the homologous TG2 residues may create a stable monomer, which one day might be used as a therapeutic for FXIII deficiency and thus combat postoperative bleeding

After exploring FXIII-A₂ during activation and comparing FXIII-A₂ to the open versus closed conformation for TG2, our focus shifted in Chapter IV to the conformational dynamics of FXIII-A₂ bound to FXIII-B₂ but before activation. It has been proposed that the two β -barrels of FXIII-A₂ interact with FXIII-B₂ in the heterotetramer since heterotetramer formation does not occur when the β -barrels are truncated (99). This evidence is supported by the significant protection from HDX observed in the β -barrels. If the FXIII-B₂ dimer assumes the structure

proposed by Souri, *et al.* its length would be $> 380 \text{ \AA}$ (98). The FXIII-A₂ structure is only 102 Å in length, and therefore, protection was observed throughout FXIII-A₂. One FXIII-A₂ region of interest during activation, the dimer interface, becomes significantly protected when bound to FXIII-B₂, which would limit FXIII-A₂ activation.

To further investigate the FXIII-A₂B₂ heterotetramer, a more intensive HDX method could be performed. Our MALDI-TOF MS method works well, but increased sensitivity and increased sequence coverage is observed using LC-ICR-MS (129-130). Develop an HDX method on an LC-FT-ICR-MS would open many new doors. Historically, LC methods were avoided during HDX experimentation due to increased hydrogen back-exchange during the aqueous separation prior to MS detection. This problem is now avoided by keeping the column and transfer lines chilled to 0 °C and also the advances in LC and column technology that have led to shorter run times (1 min). One factor limiting HDX with MALDI-TOF-MS detection is the mass of the protein and number of proteins being analyzed due to the increase in peptides produced and the inability to distinguish them. A liquid chromatography separation of peptides prior to MS detection allows for the identification of peptides of similar mass that overlap in a MALDI-MS.

Now that the regions of FXIII-A₂ which contact FXIII-B₂ in the FXIII-A₂B₂ heterotetramer have been established, it would be of interest to utilize NMR to determine the regions of αC(233 – 425) that contact on FXIII-A₂B₂. The αC(233 – 425) region is involved in the dissociation of FXIII-A₂ and FXIII-B₂, and may

adopt a unique conformation when interacting with the FXIII-A₂B₂ heterotetramer. It would be nice to compare the analysis of α C(233 – 425) in the presence of FXIII-A₂B₂ to the results reported in chapter V for α C(233 – 425) in the presence of FXIII-A₂.

Lastly, in chapter V, a method was established for expressing α C (233 – 425) in both standard LB broth for bulk protein production and minimal media for isotopically labeling the protein to enable NMR analysis. The α C (233 – 425) domain was chosen because of its known involvement in fibrin crosslinking by FXIII-A₂. Not only does α C (233 – 425) contain 3 FXIII-A₂ Gln substrate residues, it also acts as a cofactor in the dissociation of FXIII-A₂B₂ as well (74, 109). The α C (233 – 425) region is largely unstructured and has yet to be visualized via crystallography (110-111).

In an attempt to determine if the α C (233 – 425) region adopts a compact structure when interacting with FXIII-A₂, ¹⁵N- α C (233 – 425) was expressed in minimal media and subjected to ¹⁵N-HSQC NMR analysis. An increase in the total number of peaks indicates that the interaction between α C (233 – 425) and FXIII-A₂ induces a more structured α C. While these NMR studies were being developed, Smith *et al.* used SPR techniques, to demonstrate that α C (233 – 425) has low nM affinity for FXIII-A₂B₂ and FXIIIa but does not interact with FXIII-A₂ zymogen (121). These results lead to the question of whether α C (233 – 425) adopts a more defined structure when interacting with activated FXIII-A₂ or FXIII-A₂B₂ versus the FXIII-A₂ analyzed.

Once an α C (233 – 425) compact structure is defined, several different heteronuclear methods will be used to assign the HSQC spectrum. TOCSY- ^{15}N -HSQC can be run with a singly labeled ^{15}N - α C-domain. Further analysis could be conducted with doubly labeled ^{13}C - ^{15}N - α C-(233 – 425). The benefit of the doubly labeled protein is the ability to determine sequential residues using HNCO and HN(CA)CO pulse sequences. For assigning more atoms in the amino acid side chains, other 3D experiments such as: HNCA, HN(CO)CA, HN(CA)CB and HN(COCA)CB can be used (131).

To determine the structure, NOE assignments could then be elucidated using HSQC-NOESY, ^{15}N - ^{13}C edited NOESY and ^{13}C - ^{13}C -edited NOESY. The residual dipolar couplings (RDCs) of the amide backbone could be determined using ^1H - ^{15}N IPAP-HSQC with and without Pfl phage for spin alignment (102). The NOE data gathered could then be entered into XPLOR-NIH (132) for structural calculations using the angle restraints calculated by TALOS (133). It would also be possible to determine the K_d of FXIII-A₂ and α C(233 – 425) by titrating in FXIII-A₂ and monitoring the shift in resonances for the residues that contact FXIII. TG2 could also be titrated in to determine if α C(233 – 425) interacts with the two transglutaminases differently. This comparison would aid in identifying differences in TG2 and FXIII substrate specificity.

These studies have shed light upon the illusive FXIII conformational dynamics. This research presents a more global and functional understanding of how FXIII interacts with substrates and allosteric effectors like Ca^{2+} in solution. These discoveries may lead to better control of FXIII-A₂ activity during

thrombosis and atherosclerosis through a more knowledgeable route of drug discovery/design.

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APPENDIX A: A DETAILED DIAGRAM OUTLINING THE BLOOD COAGULATION CASCADE

Kindly provided by Abcam plc, Cambridge, England

APPENDIX B: ABBREVIATIONS FOR BLOOD COAGULATION ENZYMES

Abbreviation	Enzyme
II	Prothrombin
IIa	Thrombin
FV	Factor V
FVa	Activated Factor V
FVII	Factor VII
FVIIa	Activated Factor VII
FVIII	Factor VIII
FVIIIa	Activated Factor VIII
FIX	Factor IX
FIXa	Activated Factor IX
FX	Factor X
FXa	Activated Factor X
FXI	Factor XI
FXIa	Activated Factor XI
FXII	Factor XII
FXIIa	Activated Factor XII
FXIII	Factor XIII
FXIIIa	Activated Factor XIII
vWF	von Willebrand Factor

APPENDIX C: FXIII-A₂ PEPTIC PEPTIDES

Peptide	m/z ^a	Amino Acid Sequence
1-23	2431.30	SETSRATFGGRRVPPNNSNAAE
32-40	926.15	QGVVPRGVN
53-74	2819.06	FKERWDTNKVDHHTDKYENNK
75-82	963.03	IVRRGQSF
83-99	2188.48	YVQIDFSRPYDPRRDLF
88-98	1422.78	FSRPYDPRRDL
100-111	1509.78	RVEYVIGRYPQE
137-144	1031.09	REDRSVRL
145-159	1682.03	SIQSSPKCIVGKFRM
171-180	1177.20	RETSRNPETDT
194-208	1930.10	YLDNEKEREYVLND
214-230	2051.24	YGEVNDIKTRSWSYGQF
220-230	1372.70	IKTRSWSYGQF
240-247	1014.09	YVMDRAQM
248-264	1713.97	DLSGRGNPIKVS RVGSA
248-265	1845.15	DLSGRGNPIKVS RVGSAM
298-305	980.97	ILLEYRSS
300-314	1800.83	LEYRSSEN PVRYGQC
325-338	1589.88	LRCLGIPARIVTNY
328-338	1217.36	LGIPARIVTNY
364-372	1126.21	PVPIVSELQSG
380-389	1133.28	MTRPDLPVGF
407-424	1972.96	YRCGPASVQA IKGHVCF
513-522	1198.55	KSRSNVD MDF
533-550	2213.56	FKLSITFRNNSHNRYTIT
535-541	851.05	LSITFRN
607-619	1534.65	FVTARINETRDVL
632-646	1616.98	IIIKVRGTQVVGSDM

^a all m/z values represent the average mass of the undeuterated cluster.

APPENDIX D: TG2 PEPTIC PEPTIDES

Peptide	m/z ^a	Amino Acid Sequence
12-25	2431.30	ELETNGRDHHTADL
26-39	926.15	CREKLVVRRGQPFW
31-39	2819.06	VVRRGQPFW
154-160	963.03	ERQEYVL
237-253	2188.48	LGRWDNNYGDGVSPMSW
259-269	1422.78	ILRRWKNHGCQ
315-329	1509.78	FRNEFGIEIQGDKSEM
341-353	1031.09	MTRPDLQPGYEGW
369-377	1682.03	CCGPVPVRA
451-461	1177.20	ERAFTRANHL
452-461	1930.10	REAFTRANHL
475-487	2051.24	RIRVGQSMNMGSD
502-508	1372.70	YVCRLL
547-554	1014.09	YEKYRDCL
560-568	1713.97	IKVRALLVE
668-686	1845.15	ESDKLKAVKGFRNVIIGPA

^a all m/z values represent the average mass of the undeuterated cluster.

APPENDIX E: LB BROTH AND LB AGAR RECIPES

Table 1: 500 ml LB Broth

5.0 g Tryptone

2.5 g Yeast Extract

5.0 g NaCl

Add 500 ml dH₂O and stir to mix. pH the solution to 7.5 with 10 M NaOH and autoclave before use. If Ampicillin is needed, add prior to inoculation.

Table 2: 500 ml LB Agar

5.0 g Tryptone

2.5 g Yeast Extract

5.0 g NaCl

7.5 g Agar

Add 500 ml dH₂O and stir to mix. pH the solution to 7.5 with 10 M NaOH and autoclave. Let cool to ~50 °C and add 500 µl 100 mg/ml Ampicillin. Pour plates and allow them to cool at room temperature. Cap and store at 4 °C.

Table 3: 1 L SOB Medium

20.0 g Tryptone

5.0 g Yeast Extract

0.5 g NaCl

Add 1 L dH₂O and autoclave. After the SOB solution has cooled, add 10 ml filter sterilized 1 M MgCl₂ and 10 ml filter sterilized 1 M MgSO₄.

Table 4: 1 L SOC Medium (make immediately before use)

Add 2 ml filter sterilized 20 % glucose solution to 100 ml SOB Medium.

Table 5: 1 L Phosphate Buffer

23.2 g KH₂PO₄

164 g K₂HPO₄

Dilute to 1 L in volumetric flask. Transfer to autoclave safe bottle and autoclave.

Table 6: 2 L Terrific Broth

24.0 g Tryptone

48.0 g Yeast Extract

8 ml Glycerol

Add 1.8 L of dH₂O and autoclave. Before use add 200 ml Phosphate Buffer.

Appendix E (continued)

Table 7: 1 L *E. coli* Wash Buffer

20 ml 1 M Tris pH = 8

10 ml 5 M NaCl

Dilute to 1 L with dH₂O.

Table 8: 1 L Phosphate Buffered Saline (1× PBS)

8.0 g NaCl (137 mM)

0.2 g KCl (2.7 mM)

1.42 g Na₂HPO₄ (10 mM)

0.24 g KH₂PO₄ (2 mM)

187.5 g glycerol (15 %)

Add 800 ml dH₂O to the bottle and pH to 7.4 then fill to 1 L. Autoclave or filter sterilize before use.

APPENDIX F: NMR PEAKLIST FOR ac (233 – 425)

assignment	¹⁵ N ppm	¹ H ppm	assignment	¹⁵ N ppm	¹ H ppm
1	112.288	7.488	41	122.081	8.541
2	129.646	10.131	42	121.855	8.256
3	129.613	10.099	43	121.761	8.393
4	129.416	10.075	44	121.775	8.061
5	129.358	10.147	45	121.583	8.226
6	126.523	8.409	46	121.534	8.26
7	125.876	8.3	47	121.481	8.337
8	125.794	8.415	48	121.481	8.29
9	125.606	8.387	49	121.333	8.438
10	125.449	7.883	50	121.342	8.359
11	124.919	8.207	51	121.318	7.916
12	124.547	8.346	52	121.199	8.262
13	124.393	8.177	53	121.146	8.554
14	124.24	8.254	54	121.162	8.128
15	124.107	8.292	55	121.097	8.5
16	123.979	8.386	56	120.978	8.316
17	123.884	8.416	57	120.813	8.15
18	123.734	8.577	58	120.78	8.472
19	123.634	8.253	59	120.731	8.52
20	123.542	8.494	60	120.724	7.854
21	123.342	8.164	61	120.724	7.844
22	123.266	8.313	62	120.653	8.262
23	123.229	8.437	63	120.49	8.443
24	123.136	8.705	64	120.328	8.564
25	123.073	8.402	65	119.935	8.095
26	123.113	8.366	66	119.712	8.166
27	122.928	8.721	67	119.491	8.036
28	122.916	8.195	68	119.439	8.434
29	122.867	8.237	69	119.189	8.263
30	122.794	8.482	70	119.113	8.402
31	122.801	8.271	71	118.949	8.366
32	122.685	8.216	72	118.892	8.316
33	122.661	8.443	73	118.813	8.403
34	122.608	8.327	74	118.825	8.35
35	122.566	8.6	75	118.781	8.29
36	122.527	8.373	76	118.655	8.472
37	122.358	8.298	77	118.56	8.501
38	122.309	8.245	78	118.49	8.399
39	122.262	8.577	79	118.085	8.223
40	122.183	8.465	80	118.056	8.296

APPENDIX F (continued)

assignment	¹⁵ N ppm	¹ H ppm	assignment	¹⁵ N ppm	¹ H ppm
81	117.93	8.437	121	113.028	6.93
82	117.785	8.46	122	112.951	7.658
83	117.67	8.268	123	112.946	7.499
84	117.529	8.378	124	112.961	6.979
85	117.445	8.204	125	112.9	7.62
86	117.409	8.425	126	112.9	6.912
87	116.908	8.058	127	112.863	7.602
88	116.445	8.122	128	112.78	7.484
89	116.373	8.304	129	112.771	7.474
90	116.414	8.171	130	112.782	6.853
91	116.084	8.312	131	112.751	7.586
92	115.915	8.01	132	112.753	6.935
93	115.809	8.284	133	112.616	7.494
94	115.701	8.342	134	112.597	6.964
95	115.703	8.209	135	112.569	6.864
96	115.656	8.139	136	112.5	7.462
97	115.618	8.173	137	112.234	6.937
98	115.548	8.12	138	112.233	6.856
99	115.494	8.084	139	111.791	8.619
100	115.437	8.422	140	111.438	8.522
101	115.268	8.313	141	111.424	8.534
102	115.29	8.062	142	111.112	8.406
103	115.172	8.27	143	111.099	8.368
104	114.23	8.159	144	111.059	8.4
105	114.144	7.987	145	110.806	8.527
106	113.969	8.082	146	110.783	8.426
107	113.768	8.139	147	110.802	8.393
108	113.454	7.656	148	110.761	8.364
109	113.451	6.979	149	110.412	8.242
110	113.37	7.913	150	110.272	8.47
111	113.313	7.261	151	110.317	8.195
112	113.265	8.258	152	110.239	8.5
113	113.264	8.087	153	110.139	8.568
114	113.181	7.498	154	110.086	8.639
115	113.174	6.948	155	110.029	8.206
116	113.186	6.872	156	109.947	8.452
117	113.092	8.177	157	109.852	8.374
118	113.144	7.629	158	109.783	8.538
119	113.129	6.961	159	109.709	8.596
120	112.983	7.592	160	109.625	8.523

APPENDIX F (continued)

assignment	¹⁵ N ppm	¹ H ppm
161	108.948	8.506
162	108.918	8.472
163	108.909	8.334
164	108.823	8.358
165	108.815	8.446
166	107.949	8.267
167	107.658	8.205
168	132.122	8.078
169	119.788	8.254
170	113.171	7.322
171	112.27	7.589
172	129.965	10.091
173	110.822	8.569
174	110.805	8.669
175	110.651	8.637
176	115.748	8.46
177	115.782	8.262
178	121.697	8.432
179	121.084	8.644
180	121.578	8.608
181	122.942	8.325
182	113.242	7.19
183	113.396	7.694
184	129.669	10.078

APPENDIX G: NMR ASSIGNMENTS FOR α C(233 – 425) IN THE PRESENCE OF FXIII-A₂

assignment	¹⁵ N ppm	¹ H ppm	assignment	¹⁵ N ppm	¹ H ppm
1	111.454	8.522	40	122.615	8.327
2	129.976	10.092	41	122.546	8.373
3	129.693	10.154	42	122.37	8.297
4	129.664	10.13	43	122.344	8.35
5	129.684	10.075	44	122.319	8.246
6	129.628	10.097	45	122.204	8.57
7	129.425	10.074	46	122.168	8.465
8	129.368	10.146	47	122.105	8.54
9	126.534	8.409	48	121.869	8.258
10	125.884	8.299	49	121.807	8.359
11	125.802	8.414	50	121.792	8.061
12	125.615	8.387	51	121.737	8.429
13	125.458	7.883	52	121.777	8.394
14	124.927	8.208	53	121.583	8.23
15	124.561	8.346	54	121.558	8.612
16	124.404	8.177	55	121.541	8.26
17	124.251	8.253	56	121.499	8.339
18	124.122	8.292	57	121.496	8.29
19	124.001	8.385	58	121.427	8.554
20	123.902	8.416	59	121.362	8.359
21	123.742	8.576	60	121.347	8.438
22	123.644	8.254	61	121.327	7.915
23	123.559	8.493	62	121.214	8.264
24	123.359	8.163	63	121.156	8.554
25	123.332	8.188	64	121.17	8.131
26	123.236	8.437	65	121.089	8.645
27	123.278	8.312	66	121.107	8.5
28	123.149	8.706	67	120.99	8.316
29	123.152	8.367	68	120.822	8.149
30	123.089	8.4	69	120.751	8.52
31	122.939	8.72	70	120.789	8.472
32	122.931	8.324	71	120.69	8.677
33	122.924	8.195	72	120.732	7.853
34	122.796	8.481	73	120.73	7.843
35	122.835	8.27	74	120.665	8.261
36	122.703	8.358	75	120.503	8.444
37	122.723	8.218	76	120.339	8.564
38	122.667	8.442	77	119.946	8.096
39	122.568	8.601	78	119.806	8.255

APPENDIX G: (continued)

assignment	¹⁵ N ppm	¹ H ppm	assignment	¹⁵ N ppm	¹ H ppm
79	119.722	8.166	118	115.084	8.264
80	119.521	8.034	119	114.245	8.16
81	119.45	8.433	120	114.156	7.987
82	119.254	8.308	121	113.987	8.083
83	119.204	8.265	122	113.778	8.139
84	119.13	8.402	123	113.461	7.656
85	118.96	8.366	124	113.448	6.979
86	118.905	8.316	125	113.383	7.913
87	118.821	8.403	126	113.383	7.693
88	118.834	8.349	127	113.329	7.261
89	118.772	8.291	128	113.273	8.258
90	118.672	8.471	129	113.276	8.088
91	118.586	8.5	130	113.21	7.195
92	118.479	8.398	131	113.153	7.628
93	118.102	8.224	132	113.184	7.497
94	118.068	8.296	133	113.188	7.339
95	117.966	8.435	134	113.192	6.87
96	117.815	8.459	135	113.093	8.177
97	117.683	8.269	136	113.143	6.961
98	117.543	8.377	137	113.054	6.929
99	117.435	8.424	138	112.979	7.591
100	117.445	8.205	139	112.962	7.658
101	116.921	8.059	140	112.961	7.497
102	116.429	8.171	141	112.95	6.978
103	116.456	8.122	142	112.912	7.619
104	116.386	8.303	143	112.87	7.601
105	115.934	8.01	144	112.914	6.911
106	115.822	8.284	145	112.853	7.643
107	115.791	8.262	146	112.769	7.585
108	115.729	8.462	147	112.792	7.483
109	115.719	8.342	148	112.78	7.471
110	115.712	8.21	149	112.774	6.934
111	115.641	8.138	150	112.793	6.853
112	115.619	8.173	151	112.638	7.492
113	115.548	8.12	152	112.617	6.964
114	115.499	8.084	153	112.603	6.864
115	115.452	8.422	154	112.513	7.46
116	115.285	8.314	155	112.28	7.587
117	115.295	8.061	156	112.263	7.486

APPENDIX G: (continued)

assignment	¹⁵ N ppm	¹ H ppm
157	112.236	6.937
158	112.247	6.855
159	111.803	8.618
160	111.438	8.534
161	111.1	8.405
162	111.111	8.367
163	110.832	8.585
164	110.818	8.569
165	110.817	8.527
166	110.823	8.393
167	110.811	8.672
168	110.793	8.425
169	110.771	8.363
170	110.654	8.64
171	110.423	8.242
172	110.324	8.194
173	110.289	8.47
174	110.258	8.5
175	110.167	8.568
176	110.102	8.639
177	110.033	8.206
178	109.955	8.451
179	109.861	8.374
180	109.792	8.537
181	109.725	8.596
182	109.61	8.522
183	108.952	8.506
184	108.918	8.471
185	108.928	8.334
186	108.827	8.446
187	108.843	8.358
188	107.952	8.266
189	107.669	8.204
190	113.158	6.947
191	132.113	8.077

APPENDIX H: ABBREVIATIONS

(Not defined in coagulation schematic and in alphabetical order)

1D- One Dimensional
2D- Two Dimensional
 α_2 AP- α_2 -antiplasmin
 α -CHCA- α -cyano-4-hydroxycinnamic acid
AP- Activation Peptide
AUC- Analytical Ultracentrifugation
DMSO- Dimethylsulfoxide
DON- 6-diazo-5-oxo-norleucine
FXIIIa^{IIa}- Thrombin Activated FXIII
FXIIIa^{Ca}- Calcium Activated FXIII
GDH- Glutamate Dehydrogenase
GDP- Guanine Diphosphate
GEE- Glycine ethyl ester
HDX- Hydrogen Deuterium Exchange
HSQC- Heteronuclear Single Quantum Coherence
IAA- Iodoacetamide
IC50- Concentration Resulting in 50% Inhibition
IPAP- Inphase Antiphase
Kd- Dissociation Equilibrium Constant
MALDI- Matrix-Assisted Laser Desorption-Ionization
MS- Mass Spectrometry
MWCO- Molecular Weight Cut-Off
m/z- Mass to Charge ratio
NMR- Nuclear Magnetic Resonance
NADH- Nicotinamide adenine dinucleotide
TGase- Transglutaminase
TG2- Transglutaminase 2
TOF- Time-of-Flight
TOCSY- Total Correlation Spectroscopy
TFA- Trifluoroacetic acid

CURRICULUM VITAE

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EDUCATION:

PhD CHEMISTRY

Department of Chemistry
University of Louisville
Louisville, KY (December 2011)

BACHELOR OF SCIENCE DEGREE IN CHEMISTRY (ACS Certified):

College of Charleston
Charleston, SC (May 2001)

BACHELOR OF SCIENCE DEGREE IN BIOCHEMISTRY (ACS Certified):

College of Charleston
Charleston, SC (May 2000)

RESEARCH EXPERIENCE:

GRADUATE RESEARCH:

Department of Chemistry, University of Louisville, Louisville, KY

Utilized LC-ESI-MS for the analysis of volatile compounds released from A549 lung epithelial cells, 2006-2007.

THESIS:

Investigated Factor XIII structural dynamics during activation and inhibition utilizing MALDI-TOF-MS and NMR techniques, 2008-present

Utilized hydrogen-deuterium exchange (HDX) in conjunction with MALDI-TOF-MS to investigate Factor XIII conformational dynamics due to ionic concentration as well as nonproteolytically activation

Utilized HDX coupled with MALDI-TOF-MS to compare the conformational dynamics of FXIII-A₂ to transglutaminase 2 when activated, unactivated and bound to substrate.

Determine the residues on FXIII-A that are crucial for FXIII-A₂B₂ heterotetramer formation using HDX technology. Also investigate the role of the fibrinogen α C-domain in the dissociation of FXIII-B.

Utilize ¹H-¹⁵N HSQC to probe whether the α C-domain contains any compact structure when interacting with recombinant C314A FXIII-A₂.

GOVERNMENT:

Marine Biotoxins Program, Center for Coastal Environmental Health and Biomolecular Research NOAA/NOS, Charleston, SC

Developed a radioimmunoassay (RIA) for the detection of brevetoxin, a potent neurotoxin produced by the marine algae *Karenia brevis*, in the blood of exposed animals, 2001-2002

Investigated possible therapeutic effects of cholestyramine in brevetoxin exposed mice, 2002-2003

Utilized the RIA to assist the Analytical Response Team in toxin analysis of tissue samples during marine mammal mortality events, 2002-2006

Monitored the uptake and elimination of brevetoxin in the blood of striped mullet (*Mugil Cephalis*), 2003-2004

Determined the distribution of brevetoxin within specific lipoprotein fractions of mouse blood and human plasma, 2003-2006

UNDERGRADUATE RESEARCH:

Department of Chemistry and Biochemistry, College of Charleston, Charleston, SC

Synthesized silyl-substituted organometallic fluorene compounds with Dr. Jason Overby, Summer 2000

Department of Geology, College of Charleston, Charleston, SC

Observed the migrating and homing behavior of the chitin *Acanthopleura granulata* in San Salvador, Bahamas with Dr. James Carew, Spring 2001

Department of Physics and Astronomy, College of Charleston, Charleston, SC

Studied the relationship between mass and velocity in Cub Scout pinewood derby cars with Dr. Laney Mills, Spring, 1999

INDUSTRY:

Charleston Technical Center, Westvaco, North Charleston, SC

Assigned a project formulating new inks and toners as a co-op research analyst, 1999 and 2001

TEACHING EXPERIENCE:

GEMS Science Fellow, Jefferson County Public Schools, Louisville, KY
Co-taught in a 7th grade science classroom, Fall 2008-Spring 2010

Adjunct Faculty, Chemistry Department, Trident Technical College, North Charleston, SC
Chemistry 110 Lab and tutorial, Spring 2002, Spring 2003, and Fall 2004

TEACHING ASSISTANT:

Department of Chemistry, University of Louisville, Louisville, KY
Chemistry Recitation and Laboratory, 2007-2008

Department of Chemistry and Biochemistry, College of Charleston, Charleston, SC
Analytical and Organic Laboratory, 1998-2000

AWARDS AND HONORS:

- Groundwork Education in Mathematics and Science (GEMS) Fellow, National Science Foundation GK-12 Program, Partnership between University of Louisville and Jefferson County Public Schools, 2008-2010
- Departmental Fellow, University of Louisville Chemistry Department, 2006-2007
- Eagle Scout plus Bronze Palms, Troop 28 Spartanburg, SC

ACADEMIC SERVICE AND PROFESSIONAL AFFILIATIONS:

- Information Officer and founding member of the Arts and Sciences Graduate Student Union, University of Louisville, 2010-2011
- Member, Protein Society, 2009-present
- President, Chemistry Graduate Student Association, University of Louisville, 2008-present
- Member, Gamma Delta Chapter of Alpha Chi Sigma, Professional Chemistry Fraternity, 1998-present
- Chair, Social Committee, National Ocean Service Marine Biotoxins Program, 2002-2006
- Member, International Society for the Study of Harmful Algae, 2002-2006
- Mentor, South Carolina Junior Academy of Science, Academic Magnet High School, 2001-2006
- Science Fair Judge, Charleston area schools, 2000-2006
- Master Alchemist, Gamma Delta Chapter of Alpha Chi Sigma, Chemistry Fraternity, 2000-2001

PUBLICATIONS:

Woofter, R., Maurer, M. *Investigating the Importance of Calcium in the Conformational Dynamics of Factor XIII Activation by Utilizing Hydrogen/Deuterium Exchange in Conjunction with MALDI-TOF-MS.* Arch Biochem Biophys. 2011 Aug 1;512(1):87-95.

Woofter, R., Ramsdell, J. *Distribution of Brevetoxin to Lipoproteins in human plasma*. *Toxicon*, 2007. 1:49(7): p. 1010-8.

Woofter, R., Brendtro, K., Ramsdell, J. *Uptake and Elimination of Brevetoxin in Blood of Striped Mullet (*Mugil Cephalis*) after Aqueous Exposure to *Karenia Brevis**, *Environ Health Perspect*, 2005. 113(1): p. 11–16.

Woofter, R., Spiess, P., Ramsdell, J. *Distribution of brevetoxin (PbTx-3) in mouse plasma: association with high-density lipoproteins*. *Environ Health Perspect*. 2005. 113(11):1491-6.

Ramsdell, J., **Woofter, R.**, Colman, J., Dechraoui, MYB., Dover, S., Pandos, B., Gordon, C.J. *Protective effects of cholestyramine on oral exposure to the red tide toxin brevetoxin*, *FASEB JOURNAL*, 2003. 17 (4): A613-A613 Part 1 Suppl. S.

Woofter, R., Bottein Dechraoui, Y., Garthwaite, I., Towers, N., Gordon, C., Cordova, J, Ramsdell, J. *Measurement of Brevetoxin Levels by Radioimmunoassay of Blood Collection Cards after Acute, Long-Term, and Low Dose Exposure in Mice*. *Environ Health Perspect*, 2003. 111(13): p. 1595-1600.

Overby, J., **Woofter, R.**, Rheingold, A., Incarvito, C., and Sommer, R. *The synthesis and structural characterization of silyl-substituted fluorenes*. *J Chem Crystallogr*, 2003. 33(5-6): p. 357-364.

Woofter, R. Experiment XVI In: *Organic Chemistry Laboratory Manual 2nd Edition*; An Introduction to Organic Laboratory: Techniques of Synthesis and Characterization, Part B - 2nd Semester (Heldrich, Frederick J.); Wiley and Sons, Inc. 2000; p.69-71.

Woofter, R. Experiment XVII In: *Organic Chemistry Laboratory Manual 2nd Edition*; An Introduction to Organic Laboratory: Techniques of Synthesis and Characterization, Synthesis of Tetraphenylcyclopentadienone, Part B - 2nd Semester (Heldrich, Frederick J.); Wiley and Sons, Inc. 2000; p. 81-84.

PRESENTATIONS:

Invited Talks:

Woofter, R., Sexton, B., Rich, C. *Developing and Implementing Meaningful NSF GK-12 Partnerships: Some Do's and Don'ts*. American Association for the Advancement of Science/National Science Foundation Annual GK-12 Conference, Washington D C, March, 26-28, 2010.

Posters:

Woofter, R., Sexton, B., Rich, C. *GEMS Fellows and Teachers Focus on Strategies for Informed Science Instruction: Formative Assessment Activities that Complement Inquiry-Based Curricula*. American Association for the Advancement of Science/National Science Foundation Annual GK-12 Conference, Washington D C, March, 26-28, 2010.

Woofter, R., Sabo, M., Maurer, M. *Investigating Factor XIII Structural Changes in the Presence of Mono and Divalent Cations Utilizing HDX coupled with MALDI-TOF-MS*. 23rd Symposium of the Protein Society, Boston, MA. July 25-29, 2009.

Ramsdell, J., **Woofter, R.**, Brendtro, K. *Uptake and Elimination of Brevetoxin in Blood of Striped Mullet (Mugil Cephalis) after Aqueous Exposure to Karenia Brevis*. 11th International Conference on Harmful Algae, Cape Town, South Africa. November 14-19, 2004.

Spiess, P., **Woofter, R.**, Ramsdell, J. *The Distribution of Brevetoxin (PbTx-3) to Specific Lipoprotein Fractions in Mouse Blood*. Second Symposium on Harmful Marine Algae in the U.S., Woods Hole, MA. December 9-13, 2003.

Woofter, R., Brendtro, K., Miles, C., Towers, N., Ramsdell, J. *Biomonitoring Blood Brevetoxin in Striped Mullet (Mugil cephalis) after Sub-lethal Laboratory Exposure to Karenia Brevis*. Second Symposium on Harmful Marine Algae in the U.S., Woods Hole, MA. December 9-13, 2003.

Ramsdell, J., **Woofter, R.**, Colman, J., Bottein Dechraoui, M-Y, Dover, S., Pandos, B., Gordon, C. *Protective effects of cholestyramine on oral exposure to the red tide toxin brevetoxin*. Experimental Biology 2003 - Translating the Genome, San Diego, CA, April, 11-15, 2003.

Woofter, R., Bottein Dechraoui, M-Y., Garthwaite, I., Towers, N., Gordon, C., Cordova, J, Ramsdell, J. *Comparison of Receptor and Immunoassays for Brevetoxin Detection in the Blood of Exposed Animals*. Xth International Conference on Harmful Algae, St. Pete Beach, FL. October, 21-25, 2002.