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Evaluating Factor XIII Specificity for Glutamine-Containing Substrates Using a MALDI-TOF Mass Spectrometry Assay

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Abstract

Activated Factor XIII (FXIIIa) catalyzes the formation of γ -glutamyl- ϵ -lysyl cross-links within the fibrin blood clot network. Although several cross-linking targets have been identified, the characteristic features that define FXIIIa substrate specificity are not well understood. To learn more about how FXIIIa selects its targets, a matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) based assay was developed that could directly follow the consumption of a glutamine-containing substrate and the formation of a cross-linked product with glycine ethylester. This FXIIIa kinetics assay is no longer reliant on a secondary coupled reaction, on substrate labeling, or on detecting the final deacylation portion of the transglutaminase reaction. With the MALDI-TOF MS assay, glutamine-containing peptides derived from α_2 -antiplasmin, *S. Aureus* fibronectin binding protein A, and thrombin activatable fibrinolysis inhibitor were examined directly. Results suggest that the FXIIIa active site surface responds to changes in substrate residues following the reactive glutamine. The P₋₁ substrate position is sensitive to charge character and the P₋₂ and P₋₃ to the broad FXIIIa substrate specificity pockets. The more distant P₋₈ to P₋₁₁ region serves as a secondary substrate anchoring point. New knowledge on FXIIIa specificity may be used to design better substrates or inhibitors of this transglutaminase.

Keywords

Factor XIII; transglutaminase; coagulation; substrate specificity; kinetics; cross-linking; mass spectrometry

Introductory Statement

In blood coagulation, the serine protease thrombin cleaves the N-terminal portions of the fibrinogen A α and B β chains. The resultant fibrin monomers polymerize non-covalently into linear protofibrils and then laterally into fibers forming a blood clot that is held together by

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non-covalent forces [1; 2]. To make this blood clot more resistant to degradation, covalent cross-links are introduced into the fibrin network [3; 4]. Thrombin aids in this process by helping to activate Factor XIII (FXIII)¹ via cleavage of the FXIII R37-G38 activation peptide bond. The resultant transglutaminase, FXIIIa, then catalyzes the formation of γ -glutamyl- ϵ -lysyl cross-links within fibrin and fibrin-ligand complexes [5; 6]. The reaction mechanism of FXIIIa involves the thiol group of Cys314 attacking the carboxamide of a reactive glutamine (Q) side chain. Ammonia is released and a thioester is generated. Deacylation then occurs as the amino group from a Lys (K) side chain targets the thioester. The resultant product of the transglutaminase catalyzed reaction contains an isopeptide bond between the side chains of the Q and K residues [5].

FXIIIa targets several sites within the fibrin $\alpha\beta\gamma$ environment leading to formation of a rigid blood clot [7]. Other key FXIIIa substrates include α_2 -antiplasmin, fibronectin, thrombin activatable fibrinolysis inhibitor, plasminogen activation inhibitor-1, vitronectin, and *S. Aureus* fibronectin binding protein A [8]. Although a number of FXIIIa substrates have been identified, an obvious consensus sequence for the Q-containing substrate has been difficult to establish [6; 8; 9; 10; 11].

A variety of methods have been developed to study interactions between FXIIIa and its substrates. In several assays, the lysine-like substrate is detected by colorimetric [12; 13], fluorescent [14; 15], or radioactive methods [16; 17; 18]. With this approach, the glutamine-containing reaction must occur before the lysine-like reaction can be monitored. Another strategy for measuring enzymatic activity is a coupled uv/vis assay in which ammonia released from the reactive glutamines are detected via a separate, secondary colorimetric assay [19; 20; 21; 22]. This resultant ammonia is used to convert α -ketoglutarate to glutamate in an NADH dependent reaction that is monitored at 340nm. Again the glutamine-containing reaction is not being monitored directly. An additional method is to work with synthetic peptides in which the reactive glutamine is replaced with colorimetric Glu-pNA or fluorimetric Glu-AMC [23; 24]. With such reporter groups, the direct release of p-nitroaniline (pNA) or amidomethylcoumarin (AMC) can finally be monitored and is a real strength of this assay design. A drawback is that each substrate needs to have this modified Glu residue introduced into the sequence. Moreover, the bulky reporters group may hinder interactions with the FXIIIa active site surface.

A valuable advance for monitoring FXIIIa kinetics would be an assay that directly records consumption of substrate and formation of product within a single platform. The use of low sample volumes is also desirable. Our newly developed MALDI-TOF mass spectrometry kinetics assay achieves these goals (Fig 1). In the assay, the FXIIIa-catalyzed reaction between a Q-containing substrate and excess glycine ethylester (GEE) is followed. GEE is routinely used as a lysine mimic in the coupled uv/vis assays [19; 20; 21; 22]. The individual

¹Abbreviations Used

human cellular factor XIII, FXIII; activated factor XIII, FXIIIa; α_2 -antiplasmin, α_2 AP; fibronectin binding protein A, Fnb A; thrombin activatable fibrinolysis inhibitor, TAFI; glycine ethylester, GEE; trifluoroacetic acid, TFA; α -cyano-4-hydroxycinnamic acid, α CHCA; matrix assisted laser desorption ionization time of flight mass spectrometry, MALDI-TOF MS; transglutaminase, TGase; FXIIIa inhibitory peptide (Ac-Asp-MA(Michael Acceptor)-Nle-Nle-Leu-Pro-Trp- Pro-OH), ZED1301; Michael Acceptor unit; MA.

transglutaminase reactions are quenched at distinct time points and MALDI-TOF mass spectra are collected to monitor for loss of Q-substrate and gain of the cross-linked product (Q-substrate)-GEE (gain of 86 m/z). Overall, the MALDI-TOF MS kinetics assay is no longer reliant on a secondary coupled reaction, on substrate labeling, or on only detecting the final deacylation portions of the transglutaminase reaction [12; 22; 23].

A series of glutamine containing substrates were chosen to further test the MALDI-TOF MS assay. Using a nomenclature similar to that of the proteases, the reactive Q of FXIIIa substrates occupies the P₁ position. Amino acids can be assigned as ...P₄ P₃ P₂ P₁^(reactive Q) and then to the right of the reactive Q as P₋₁ P₋₂ P₋₃ P₋₄.... (Table 1). Regions to explore in kinetic studies include the P₋₁ and P₋₂ positions located near the reactive Q residue and also the putative substrate recognition segment P₋₈ to P₋₁₁ [20; 21; 25]. This P₋₈ to P₋₁₁ segment is proposed to serve as an extra substrate anchor on to the extended FXIIIa active site region. Three FXIIIa substrates containing reactive Q residues include α₂-antiplasmin (α₂AP), *S. Aureus* fibronectin binding protein A (*S. Aureus* Fnb A), and thrombin activatable fibrinolysis inhibitor (TAFI) (Table 1). Characteristics of these substrates are described below.

α₂AP (464 residues) serves as a potent inhibitor of the fibrinolytic agent plasmin. Cleavage of the P12–N13 amide bond generates a 452 residue protein starting with an Asn [26; 27]. FXIIIa rapidly cross-links Q2 of N1-α₂AP to K303 of the fibrin(ogen) α-chain [26; 28; 29]. While this N1-α₂AP is tethered to a blood clot, its C-terminal domain remains free to inhibit plasmin [26]. Previous coupled uv/vis kinetic assays with α₂ AP (1–15) (Table 1) have indicated that the Q2 at the P₁ position is the reactive glutamine of this peptide with the Q4 at the P₋₂ position serving a supporting role in binding [20]. α₂ AP also contains an ¹⁰LLKL¹² stretch (P₋₈ to P₋₁₁ region) that is part of a putative substrate recognition exosite [20; 25].

S. Aureus colonizes human tissue during vascular injury by linking its Fnb A to fibrinogen [30; 31]. Similar to the α₂AP (1–15) sequence, Fnb A contains glutamines at the P₁ and P₋₂ positions (Table 1) [32; 33; 34]. By contrast, *S. Aureus* Fnb A has a basic R at the P₋₁ position whereas α₂AP (1–15) has an acidic E. As found in α₂AP, *S. Aureus* Fnb A possesses K residues at the P₋₈ and P₋₉ positions.

TAFI modifies lysine residues within the fibrin network thereby reducing the affinity of plasminogen for the blood clot. As a result, less plasmin is generated for fibrinolysis [14; 35; 36]. Similar to α₂AP and *S. Aureus* Fnb A, TAFI also contains two glutamines (Table 1). However with TAFI, a polar serine and the small, flexible glycine are situated between the two glutamines. The second Q residue is thus switched from the P₋₂ to P₋₃ position. As observed with α₂AP and *S. Aureus* Fnb A, TAFI (1–15) contains a basic arginine within the P₋₈ to P₋₁₁ region.

Studies to characterize how FXIIIa selects its substrate targets will benefit from the development of assays that directly follow the Q-containing substrate step. Our newly developed MALDI-TOF mass spectrometry kinetics assay achieves this goal by recording the consumption of the Q-containing substrate and formation of the (Q- glycine ethylester)

product within a single assay platform. This mass spectrometry based assay is no longer reliant on a secondary coupled reaction, on substrate labeling, or on only detecting the final deacylation portions of the transglutaminase reaction. Using our direct monitoring assay, the kinetic properties of α_2 AP (1–15), *S. Aureus* Fnb A (100–110; 100–114), and TAFI (1–15) were successfully determined. Aside from native peptide sequences, individual E, S, and/or R substitutions were also examined. Results indicate that the FXIIIa active site surface responds to changes in the substrate residue positions P₋₁ and P₋₂ and also to those of a more distant exosite involving the P₋₈ to P₋₁₁ residues. Additional studies with this MALDI-TOF mass spectrometry assay will aid in further deciphering the key features of a good FXIIIa substrate or inhibitor.

Materials and Methods

Materials for MALDI-TOF Assay

Human cellular FXIII expressed in *Saccharomyces cerevisiae* was kindly provided by Dr. Paul Bishop (ZymoGenetics Inc, Seattle, WA). The lyophilized FXIII was reconstituted in 18 M Ω deionized water, aliquoted, and stored at –70 °C until future use. The concentration of the FXIII A₂ stock solutions were determined on a Cary 100 uv/vis spectrophotometer using an extinction coefficient of 1.49 (ml mg⁻¹ cm⁻¹) at 280 nm. Plasma derived bovine thrombin (Sigma Aldrich) was used to activate the FXIII A₂. All the Q-containing substrate peptides (Table 1) were synthesized and purified by New England Peptide (Gardner, MA). The α_2 AP and TAFI based peptides contained a free N-terminus and an amidated C-terminus. The *S. Aureus* fibronectin binding A based peptides had an acetylated N-terminus and an amidated C-terminus. Stock peptide concentrations were determined by quantitative amino acid analysis (AAA Service Laboratory, Damascus, OR). Peptide purity was verified in house as being 90% or higher using an Applied Biosystems Voyager DE-PRO MALDI-TOF mass spectrometer.

MALDI-TOF MS assay for kinetics

The final assay volume for each trial was 500 μ l. The reaction mix was composed of 2.5 mM CaCl₂, 5 mM glycine ethylester, and MALDI Assay Buffer (100 mM Tris-Acetate, 150 mM NaCl, 0.1% PEG, pH 7.4). The reaction components listed above were prepared in a 1.5 ml reaction tube and incubated at 37°C. FXIII and thrombin were then added to the reaction mix at final assay concentrations of 24–100 nM FXIII and 1–2 nM thrombin. With this assay design, a fresh FXIII activation was carried out for each peptide concentration examined. Consistent activation conditions and quench times were employed across each peptide series. Any losses in transglutaminase activity that may occur upon freeze/thawing of previously activated FXIII substocks would be avoided. For each assay, the FXIII activation was allowed to proceed for 12 minutes and then 140 to 180 nM PPACK were added to inhibit the thrombin. After 5 minutes of incubation at 37°C, a specific concentration of Q-containing peptide ranging from 200 μ M to 2000 μ M was added to the reaction. Every 3 min up to 18 min or every 5 min up to 30 min, 75 μ l of reaction mix were removed and quenched in 5 μ l of 160 mM EDTA (10 mM final). After the sixth time point, quenched reactions were incubated at 37 °C for 15 minutes.

Samples were then prepared for MALDI-TOF MS analysis on an Applied Biosystems Voyager DE-PRO mass spectrometer. C₁₈ reverse phase resin ziptips (Millipore) were employed to reduce buffer salts that can hinder peptide ionization in a MALDI-TOF MS run. The C₁₈ resin in these tips were first wetted with 100% acetonitrile and then rinsed and equilibrated with 0.1% trifluoroacetic acid (TFA). Next, 15 µl of the FXIIIa reaction mix were bound to the C₁₈ resin and then followed by four washes with 0.1% TFA. The sample was released from the C₁₈ resin with 10 mg/ml α-cyano-4-hydroxycinnamic acid matrix (α-CHCA) containing 1:1:1 ethanol, 0.1% TFA, and acetonitrile. The eluted FXIIIa catalyzed reactions were spotted as one µl aliquots on a stainless steel MALDI plate. Four wells were spotted for each time point. All mass spectral analysis was done in positive, reflector ion mode, 256 shots per spectrum over an *m/z* range of 800 to 2000 Daltons. A relative intensity of 1×10^4 above the noise was desired. Of the four wells examined, three spectral results were selected for analysis. Moreover, each substrate concentration range was examined in triplicate.

Control experiments were carried out with solutions that contained peptide dissolved in reaction mixes that lacked FXIII. These zero minute time experiments were used to demonstrate the need for using the C₁₈ resin tips for MALDI-TOF mass spectrometry analysis (Fig 2). Furthermore, the control studies verified that the peptides were not altered by this added sample preparation procedure.

To aid in quantification of the MALDI-TOF MS results, an internal standard composed of 220 µM α₂AP (1–15) Q2N was tested in the assay design [37; 38]. This non-reactive peptide was added to all the time points after the transglutaminase reaction was quenched with EDTA. As described above, the reaction mixes were subjected to the C₁₈ resin protocol to reduce any interfering buffer salts. MALDI-TOF mass spectra were then collected, and a calibration plot of peak height ratios (substrate peak height /internal standard peak height) versus the substrate concentration was prepared. The slope of the line was used to convert experimental glutamine-containing peptide heights into concentrations. Changes in concentration over time would then be used to determine velocities. Subsequently, we discovered that the internal standard method could not be applied toward all the peptides being investigated. Some of the Q-containing substrate peptides were found to have quite different ionization efficiencies than the non-reactive internal standard α₂AP (1–15) Q2N. As a result, it was challenging to use a single peptide-based internal standard for all assays

As an alternative quantification strategy, the following peak height ratio formula was used to determine the amount of reactant remaining. As needed, any residual sodiated and potassiated peptide species still present after the C₁₈ resin zip tip procedure were added to the cation-free peptide peaks to provide the sum of Reactant Peak Heights and/or the sum of Product Peak Heights.

$$\frac{\sum \text{Reactant Peak Heights}}{\sum \text{Reactant Peak Heights} + \sum \text{Product Peak Heights}} * \text{Concentration of the Peptide}$$

The peptide α₂ AP (1–15) Q4K was used as a control sequence to test out the peak height ratio method as a means to quantitate the MALDI-TOF MS kinetic data. Velocities

corresponding to loss of Q-containing reactant over time were calculated, and the kinetic data were fit to the Michaelis-Menten equation. The K_m value for this Q-containing peptide was within statistical error of that determined using the traditional coupled uv-vis assay [20]. Moreover, these two K_m values were both within statistical error of the value determined using the internal standard α_2 AP (1–15) Q2N [20].

Since the peak-height ratio method provided comparable results to the other quantification approaches tested, this ratio method was used throughout the kinetics project. Consumption of the Q-substrates could be followed directly and thus velocities calculated. Excel and Sigma Plot were then employed to determine kinetic parameters from the Michaelis-Menten plots and to calculate standard errors of the mean. Each substrate concentration range was independently examined in triplicate. Data were further evaluated with a two-tailed t-test and P-values reported.

Results and Discussions

Although a number of FXIIIa substrates have been identified, how this transglutaminase selects its Q-containing substrate is still not well understood. To further characterize the features that define FXIII substrate specificity, a MALDI-TOF mass spectrometry kinetics assay was developed. This assay can directly monitor loss of Q-containing substrate and formation of glycine ethylester (GEE) linked product (Fig 1). Such a single assay platform was applied successfully to probe new peptide substrates with an emphasis on the P₋₁ and P₋₂ positions and the more distant region P₋₈ to P₋₁₁.

Validating the MALDI-TOF MS Assay

The Q-reactive substrate α_2 AP (1–15) Q4K, ¹**NQEKVSPLTLLKLG**N¹⁵ (the reactive glutamine is bold and underlined), was selected to confirm that the K_m values calculated from the MS assay would be comparable to those obtained with our previously optimized coupled uv/vis approach [20]. In that earlier study, the k_{cat}/K_m rankings for a series of α_2 AP (1–15) Q4X peptides were observed to follow the same trends as those observed for the K_m values. The K_m values were thus used as a kinetic parameter to compare the two assay formats.

The MALDI-TOF MS method has the advantage of directly monitoring all components in a sample without having to introduce a liquid chromatography step to separate out the species. However with this MS method, it may be necessary to introduce a C₁₈ zip tip step to remove buffer salts that can hinder peptide ionization. Control experiments were thus carried out with free α_2 AP (1–15) Q4K to evaluate the need for this C₁₈ resin step and to verify that the peptide had not been compromised by this procedure. α_2 AP (1–15) Q4K was dissolved in all the reaction mix components except the transglutaminase FXIII. This control sample was examined by MALDI-TOF MS in the absence (no Zip) and presence of zip tipping (Zip Tip) (Fig 2). Without the C₁₈ resin wash, it was challenging to observe the α_2 AP (1–15) Q4K by MALDI-TOF MS and the sodiated peptide was the predominant species in the reaction mix. Following the zip tipping procedure, overall peak intensity increased, S/N levels improved, and the cation-free form of α_2 AP (1–15) Q4K predominated. There was no evidence that the Q-containing peptide had been compromised by this extra processing step.

An initial screening of the full mass spectrometry method was thus carried out with the α_2 AP (1–15) Q4K. The peptide at a final concentration of 400 μ M was added to a mixture of 35 nM thrombin-activated FXIIIa, 2.5 mM CaCl_2 , and 5 mM GEE, all in Tris-acetate buffer. Aliquots were removed every 3 minutes and quenched with EDTA. The EDTA serves to scavenge the calcium away from the catalytic core domain of FXIIIa. Kinetic reaction samples were then subjected to the C_{18} resin procedure to remove interfering buffer and salts from the samples. Fig 3 displays representative MALDI-TOF mass spectra for the 3, 9, and 18 minute time points. In Fig 3A, the peak at 1653 m/z corresponds to the original reactant α_2 AP (1–15) Q4K whereas the cross-linked product α_2 AP (1–15) Q4K – GEE is observed at 1739 m/z . The more minor populations of sodiated and potassiated peptides are also marked in the spectrum. As the reaction proceeds, the intensity (or amount) of the Q-containing substrate decreases whereas the cross-linked product increases.

With an HPLC based assay, the sum of reactant and product peak areas should be a constant value for each substrate concentration. By contrast with MALDI-TOF MS, there may be ionization efficiency and/or matrix crystallization differences across a series of sample spots. As a result, the maximal peak intensity may not be the same for all the sample spots. Consequently, the sum of reactant and product peak heights is not always a constant for a particular substrate concentration. To address this problem, we turned to the peak height ratio method [$(\sum \text{reactant peak heights} / \sum \text{reactant peak heights} + \sum \text{product peak heights}) \times \text{peptide concentration}$] to determine quantitatively the amount of reactant remaining after each time point. This approach was found to be a valuable strategy to increase the reproducibility of kinetic data derived from a MALDI-TOF MS based assay. Such a peak height ratio approach has also been used successfully by Jennings et al. to follow production of organophosphate-acetylcholinesterase adducts by MALDI-TOF MS. Moreover, their adduct ratios could be directly correlated with a separate enzyme activity assay [39].

The peak height ratio method was thus used throughout the current FXIIIa kinetics project to determine the concentration of α_2 AP (1–15) Q4K remaining after each quenched time point. A more extensive plot of Q-substrate concentration remaining versus time (3, 6, 9, 12, 15, 18 min) was employed to determine the velocity associated with the 400 μ M α_2 AP (1–15) Q4K reaction (Fig 3B). The MALDI-TOF MS kinetics assay was repeated in triplicate over a series of peptide concentrations, and the velocities corresponding to each peptide concentration were determined. A K_m of $316 \pm 45 \mu\text{M}$ was calculated from a Michaelis-Menten plot. This measure of binding interactions was comparable to the K_m value of $426 \pm 93 \mu\text{M}$ derived earlier from the coupled uv/vis assay [20]. The MALDI-TOF MS assay could thus be used as an alternative to the coupled uv/vis approach. This MS based assay has the advantage of being able to directly monitor the consumption of Q-containing substrate and production of GEE-linked product.

Examining α_2 AP (1–15) K12R

A series of peptides based on the α_2 -antiplasmin sequence α_2 AP ($^1\text{NQEQVSPLTLLKLGN}^{15}$) has been used successfully to explore FXIIIa substrate positions surrounding the reactive glutamine Q2 residue [20; 21; 25]. Some blood coagulation substrates can also take advantage of one or more exosites on their respective

enzyme targets [40]. Studies by Gorman and Folk had suggested that residues within the P₋₈ to P₋₁₀ region could promote binding interactions with the FXIIIa active site [41; 42] (Table 1). For the current project, there was interest in further probing the P₋₁₀ position that is part of this putative substrate recognition region.

MALDI-TOF MS kinetic studies were carried out with α_2 AP (1–15) K12R, a substrate sequence where the lysine at the P₋₁₀ position is substituted with an arginine containing an ϵ -amino guanidinium group. MALDI-TOF MS spectra for this Q-containing substrate exhibited an initial reactant peak at 1681 *m/z* and a product peak at 1767 *m/z*. Only one glutamine to GEE linked product peak could be followed kinetically (Fig 4). If two GEE units had been cross-linked into the sequence, a product of 1853 *m/z* (1681 + 172 *m/z*) would have been observed.

As with the α_2 AP (1–15) Q4K peptide, a timed reaction series (3, 5, 7, 9, 12, 15, 18 min) for α_2 AP (1–15) K12R was examined by MALDI-TOF MS (highlights in Fig 5). The kinetic results for the K12R peptide indicate a K_m of $108 \pm 27 \mu\text{M}$ and a k_{cat} of $283 \pm 28 \text{ s}^{-1}$ (Table 2). Although R12 and the original K12 are both basic, the R12 exhibited a 4-fold improvement in K_m relative to the K12 sequence examined previously ($108 \pm 27 \mu\text{M}$ for R12 versus $459 \pm 50 \mu\text{M}$ for K12) ($P < 0.01$) [20]. The guanidinium group of arginine 12 may enhance binding interactions by utilizing conjugation between the lone pairs on the nitrogen and the double bond to delocalize the positive charge. As a result, different H-bonding scenarios become available to promote contact with the extended FXIIIa active site region. The single amine at the end of the K12 would have fewer options for manipulating how its side chain character interacts with the FXIIIa surface. In contrast to the K_m benefits, the α_2 AP (1–15) K12R peptide exhibited the lowest k_{cat} value for the peptides examined in this project. Even though the α_2 AP (1–15) R12 binds more effectively to the FXIIIa surface, this α_2 AP sequence is not as well oriented for promoting the transglutaminase reaction.

Examining sequences related to *S. Aureus* Fnb A

Reactive glutamines have already been proposed for *S. Aureus* Fnb A, but the FXIIIa kinetics have not been reported [32; 33; 34]. Studies began with *S. Aureus* Fnb A (100–110), a sequence without a potentially reactive lysine, ¹⁰⁰SGDQRQVDLIP¹¹⁰. Unlike α_2 AP (1–15) with its acidic Glu in the ²QEQ⁴ sequence, the *S. Aureus* Fnb A (100–110) peptide contains a basic Arg between the two Gln residues, ¹⁰³QRQ¹⁰⁵. MALDI-TOF MS spectra of the quenched reactions revealed that only a single Q-containing GEE linked product could be observed and monitored (Fig 4). The reactant at 1269 *m/z* increased 86 *m/z* to generate a product at 1355 *m/z*. The K_m value for this 11 residue peptide was $971 \pm 175 \mu\text{M}$ (Table 2).

The peptide sequence was then extended to *S. Aureus* Fnb A (100–114), ¹⁰⁰SGDQRQVDLIPKKAT¹¹⁴. Similar to α_2 AP (1–15) with a Lys at the P₋₁₀ position, the *S. Aureus* Fnb A (100–114) contains basic Lys residues at the P₋₈ and P₋₉ positions. Control studies with free *S. Aureus* Fnb A (100–114) in the reaction mix demonstrated how readily this peptide could be sodiated (Fig 2). The zip tipping procedure diminished the cationic adducts, substantially increased the peptide intensity relative to the baseline, and did not compromise the integrity of the peptide. Subsequent FXIIIa-catalyzed reactions indicated that only one Q-containing GEE linked product peak was observed (free peptide,

1269 m/z, +GEE, 1355 m/z) (Fig 4). In addition, only the glycine ethylester was serving as a lysine mimic in the transglutaminase reaction. The two Lys residues did not participate in intra- or intermolecular crosslinks.

A full series of kinetic time points (3, 5, 7, 9, 12, 15, 18 min) was collected for *S. Aureus* Fnb A (100–114) (highlights in Fig 6). This longer peptide exhibited a K_m of $571 \pm 109 \mu\text{M}$, almost two-fold better than the *S. Aureus* Fnb A (100–110) ($P < 0.05$). There was also a k_{cat} increase from $387 \pm 40 \text{ s}^{-1}$ to $535 \pm 46 \text{ s}^{-1}$ when the FnbA sequence was extended to 100–114 ($P < 0.05$). A clear improvement in binding interactions thus occurs when *S. Aureus* Fnb A $^{100}\text{SGDQRQVDLIP}^{110}$ is increased to $^{100}\text{SGDQRQVDLIPKKAT}^{114}$. The k_{cat} also undergoes an increase reflecting better turnover into product.

Earlier studies by Penzes et al. [21] on $\alpha_2\text{AP}$ (1–12) peptides revealed that a 3-fold increase in K_m occurred when the K12 at the P₋₁₀ position was removed to produce $\alpha_2\text{AP}$ (1–11). Subsequent deletions led to further increases in K_m , but the fold differences were not as great as with the loss of K12. The minimal length for $\alpha_2\text{AP}$ to serve as a substrate was 7 residues [21]. Alanine substitutions to the $\alpha_2\text{AP}$ (1–12) sequence revealed that L10A at the P₋₈ position hindered the K_m the most [21]. With the current *S. Aureus* Fnb A studies, peptide 100–110 was increased to 100–114. The extra segment (111–114) is proposed to be part of the same critical anchoring segment at the P₋₈ to P₋₁₀ positions as observed with $\alpha_2\text{AP}$ peptides. Moreover, the beneficial K residues at the P₋₈ and P₋₉ positions are also introduced with the longer peptide. The improvements in K_m values observed for both the $\alpha_2\text{AP}$ and the *S. Aureus* Fnb A peptides support the proposal that FXIIIa does contain a distant exosite that can promote binding interactions separate from the active site. Two substrates with quite *different* functions take advantage of targeting a FXIIIa exosite. α_2 -antiplasmin is involved in controlling fibrinolysis whereas Fnb A is used by *S. Aureus* to anchor on to fibrinogen for colonization [31; 43].

In a prior $\alpha_2\text{AP}$ (1–15) series, a substrate with improved binding properties could be generated when the Q4 position was substituted with serine ($\alpha_2\text{AP}$ (1–15) Q4S) [25]. To test if the same effect could be observed with the *S. Aureus* Fnb A series, a serine was substituted in place of the glutamine at the Q105 position for *S. Aureus* Fnb A (100–114). *S. Aureus* Fnb A (100–114) Q105S substitution gave a K_m of $644 \pm 122 \mu\text{M}$ showing a modest decrease in binding relative to wild-type (Table 2). The two k_{cat} values were similar at $535 \pm 46 \text{ s}^{-1}$ for *S. Aureus* Fnb A (100–114) and $453 \pm 38 \text{ s}^{-1}$ for *S. Aureus* Fnb A (100–114) Q105S. A Q to S substitution at the P₋₂ position of *S. Aureus* Fnb A thus could not generate the 3-fold improvement in K_m observed previously for $\alpha_2\text{AP}$ (1–15, Q4S) [25]. The P₋₂ to P₋₇ residues on *S. Aureus* Fnb A must have greater difficulties accommodating the small polar serine than with $\alpha_2\text{AP}$ (1–15, Q4S).

In contrast to the $^{103}\text{QRQ}^{105}$ sequence (P₁ to P₋₂) of *S. Aureus* Fnb A (100–114), $\alpha_2\text{AP}$ (1–15) has a glutamic acid between the two glutamines ($^2\text{QEQ}^4$). To test if introducing a more acidic environment into the *S. Aureus* Fnb A (100–114) would promote binding interactions, the *S. Aureus* Fnb A R104 was replaced with E104. Unexpectedly, *S. Aureus* Fnb A (100–114) R104E and its GEE-linked products were quite difficult to detect by MALDI-TOF mass spectrometry suggesting that ionization efficiency problems were occurring with these

reaction mixes. An alternative buffer system with lower salt content was found to help out with this issue (data not shown). However for the current project, the focus was on using the same Tris-acetate, NaCl containing buffer as employed in the previously published coupled uv-vis assays [20].

Because of issues associated with detecting *S. Aureus* Fnb A (100–114) R104E containing species, the opposite substitution in α_2 AP (1–15) was tested. The $^2\text{QEQ}^4$ of α_2 AP (1–15) was replaced with an E3R substitution to give a $^2\text{QRQ}^4$ sequence. The basic arginine at the P₋₁ position showed an increase in K_m ($594 \pm 144 \mu\text{M}$ versus the $316 \pm 45 \mu\text{M}$ for α_2 AP E3) reflecting a decrease in binding interactions. Interestingly, the K_m of α_2 AP (1–15) E3R ($594 \pm 144 \mu\text{M}$) now approached that of *S. Aureus* Fnb A (100–114) ($571 \pm 109 \mu\text{M}$). The k_{cat} value of $484 \pm 44 \text{ s}^{-1}$ was also comparable to the $535 \pm 46 \text{ s}^{-1}$ of Fnb A 100–114 (Table 2).

Examining sequences related to TAFI

Thrombin activatable fibrinolysis inhibitor (TAFI) was the next candidate to be investigated [14; 35; 36]. The TAFI (1–15) sequence $^1\text{FQSGQVLAALPRTSR}^{15}$ has a serine and glycine between the two glutamines. The P₋₂ position glutamine that was found to be valuable for α_2 AP binding interactions is now shifted to the P₋₃ position. Interestingly, there is an arginine at the P₋₁₀ position.

A review of the MALDI kinetic spectra indicates that only one of the Q residues could be monitored kinetically (Fig 3, and highlighted time series in Figure 7). The TAFI peptide at 1630 m/z increased by a single 86 m/z to 1716 m/z . A second product at 1802 m/z was not detected. A K_m of $3330 \pm 905 \mu\text{M}$ was obtained for TAFI (1–15), a value more than 5-fold weaker than what was observed for α_2 AP (1–15) K12R (K_m of $108 \pm 27 \mu\text{M}$) and *S. Aureus* Fnb A (100–114) (K_m of $571 \pm 109 \mu\text{M}$) (Table 2). The higher K_m for TAFI (1–15) indicates that this sequence exhibits much weaker binding interactions with the FXIIIa active site region than the two other substrates tested. The k_{cat} was comparable to that of *S. Aureus* Fnb A (100–114). A Q5S substitution generated a TAFI sequence that exhibited an even weaker K_m and full scale kinetics were not performed.

α_2 AP (1–15) and *S. Aureus* Fnb A (100–114) both contain a single, charged amino acid between the two glutamines. By contrast, TAFI has the polar serine and the small and flexible glycine between the two glutamines. Moreover, a review of TRANSDAB [8] reveals few examples of substrates with a Q at this P₋₃ position. It is important to point out that the loss in binding interactions occurs even though TAFI contains a beneficial R at the P₋₁₀ position. The higher than expected K_m value is proposed to be due either to the non-optimal $^2\text{QSGQ}^5$ sequence and/or difficulties anchoring the $^{10}\text{LPRTSR}^{15}$ sequence to a distant FXIIIa surface. Physiologically, α_2 -antiplasmin is a major substrate for FXIIIa and plays a vital role in regulating fibrinolysis and fibrin architecture [43]. Amino acid properties within the TAFI (1–15) sequence likely contribute to making this protein a significantly weaker transglutaminase catalyzed substrate.

Examining the X-ray Crystal Structures of FXIII_{A2}

More structural information on the FXIII_A active site region will help in defining the features that control the substrate specificity of transglutaminases. The X-ray crystal structures reported thus far for the activated FXIII A₂ dimer, however, have all been in the closed conformation with access to the active site region obstructed [44; 45]. The first crystal structure of an inhibitory peptide bound to nonproteolytically activated FXIII A₂ was published in late 2013 by Stieler *et al.* [46]. The inhibitory peptide contains a glutamine side chain analog that attaches covalently to the catalytic Cys 314 via Michael Addition [46]. The sequence of the ZED1301 inhibitory peptide includes Ac-Asp-MA(Michael Acceptor)-Nle-Nle-Leu-Pro-Trp-Pro-OH. The MA unit is composed of an electrophilic α , β unsaturated carbonyl compound that serves as a glutamine side chain mimetic.

For the crystallization project, the FXIII A₂ unit was nonproteolytically activated with 100 mM calcium [46]. Three calcium ions were found to be bound to a single FXIII A subunit, and these cations assisted in maintaining the final transglutaminase conformation. In the presence of the Michael Acceptor containing peptide, the FXIII A₂ dimer unit dissociated into two monomeric A subunits (Fig 8, Panel A, front view of FXIII). A side view of the active site- inhibited monomer (Fig 8, Panel B) reveals that in response to adding ZED1301, the FXIII β -barrel-1 and β -barrel-2 domains rotated horizontally (90°) away from the catalytic core region and were directed upwards closer to the β -sandwich domain. For the first time, it was possible to see an exposed FXIII_A active site region containing a bound ligand. A review of the catalytic site (accommodating ligand residues P₂ to P₋₂) confirms the presence of the Michael Acceptor group at the P₁ site covalently bound to the catalytic FXIII_A C314 located within the enzyme's S₁ subsite. The remainder of ZED1301 becomes directed toward what Stieler *et al.* call the hydrophobic site (contains S₋₃ to S₋₆ enzyme subsites that accommodate the P₋₃ to P₋₆ ligand residues) [46]. FXIII_A residues that interact at or near the ZED1301 peptide include W279, Y283, Q313, Y315, R223, V369, Y370, N371, Y372 (highlighted in green in Fig 8). A review of these FXIII_A residues confirms that the substrate binding region for the ZED1301 inhibitory peptide (Ac-Asp-MA(Michael Acceptor)-Nle-Nle-Leu-Pro-Trp- Pro-OH) is quite hydrophobic with some additional polar character.

The kinetic studies involving our MALDI-TOF MS assay reveal that amino acid substitutions at the P₋₁ to P₋₃ positions can hinder optimal binding within the FXIII_A active site region S₋₁ to S₋₃. For example, changing the P₋₁ position of α_2 AP from an acidic E3 to a basic residue R3 causes this peptide to now exhibit the weaker binding properties of *S. Aureus* FnbA (100–114). A review of the crystal structure of the inhibited complex indicates that FXIII_A residues that are located near the corresponding positions on bound ZED1301 include Y370, N371, and V369. This FXIII_A active site region may have a harder time accommodating the positively charged environment from the R3 guanidinium group at the P₋₁ position. Interestingly, *S. Aureus* Fnb A (100–114) has difficulty accepting a Q105S substitution. Somehow the small polar S105 cannot be as well accommodated by the broad FXIII_A S₋₂ substrate specificity pocket as α_2 AP (1–15) Q4S. Studies with the TAFI sequence reveal that Q5 at the P₋₃ instead of the P₋₂ position produces one of the weakest

K_m values to date. There may be trouble accepting the more polar TAFI Q5 within the hydrophobic binding area (FXIIIa S-3 to S-6 positions) as opposed to the L5 of ZED1301.

There must be additional subsites on FXIIIa that interact with the P-8 to P-10 substrate positions. α_2 AP (1–15) and *S. Aur* FnbA (100–114) could both take advantage of this distant FXIIIa surface to anchor on to and thus help lower the K_m value. α_2 AP (1–15) K12R generated the best K_m value to date. The newly reported FXIII-ZED1301 complex extends to the P-6 position [46]. Assessing where to direct the P-8 to P-10 ligand residues on to the transglutaminase surface may however be challenging. Computational studies have been performed on the docking of inhibitors to FXIII, TGase2, TGase3, and microbial transglutaminase [47; 48; 49]. Similar to ZED1301, the synthetic inhibitors have not extended all the way to the P-8 to P-10 substrate positions. There is often more than one putative binding region to direct a ligand to beyond the P-4 position. X-ray crystal structures of FXIIIa bound to longer ligands will be beneficial to the field.

Conclusions

Although several FXIIIa targets have been identified, the characteristics that help produce a good glutamine-containing substrate are not well understood. A MALDI-TOF mass spectrometry assay was developed to help better decipher how FXIIIa selects these substrates. The knowledge gained may be used later to design more potent substrates and inhibitors of this transglutaminase. The MALDI-TOF mass spectrometry assay has the advantage of directly monitoring consumption of substrate and formation of product within a single platform. Kinetic analysis using this new assay indicates that the $^2\text{QEQ}^4$ (P₁, P₋₁, P₋₂ positions) residues of α_2 AP can be well accommodated within the FXIIIa active site. An α_2 AP K12R substitution further enhances secondary anchoring within the P-8 to P₋₁₁ region thus generating the best K_m value to date. *S. Aureus* Fnb A 100–114 was examined for the first time and serves to prove that FXIIIa substrates do benefit from a distant exosite. Kinetic properties for this more basic substrate are, however, not as good as those of α_2 AP. TAFI is likely such a weak FXIIIa substrate because the second Q is moved from the common P₋₂ position to the P₋₃ position that is now at the entrance to the FXIII hydrophobic binding site. Further clues about the amino acid features that govern the substrate specificity of FXIIIa will be obtained from employing our single platform MALDI-TOF mass spectrometry assay.

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MALDI-TOF Mass Spectrometry Based Kinetic Assay

Figure 1. Flow chart describing the MALDI-TOF mass spectrometry assay

In this assay, Factor XIII is activated by thrombin in the presence of calcium. The resultant FXIIIa is then responsible for catalyzing the reaction between the Q-containing substrate and glycine ethylester that serves as the lysine mimic. EDTA is added to scavenge calcium away from the FXIIIa and quench the transglutaminase reaction. MALDI-TOF mass spectrometry is used to monitor loss of the Q-substrate reactant and formation of the Q-substrate-GEE product.

Figure 2. Evaluating the influence of C₁₈ resin treatment on the quality and integrity of MALDI-TOF mass spectra of Q-containing substrate peptides

A kinetic reaction mix containing 400 μ M α_2 AP (1–15) Q4K (no FXIII, thus the zero time point) yielded a MALDI-TOF mass spectrum that showed predominantly the sodiated adduct of the peptide. C₁₈ resin treatment using a zip tip reduced the levels of sodiated adducts. Moreover, the S/N of the spectrum increased. A zero time point kinetic reaction mix containing 600 μ M *S. Aur FnbA* (100–114) exhibited similar improvements in MALDI-TOF mass spectra following zip tipping. The integrities of both peptides were maintained. The C₁₈ resin strategy could thus be employed successfully for this kinetic assay project.

Figure 3. Measurement of velocity from a MALDI-TOF mass spectrometry assay

A) Enzymatic reactions for 400 μ M α_2 AP (1–15) Q4K were quenched at 3, 9, and 18 minutes. The peak at 1653 m/z corresponds to the amount of Q-containing substrate α_2 AP (1–15) Q4K present after each quench point. The peak at 1739 m/z corresponds to the increasing amount of α_2 AP (1–15) Q4K cross-linked with glycine ethylester (α_2 AP (1–15) Q4K – GEE). Sodiated and potassiated species are also labeled. B) A more extensive plot of remaining α_2 AP (1–15) Q4K concentrations as a function of time (3, 6, 9, 12, 15, and 18 min). MALDI peaks were converted into concentrations using the peak height ratio method described in Materials and Methods. The slope of the substrate concentration versus time plot was used to calculate the velocity associated with the 400 μ M α_2 AP (1–15) Q4K reaction. Velocities were then assessed for a series of peptide substrate concentrations. Each substrate series was examined in triplicate

Figure 4. MALDI-TOF mass spectra displaying single Q-peptide - GEE crosslinking reactions that can be followed kinetically

A) FXIIIa catalyzed reaction involving 175 μ M α_2 AP (1–15) K12R and 5 mM GEE quenched after 18 minutes. The Q-containing peptide (reactant) appears at 1681 m/z and the peptide-GEE (product) at 1767 m/z (1681 + 86). A second product involving both Q residues (1681 + 172 = 1853) is not observed. For the different Q-containing peptides examined in this project, only a single crosslinking reaction could be followed in the MALDI-TOF kinetic assay. B) FXIIIa catalyzed reaction involving 500 μ M *S. Aur* Fbn A

(100–110) and 5 mM GEE quenched after 20 minutes. The Q-containing peptide substrate (reactant) appears at 1269 m/z and the peptide-GEE (product) at 1355 m/z. C) FXIIIa catalyzed reaction involving 400 μ M *S. Aur* Fnb A (100–114) and 5 mM GEE quenched after 18 minutes. The Q-containing peptide substrate (reactant) appears at 1697 and the peptide-GEE (product) at 1783. D) FXIIIa catalyzed reaction involving 1200 μ M TAFI (1–15) and 5 mM GEE quenched after 25 minutes. The Q-containing peptide (reactant) appears at 1630 m/z and the peptide-GEE (product) at 1716 m/z.

Figure 5. Following FXIIIa catalyzed reactions involving $\alpha_2\text{AP}$ (1–15) K12R and GEE
 Enzymatic reactions for 100 μM $\alpha_2\text{AP}$ (1–15) K12R were quenched at 3, 9, and 18 minutes. The peak at 1681 m/z corresponds to the amount of Q-containing substrate $\alpha_2\text{AP}$ (1–15) K12R present after each quench point. The peak at 1767 m/z corresponds to the increasing amount of $\alpha_2\text{AP}$ (1–15) K12R cross-linked with glycine ethylester ($\alpha_2\text{AP}$ (1–15) K12R – GEE).

Figure 6. Following FXIIIa catalyzed reactions involving *S. Aureus* FnbA (100–114) and GEE Enzymatic reactions for 600 μ M *S. Aureus* FnbA (100–114) were quenched at 3, 9, and 18 minutes. The peak at 1698 m/z corresponds to the amount of Q-containing substrate *S. Aureus* Fnb A (100–114) present after each quench point. The peak at 1783 m/z corresponds to the increasing amount of *S. Aureus* FnbA (100–114) cross-linked with glycine ethylester (*S. Aureus* Fnb A (100–114) – GEE).

Figure 7. Following FXIIIa catalyzed reactions involving TAFI (1–15) and GEE
Enzymatic reactions for 1200 μ M TAFI (1–15) were quenched at 3, 9, and 18 minutes. The peak at 1630 m/z corresponds to the amount of Q-containing substrate TAFI (1–15) present after each quench point. The peak at 1716 m/z corresponds to the increasing amount of TAFI (1–15) cross-linked with glycine ethylester (TAFI (1–15) – GEE).

Figure 8. Examining the effects of the Michael acceptor inhibitor ZED1301 on the structural properties of Factor XIIIa

Unactivated cellular FXIII in its ligand free state exists as an A₂ dimer. β -barrels 1 (yellow, β B1) and β -barrels 2 (orange, β B2) are labeled. Nonproteolytically activated FXIIIa dissociates into two monomers upon binding the Michael acceptor ZED1301 [46]. The β B1 & β B2 domains shift 90° and then move upwards in response to the inhibitor. For the first time, the active site region of FXIIIa can be examined. In the highlighted panel, the ZED1301 ligand (Asp1-MA2-Nle3-Nle4-Leu5-Pro6-Trp7-Pro8) is blue and the FXIIIa catalytic triad (Cys 314, Asp396, and His373) is red. Cys 314 forms a covalent complex with the MA2 group. The FXIIIa residues that are located in the vicinity of ZED1301 are shown in green. All panels were made in Pymol (DeLano Scientific Inc). PDB code 4KTY.

TABLE 1

FXIIIa Substrate Sequences Aligned Along the Reactive Glutamine Position^a

Q-containing FXIIIa Substrate	Substrate Sequence Aligned Along the P ₁ residues	Masses [M+ H ⁺] m/z	
	P ₂ P ₁ P ₋₁ P ₋₂P ₋₈ P ₋₉ P ₋₁₀	Free	+GEE
α ₂ AP (1–15) Q4	N <u>Q</u> E Q V S P L T L L K L G N	1653	1739
α ₂ AP (1–15) Q4K	N <u>Q</u> E K V S P L T L L K L G N	1653	1739
α ₂ AP (1–15) K12R	N <u>Q</u> E Q V S P L T L L R L G N	1681	1767
α ₂ AP (1–15) E3R	N <u>Q</u> R Q V S P L T L L K L G N	1680	1766
<i>S. Aureus</i> Fnb A (100–110)	S G D <u>Q</u> R Q V D L I P	1269	1355
<i>S. Aureus</i> Fnb A (100–114)	S G D <u>Q</u> R Q V D L I P K K A T	1697	1783
<i>S. Aureus</i> Fnb A (100–114) Q105S	S G D <u>Q</u> R S V D L I P K K A T	1656	1742
<i>S. Aureus</i> Fnb A (100–114) R104E	S G D <u>Q</u> E Q V D L I P K K A T	1670	1756
TAFI (1–15)	F <u>Q</u> S G Q V L A A L P R T S R	1630	1716
TAFI (1–15) Q5S	F <u>Q</u> S G S V L A A L P R T S R	1589	1675

^aFXIIIa substrate peptides derived from the proteins α₂-antiplasmin (α₂ AP), *S. Aureus* fibronectin binding protein A (Fnb A), and thrombin activatable fibrinolysis inhibitor (TAFI). The reactive glutamines are bold/underlined and located at the P₁ position. The P₋₁ to P₋₃ and the P₋₈ to P₋₁₀ positions may participate in promoting binding interactions at the active site.

TABLE 2Binding Interactions Associated with the Reaction of FXIIIa with Q-Substrates^a

Q- Containing FXIIIa Substrate	K _m (μM)	k _{cat} (s ⁻¹)
α ₂ AP (1–15) K12R	108 ± 27	283 ± 28
α ₂ AP (1–15) E3R	594 ± 114	484 ± 44
<i>S. Aureus</i> Fnb A (100–110)	971 ± 175	387 ± 40
<i>S. Aureus</i> Fnb A (100–114)	571 ± 109	535 ± 46
<i>S. Aureus</i> Fnb A (100–114) Q105S	644 ± 122	453 ± 38
TAFI (1–15)	3330 ± 905	559 ± 109

^aK_m values were derived from a MALDI-TOF mass spectrometry assay involving Q-containing substrate peptides and glycine ethylester. The assay design is described in Materials and Methods. The results shown represent averages from three independent trials of the concentration ranges. Kinetic values were calculated using nonlinear regression analysis methods using SigmaPlot. The error values correspond to standard error of the mean.