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Evaluating factor XIII specificity for glutamine-containing substrates using a matrix-assisted laser desorption/ionization time-of-flight 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 kinetic assay is no longer reliant on a secondary coupled reaction, on substrate labeling, or on detecting only the final deacylation portion of the transglutaminase reaction. With the MALDI–TOF MS assay, glutamine-containing peptides derived from α_2-antiplasmin, *Staphylococcus 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} substrate positions are sensitive 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.

In blood coagulation, the serine protease thrombin cleaves the N-terminal portions of the fibrinogen Aα and Bβ chains. The resultant fibrin monomers polymerize noncovalently into linear protofibrils and then laterally into fibers, forming a blood clot that is held together by noncovalent 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 carboxyamide 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 αβγ environment, leading to formation of a rigid blood clot [7]. Other key FXIIIa substrates include α_2-antiplasmin (α_2AP), fibronectin, thrombin-activatable fibrinolysis inhibitor (TAFI), plasminogen activation inhibitor-1, vitronectin, and *Staphylococcus 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 [6,8–11].

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 [16–18] methods. 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 ultraviolet–visible (UV–Vis) assay in which ammonia released from the reactive glutamines is detected via a separate

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[1] *Abbreviations used:* FXIII, factor XIII; FXIIIa, activated FXIII; α_2AP, α_2-antiplasmin; TAFI, thrombin-activatable fibrinolysis inhibitor; UV–Vis, ultraviolet–visible; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; GEE, glycine ethylester; Fnb A, fibronectin binding protein A; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; α-CHCA, α-cyano-4-hydroxycinnamic acid; ZED1301, FXIIIa inhibitory peptide (Ac-Asp-MA-Nle-Nle-Leu-Pro-Trp-Pro-OH); MA, Michael acceptor unit.

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secondary colorimetric assay [19–22]. This resultant ammonia is used to convert α-ketoglutarate to glutamate in a nicotinamide adenine dinucleotide (NADH)-dependent reaction that is monitored at 340 nm. 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 reporter groups 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 matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) kinetic 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 [19–22]. The individual transglutaminase reactions are quenched at distinct time points, and MALDI–TOF mass spectra are collected to monitor for loss of Q-containing substrate and gain of the cross-linked product (Q-containing substrate)–GEE (gain of 86 *m*/*z*). Overall, the MALDI–TOF MS kinetic assay is no longer reliant on a secondary coupled reaction, on substrate labeling, or on detecting only 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 P_1 position. Amino acids can be assigned as $\ldots P_4\ P_3\ P_2\ P_1^{(\text{reactive Q})}$ and then to the right of the reactive Q as $P_{-1}\ P_{-2}\ P_{-3}\ P_{-4}\ldots$ (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 onto the extended FXIIIa active site region. Three FXIIIa substrates containing reactive Q residues are α_2AP, *S. aureus* fibronectin binding protein A (Fnb A), and TAFI (Table 1). Characteristics of these substrates are described below.

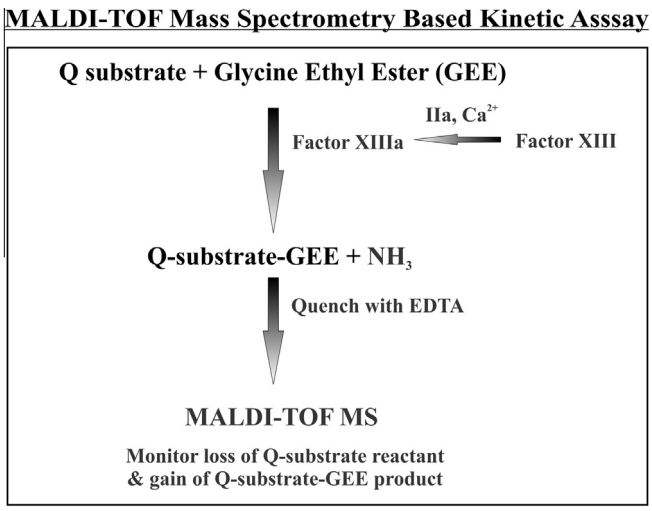

Fig.1. Flow chart describing the MALDI-TOF MS assay. In this assay, factor XIII is activated by thrombin in the presence of calcium. The resultant FXIIIa is then responsible for catalyzing the reaction between the Q-containing substrate (Q-substrate) and glycine ethylester (GEE) that serves as the lysine mimic. Ethylenediaminetetraacetic acid (EDTA) is added to scavenge calcium away from the FXIIIa and quench the transglutaminase reaction. MALDI–TOF MS is used to monitor loss of the Q-substrate reactant and formation of the Q-substrate–GEE product.

α_2AP (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–α_2AP to K303 of the fibrin(ogen) α-chain [26,28,29]. While this N1–α_2AP is tethered to a blood clot, its C-terminal domain remains free to inhibit plasmin [26]. Previous coupled UV–Vis kinetic assays with α_2AP (1–15) (Table 1) have indicated that the Q2 at the P_1 position is the reactive glutamine of this peptide with the Q4 at the P_{-2} position, serving a supporting role in binding [20]. α_2AP also contains a ^{10}LLKL^{13} 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 α_2AP (1–15) sequence, Fnb A contains glutamines at the P_1 and P_{-2} positions (Table 1) [32–34]. By contrast, *S. aureus* Fnb A has a basic R at the P_{-1} position, whereas α_2AP (1–15) has an acidic E. As found in α_2AP, *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 α_2AP 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. Thus, the second Q residue is switched from the P_{-2} position to the P_{-3} position. As observed with α_2AP 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 MS kinetic assay achieves this goal by recording the consumption of the Q-containing substrate and formation of the Q–GEE product within a single assay platform. This MS-based assay is no longer reliant on a secondary coupled reaction, on substrate labeling, or on detecting only the final deacylation portions of the transglutaminase reaction. Using our direct monitoring assay, the kinetic properties of α_2AP (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 MS 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 Paul Bishop (ZymoGenetics, Seattle, WA, USA). The lyophilized FXIII was reconstituted in 18 MΩ deionized water, aliquoted, and stored at −70 °C until future use. The concentration of the FXIII A_2 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 A_2. All of the Q-containing substrate peptides (Table 1) were synthesized and purified by New England Peptide (Gardner, MA, USA). The