Site-directed Mutagenesis of the Calcium-binding Site of Blood Coagulation Factor XIIIa*

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Blood coagulation factor XIIIa is a calcium-dependent enzyme that covalently ligates fibrin molecules during blood coagulation. X-ray crystallography studies identified a major calcium-binding site involving Asp438, Ala457, Glu485, and Glu490. We mutated two glutamic acid residues (Glu485 and Glu490) and three aspartic acid residues (Asp472, Asp476, and Asp479) that are in close proximity. Alanine substitution mutants of these residues were constructed, expressed, and purified from Escherichia coli. The $K_{act}$ values for calcium ions increased by 3-, 8-, and 21-fold for E485A, E490A, and E485A,E490A, respectively. In addition, susceptibility to proteolysis was increased by 4-, 9-, and 10-fold for E485A, E490A, and E485A,E490A, respectively. Aspartic acids 472, 476, and 479 are not involved directly in calcium binding since the $K_{act}$ values were not changed by mutagenesis. However, Asp476 and Asp479 are involved in regulating the conformation for exposure of the secondary thrombin cleavage site. This study provides biochemical evidence that Glu485 and Glu490 are Ca$^{2+}$-binding ligands that regulate catalysis. The binding of calcium ion to this site protects the molecule from proteolysis. Furthermore, Asp476 and Asp479 play a role in modulating calcium-dependent conformational changes that cause factor XIIIa to switch from a protease-sensitive to a protease-resistant molecule.

The plasma factor XIII (FXIII)$^1$ molecule is a tetramericzymogen ($A_2B_2$) that circulates in human plasma and that is composed of two A-chains and two glycosylated B-chains (1–3). In contrast, monocytes and platelet FXIII exist as an intracellular dimer composed of two A-chains (1–3). X-ray crystallography studies revealed that the FXIII A-chain is composed of four distinct structural domains. Starting from the N terminus, there is a $\beta$-sandwich domain (residues 43–184) followed by a catalytic core (residues 185–515) and two $\beta$-barrels (residues 516–628 and 629–727) at the C terminus (4). The activation peptide (residues 1–37) of each A-subunit crosses the dimer interface and partially occludes the opening to the active site in the catalytic core of the other subunit. Thrombin cleavage of the A-chains at the Arg$^{27}$–Gly$^{38}$ bond is required for the A-chains to express FXIIIa (the FXIII A-chain with the activation peptide (Met$^1$–Arg$^{27}$) removed) activity in vivo (1–3). Thrombin cleavage of plasma FXIII is a calcium-independent reaction (1–3) that is accelerated by fibrin polymers (1–3). After proteolysis, all subsequent steps in the formation and function of FXIIla are calcium-dependent (5–8).

Detailed biochemical studies of purified plasma XIII established that calcium ions dissociate B-chains from the thrombin-cleaved A-chains of plasma FXIII (1). Then, a calcium-dependent conformational change in the thrombin-cleaved A-chain is required to expose the active-site Cys$^{314}$ (1). Calcium ions are essential for FXIIla to catalyze intermolecular isopeptide bonds between protein molecules (1, 3, 5). There are two distinct biochemical steps that occur during FXIIla catalysis. In the first step, the active-site Cys$^{314}$ of FXIIla binds the peptidobound glutamine substrate, forming a thioester bond intermediate and releasing ammonia (5). Then, the enzyme-substrate complex interacts with either a primary amine or a peptidobound lysine residue, producing an isopeptide bond (5). Calcium ions are required for both steps of catalysis (6–8). Calcium ions also protect FXIIla from proteolysis at the Lys$^{513}$--Ser$^{514}$ site (9–11).

The precise location and number of calcium-binding sites in factor XIII A-chains are hampered by the relative low affinity of the calcium binding (12). Studies performed by Lewis et al. (12) demonstrated that the FXIII A-chain binds 1.2–1.5 calcium ions with low affinity ($K_d \sim 10^{-4}$ M) and up to 8 calcium ions/molecule of plasma FXIII at higher calcium concentrations. A putative calcium-binding site was first proposed by Taka-hashi et al. (11) as an EF-hand-like structure located in a fragment between Glu$^{467}$ and Asp$^{475}$. This area is rich in the negative charged amino acid residues that are postulated to bind calcium ions. Recent x-ray crystallography studies of factor XIII A-chain crystals grown in the presence of Sr$^{2+}$ or Yb$^{3+}$ demonstrated there is no EF-hand-like motif (13, 14). X-ray crystallography identified a major cation-binding site containing Asp$^{438}$, Ala$^{457}$, Glu$^{485}$, and Glu$^{490}$ and a minor site containing Asp$^{476}$ and Glu$^{479}$ (13–15). Interestingly, there were no major conformational changes between the cation-binding factor XIIIa and thezymogen structures (13–15). The role of these cation-binding sites in expression of FXIIla activity and regulation of the conformation of the protein in solution remains unclear. In addition, there were differences in coordinating ligands between the Sr$^{2+}$- and Yb$^{3+}$-bound structures, and fewer than the ideal number of coordinating ligands were reported (14, 15). These data suggest that other amino acids are also involved in the binding of cations. Since high calcium concentrations (>50 mM) enable the platelet factor XIII zymogen to express enzymatic activity in the absence of thrombin cleavage, there may be other lower affinity calcium-binding sites that play a role in regulating catalysis and protein con-

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¶¶ The abbreviations used are: FXIII, factor XIII; TGase, transglutaminase; GST, glutathione S-transferase.
formation. Therefore, site-directed mutagenesis of amino acid residues identified by x-ray crystallography studies and other potential calcium-binding residues is necessary to confirm their role in regulating catalysis and protein conformation.

In this study, we performed site-directed mutagenesis analysis of two calcium-binding ligands (Glu485 and Glu490) identified by x-ray crystallography. These amino acid sequences are conserved in other transglutaminases such as human tissue transglutaminase (TGase), bovine endothelial TGase, and guinea pig TGase (16–18). In addition, three aspartic residues (Asp472, Asp476, and Asp479) located in close proximity to Glu485 and Glu490 were mutated. The substitution mutants were expressed, purified, and analyzed for their catalytic properties and sensitivity to thrombin proteolysis. The effects of these mutations on the structure and function of FXIIIa will be discussed.

EXPERIMENTAL PROCEDURES

Materials—All restriction enzymes, T4 DNA ligase, bacteria alkaline phosphatase, LB medium, and yeast extract were obtained from Life Technologies, Inc. Human α-thrombin was supplied by Dr. J. W. Fenton II (New York State Department of Health, Albany, NY). Oligonucleotides were synthesized by Bio- synthesis, Inc. (Lewisville, TX) or Life Technologies, Inc. All other reagents used in this study were purchased from Sigma unless stated otherwise.

Construction of Factor XIII A-chain Mutants—All factor XIII A-chain mutants were constructed using oligonucleotide-mediated mutagenesis (T7-Gen mutagenesis kit, Amersham Pharmacia Biotech) as described previously (19, 20). The single point mutations E485A, E490A, D472A, D476A, and D479A were constructed using oligonucleotides 1 (GATA CTTACA AATTC CGGTG AAGGA GAAGA GAG), 2 (GAAG TCAGA AAGGA GGAGG ATTGG CCTTA AACAG TGG), 3 (AATTT GTTGG GGCGG CATGA TGG), 4 (GGCAT GATGG CGATT ACTGA GTTACA AATTC CAAGC CGGTC AAGAA GAAGA GAG), 2 (GAAGG TAC), and 5 (GGATA TTACT GCGAC TTACA AATT), respectively. In addition to the single point mutations, four double mutants (E485A,E490A, GST-D476A,D479A, and GST-D472A,D476A,D479A) were also constructed. The DNA sequences for each mutant were confirmed by DNA sequencing. The Ncol-PstI fragments of these constructs were then subcloned into the Ncol and PstI sites of pKK233-2 as described previously (20). The E485A, E490A, E485A,E490A, D476A,D479A, and D472A,D476A,D479A triple mutant A-chains or mutants, 10 mM CaCl2,

Expression of Factor XIII A-chain Mutants—The expression and preparation of Escherichia coli lysates were as described (19, 20). Briefly, after resuspending E. coli pellets in 20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM diithiothreitol/EDTA, and 15% glycerol, the cells were lysed by lysozyme and sonication as described (Cell debris was removed by centrifugation at 22,000 × g for 20 min, and the supernatants were aliquoted and stored at −70 °C. Fresh aliquots were used for each experiment, and unused samples were discarded.

Purification of Factor XIII A-chains—The glutathione S-transferase (GST)-factor XIII A-chain fusion proteins were purified using glutathione-agarose affinity as described previously (19).

Protein Determination—The amount of protein was determined by the Bradford method (21) using a commercial reagent (Bio-Rad). Bovine serum albumin was used as the protein standard. The concentrations of affinity-purified GST-factor XIII A-chains, GST-E485A, GST-E490A, GST-E485A,E490A, GST-D476A,D479A, and GST-D472A,D476A,D479A were further analyzed by scanning densitometry of the Coomassie Blue-stained gel of each protein band.

Thrombin-dependent Transglutaminase Assay—The transglutaminase activities of E. coli lysates containing wild-type factor XIII A-chains or mutants were quantitated by measuring the incorporation of [3H]putrescine (NE Laboratories) or 5-(biotinamido)pentylamine into N,N′-dimethylglycinol as described previously (22, 23). Increasing concentrations (2.5–150 μg/ml) of the thrombin-activated mixture were used to estimate the specific activity, and all assays were performed in triplicate. Aliquots were taken to analyze FXIIIa formation by quantitative immunoblotting and were used to normalize the FXIIIa concentration in the reaction (19). The thrombin-activated mixtures were added to the reaction mixture containing glycine ethyl ester, synthetic peptide substrate, NADH, α-ketoglutarate, and bovine glutamate dehydrogenase provided by the manufacturer (Behring Diagnostics, Inc., Somerville, NJ) and incubated at 37 °C for 10 min. The release of ammonia was measured by the decrease in NADH and was quantitated by goldfish ammonia substrate (5-(biotinamido)pentylamine) was determined using an assay as described earlier (22–24). The transglutaminase activity data were transformed by an Eadie-Hofstee plot to determine the Kcat.

RESULTS

The locations of the amino acid residues selected for mutagenesis are shown in relationship to the domain structure of the factor XIII A-chain (Fig. 1A). In this model, the calcium-binding ligands Glu485 and Glu490 are located at the junction between the catalytic core domain and the first β-barrel do-
A model was constructed based on the three-dimensional coordinates of the factor XIII A-chain, including the activation peptide (Asp 479 and Glu 485 and Glu 490 are 29.5, 16, and 10.6 Å, respectively (Fig. 1). The shortest distances between Asp 472, Asp 476, and Asp 479 are similar to that of E490A and was reduced by 47 and 49%. The activity of the E485A, E490A double mutant was reduced by 79%. The mutants that had reduced activity had the activation peptide cleaved by thrombin when analyzed by immunoblotting (data not shown). Therefore, the loss of FXIIIa activity displayed by these mutants was not due to a defect in cleaving the activation peptide or in cleavage at another site within the catalytic core that would inactivate FXIIIa.

The apparent $K_m$ was unchanged when the glutamine substrate ($N\text{-}N'\text{-dimethylcasein}$) was studied for the single and double mutants E485A, E490A, E485A,E490A, D472A, D476A, D479A, D472A,D476A, D472A,D479A, and D476A,D479A. The $K_m$ for the triple mutant D472A,D476A,D479A was only 3-fold higher than that for wild-type FXIIIa (Table I). The apparent $K_m$ for the primary amine substrate (5-biotinamido)pentylamine) was similar for all the thrombin-activated FXIII A-chain mutant molecules studied (75–107 μM).

We examined whether the loss of activity was due to a defect in the formation of the thioester bond or in the catalysis of the amide transfer reaction for the E485A, E490A, E485A,E490A, D476A, D479A, D472A,D476A, D472A,D479A, and D476A,D479A mutants. The formation of the thioester bond was measured by quantitating ammonia release using a glutamine peptide substrate as described previously (16, 22). The affinity-purified E485A, E490A, E485A,E490A, D476A,D479A, and D472A,D476A,D479A mutants were thrombin-activated and found to have thioester bond formation reduced by 1.8-, 1.9-, 1.9-, 4.5-, and 16-fold, respectively, when compared with wild-type FXIIIa.

The $K_{cat}$ values for calcium for the E485A, E490A, and E485A,E490A mutants increased by 2.8-, 8-, and 21-fold, respectively, whereas the D476A and D479A mutants had similar $K_{cat}$ values (—337 μM) for calcium ions compared with wild-type FXIIIa. The $K_{cat}$ values for the D472A, D472A,D479A, and D472A,D479A,D479A mutants were only 1.3–1.7-fold higher than that for wild-type FXIIIa. The $K_{cat}$ values for the D472A,D476A,D479A mutants were slightly reduced by 2.1- and 1.6-fold, respectively (Table I).

All mutants bound to a fibrin clot to the same extent as wild-type FXIIIa (84–92%) (Table I). The FXIIIa mutant molecules also displayed the same pattern of fibrin cross-linking as wild-type FXIIIa, with $gamma$-chain dimer formation occurring prior to $alpha$-chain polymer formation. However, the rate of cross-linking was reduced for mutants that had reduced activity as measured by the 5-(biotinamido)pentylamine incorporation assay (data not shown).

In the presence of 5 mM Ca$^{2+}$, we found that the thrombin proteolysis patterns of different mutants were the same as that of the wild-type FXIII A-chain. The generation of FXIIIa was followed by cleavage at the Lys<sup>515</sup>-Ser<sup>514</sup> site with the formation of the 50-kDa fragment (Arg<sup>38</sup>–Lys<sup>513</sup>). The amount (intensity) of the 50-kDa fragment formed in the D472A, D476A, D479A, D472A,D476A, D472A,D479A, D476A,D479A, and

All FXIII mutants were expressed in E. coli to the same level as wild-type FXIII A-chains (20 mg/liter). When analyzed by SDS-polyacrylamide gel electrophoresis, they migrated with the same mobility as the wild-type FXIII A-chains. The summary of the biochemical data derived from the study of each mutant is shown in Table I. The specific activity of the D472A mutant was unchanged, whereas the specific activities of the point mutations D476A and D479A were reduced by only ~15%. An additional Ala substitution at Asp<sup>472</sup> to the D476A and D479A mutants did not further modify the activity. In contrast, there was a significant inhibition of activity if both aspartic acids 476 and 479 were converted to alanine. The activity of the D476A,D479A double mutant was reduced by 69%, and an additional Ala substitution at Asp<sup>472</sup> reduced activity by 86%. The specific activity of E485A was similar to that of E490A and was reduced by 47 and 49%. The activity of the E485A,E490A double mutant was reduced by 79%. The mutants that had reduced activity had the activation peptide cleaved by thrombin when analyzed by immunoblotting (data not shown). Therefore, the loss of FXIIIa activity displayed by these mutants was not due to a defect in cleaving the activation peptide or in cleavage at another site within the catalytic core that would inactivate FXIIIa.

The apparent $K_m$ was unchanged when the glutamine substrate ($N\text{-}N'\text{-dimethylcasein}$) was studied for the single and double mutants E485A, E490A, E485A,E490A, D472A, D476A, D479A, D472A,D476A, D472A,D479A, and D476A,D479A. The $K_m$ for the triple mutant D472A,D476A,D479A was only 3-fold higher than that for wild-type FXIIIa (Table I). The apparent $K_m$ for the primary amine substrate (5-biotinamido)pentylamine) was similar for all the thrombin-activated FXIII A-chain mutant molecules studied (75–107 μM).

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In the presence of 5 mM Ca$^{2+}$, we found that the thrombin proteolysis patterns of different mutants were the same as that of the wild-type FXIII A-chain. The generation of FXIIIa was followed by cleavage at the Lys<sup>515</sup>-Ser<sup>514</sup> site with the formation of the 50-kDa fragment (Arg<sup>38</sup>–Lys<sup>513</sup>). The amount (intensity) of the 50-kDa fragment formed in the D472A, D476A, D479A, D472A,D476A, D472A,D479A, D476A,D479A, and
Calcium-binding Site of Factor XIII A-chains

| Table I | Summary of biochemical data for each mutant |
|---------|--------------------------------------------|
|         | $K_{m(Ca)}$ | $K_{m(calamine)}$ | $K_{act}(C_{aluminium})$ | Specific activity | Fibrin binding | Ammonia release |
| Wild-type | $75 \pm 13$ | $3 \pm 1$ | $337 \pm 40$ | $100$ | $87$ | $17.8 \pm 1.6$ |
| E485A | $99 \pm 19$ | $2.7 \pm 1.5$ | $934 \pm 80$ | $53 \pm 6$ | $91$ | $10.1 \pm 1.1$ |
| E490A | $90 \pm 15$ | $2.5 \pm 2$ | $2688 \pm 381$ | $51 \pm 8$ | $92$ | $9.3 \pm 0.8$ |
| E485A,E490A | $77 \pm 22$ | $3.5 \pm 1.6$ | $7170 \pm 560$ | $21 \pm 10$ | $85$ | $9.2 \pm 1.5$ |
| D472A, D476A, D479A | $89 \pm 27$ | $6.1 \pm 1$ | $451 \pm 64$ | $106 \pm 16$ | $84$ | ND |
| D472A, D476A | $75 \pm 1$ | $4.1 \pm 1.5$ | $307 \pm 86$ | $84 \pm 2$ | $88$ | ND |
| D479A | $77 \pm 2$ | $5 \pm 2$ | $268 \pm 182$ | $84 \pm 6$ | $88$ | ND |
| D472A,D476A, D479A | $73 \pm 2$ | $5.5 \pm 2$ | $579 \pm 136$ | $77 \pm 9$ | $87$ | ND |
| D472A,D479A | $70 \pm 7$ | $4.8 \pm 1.5$ | $424 \pm 18$ | $82 \pm 0.4$ | $91$ | ND |
| D476A,D479A | $107 \pm 1$ | $4.3 \pm 0.7$ | $159 \pm 40$ | $31 \pm 5$ | $90$ | $4.0 \pm 1.3$ |
| D472A,D476A,D479A | $103 \pm 8$ | $9 \pm 2$ | $213 \pm 165$ | $14 \pm 2$ | $88$ | $1.1 \pm 0.5$ |

* BP, 5-biotinamido-pentylamine; mAU, mill-absorbance units; ND, not determined.
* The specific activity was determined in reactions containing 1 mM CaCl$_2$.

D472A,D476A,D479A mutants were similar to that formed in the wild-type FXIII A-chain, with only 5% generated at 9.1 μM thrombin (Fig. 2A). In contrast, the formation of the 50-kDa fragment in the E485A, E490A, and E485A,E490A mutants was detected at thrombin concentrations as low as 0.54 μM. The formation of the 50-kDa fragment increased to 40% at 9.1 μM thrombin in the E485A, E490A, and E485A,E490A mutants.

In the absence of calcium ions (5 mM EDTA), thrombin concentrations as low as 0.56 μM initiated the formation of the 50-kDa degradation product for wild-type FXIIIa (Fig. 2B). The thrombin degradation patterns of D472A, D476A, D479A, D472A,D476A, D472A,D479A, E485A, E490A, and E485A,E490A were similar to that of the wild-type FXIII A-chain, with ~60% of the 50-kDa fragment generated at 9.1 μM. However, FXIIIa formed in the D476A,D479A and D472A,D476A,D479A mutants resisted degradation (Fig. 2B). In the absence of calcium ions, there was <10% of the 50-kDa fragment formed at 9.1 μM thrombin.

**DISCUSSION**

Calcium ions play an important role at several stages of plasma FXIII activation, catalysis, and degradation (1–3). Efforts to identify the effect of various calcium-binding ligands in plasma FXIII activation, catalysis, and degradation (1–3). Efforts to identify the effect of various calcium-binding ligands in plasma FXIII activation, catalysis, and degradation (1–3). Efforts to identify the effect of various calcium-binding ligands in plasma FXIII activation, catalysis, and degradation (1–3). Efforts to identify the effect of various calcium-binding ligands in plasma FXIII activation, catalysis, and degradation (1–3). Efforts to identify the effect of various calcium-binding ligands in plasma FXIII activation, catalysis, and degradation (1–3).

![Thrombin proteolysis of wild-type factor XIII A-chains and mutants](image)

**Fig. 2. Thrombin proteolysis of wild-type factor XIII A-chains and mutants.** A, the affinity-purified GST-wild-type FXIII (WT), GST-E485A, GST-E490A, and GST-E485A,E490A fusion proteins (4 μg each) were incubated with increasing concentrations of α-thrombin (0–9.1 μM) and 5 mM CaCl$_2$ at 37 °C for 15 min as described under “Experimental Procedures.” The reaction was stopped by SDS-polyacrylamide gel loading buffer, and samples were separated on an 8.5% polyacrylamide gel. The relative effect of calcium ions on catalysis is similar to the role that calcium ions play in catalyzing the transglutaminase reaction. The relatively indirect effect of calcium ions on catalysis is similar to the role that calcium ions play in catalyzing the transglutaminase reaction (24). The E485A, E490A, and E485A,E490A mutants had a marked reduction in the rate of ammonia release, suggesting the mutations interfered with calcium-dependent alignment of the catalytic triad with the protein-bound glutamine substrate. The loss of overall TGase activity correlated with a loss of the ability of FXIIIa to form the thioester intermediate. The results suggest that Glu$^{485}$ and Glu$^{490}$ are important for the generation of the calcium-dependent thioester intermediate. However, even when both of the acidic side chains were removed, the double mutant E485A,E490A still displayed calcium-dependent activation of...
TGase activity and could protect the secondary thrombin cleavage site when higher calcium concentrations were present. This suggests that the remaining calcium-binding coordinates or other lower affinity sites are sufficient to bind calcium and allow the mutants to display calcium-dependent TGase activity and a protease-resistant conformation.

Inspection of the three-dimensional model of the FXIII A-chain indicates that Glu485 and Glu490 are located on one side of the calcium-binding pocket next to a helix turn, whereas the other residues (Asp438 and Ala457) that coordinate with calcium are located on the other side (Fig. 1B). The side chains of three acidic residues swing in to participate in the binding of calcium or strontium ions (13–15). No large conformational change in the protein could be observed by x-ray crystallography after thrombin cleavage of the activation peptide and calcium binding (14, 27), and these may be an effect caused by constraints placed on the protein during crystallization (15). The cleaved molecule (i.e. FXIIIa) becomes susceptible to cleavage at the Lys513-Ser514 site (10). Failure to cleave the activation peptide makes the molecule resistant to cleavage at the Lys513-Ser514 site (28). The binding of calcium ions is sufficient to protect the Lys513-Ser514 site from proteolysis by thrombin (10). All these data indicate that there are limited local conformational changes around the Lys513-Ser514 site induced by binding to calcium ions. Mutants E485A, E490A, and E485A,E490A either lack one or two acidic side chain(s), could no longer undergo such conformational changes, and therefore were more susceptible to thrombin cleavage.

We then tested the effects that mutations at Asp472, Asp476, and/or Asp479 had on FXIIIa activity, substrate binding, and thrombin cleavage at the Lys513-Ser514 site. These three residues are located close in sequence and space to Glu485 and Glu490 (Fig. 1). We postulated that mutation(s) in these residues may cause a change in protein conformation that extends to Glu485 and Glu490 and that could then affect both transfugaminase activity and also proteolytic cleavage at the Lys513-Ser514 site. The conversion of Asp475 to alanine did not affect the transfugaminase activity and also proteolytic cleavage at the Lys513-Ser514 site. The conversion of Asp475 to alanine did not affect the transfugaminase activity, whereas a similar point mutation at either Asp476 or Asp479 caused only ~16% loss of FXIIIa activity (Table I). Based on the three-dimensional structure of the FXIII A-chain, the Asp476 side chain forms a hydrogen bond with Lys704, which is located in the second [β-barrel] domain and provides a link between these two structural domains. The position at Lys704 in 10 transfugaminases is always positively charged. Therefore, the role of Asp476 appears to be important in maintaining the overall conformation of the proteins rather than directly participating in catalysis. Asp476 forms a hydrogen bond with Thr478, which also forms a hydrogen-bond with Ile464. The positions at Asp476, Thr478, and Ile464 are absolutely conserved among 10 transfugaminases (Fig. 3). This raises the possibility that Asp476 plays an important role in protein folding and stability rather than calcium binding. Asp479 is less conserved among transfugaminases (Fig. 3) and does not participate in any interaction with other residues. This residue plays a minor role in catalysis since the single point mutation at Asp479 did not cause any major reduction in transfugaminase activity.

The only double alanine substitution mutant that had a further reduction in FXIIIa activity had aspartic acids 476 and 479 converted to alanine. This suggests that these two amino acid side chains have a greater effect on FXIIIa function than any of the other combinations. The addition of an alanine substitution mutation to the D476A,D479A double mutant caused a further loss of FXIIIa activity, although the protein was still active. The single, double, and triple mutations at Asp472, Asp476, and Asp479 did not significantly modify the Kₘ for calcium ions, suggesting that these residues do not participate directly in calcium-dependent catalysis of the isopeptide bond. However, the double mutant D476A,D479A and the triple mutant D472A,D476A,D479A have only 31 and 14% of FXIIIa activity, respectively, and are resistant to thrombin cleavage at the Lys513-Ser514 site even in the absence of calcium ions. The loss of FXIIIa activity correlated with the reduction in the rate of ammonia release, suggesting the mutations interfered with calcium-dependent alignment of the catalytic triad with the protein-bound glutamine substrate. This finding would suggest that this is an indirect effect caused by these residues and the calcium-binding site (Fig. 1A). These mutants apparently had an effect on the alignment of the glutamine substrate with the catalytic triad. There was also a significant change in the conformation of the molecule since the mutants adopted the protease-resistant conformation even after cleavage of the activation peptide.

Thrombin cleavage of factor XIII A-chains occurs by an ordered sequence of proteolysis (10). The cleavage of the N-terminal activation peptide at Arg477-Gly478 occurs first, followed by a secondary cleavage at the Lys513-Ser514 site, and this secondary cleavage is protected by calcium ions (10). When the Asp476 and Asp479 sites were both mutated to alanine, thrombin cleavage at the N-terminal activation peptide was not modified. In contrast to wild-type FXIII, the secondary thrombin cleavage site remained resistant to degradation in the presence of EDTA (Fig. 2). The conformation of the secondary cleavage site behaved similar to the calcium-protected form of FXIIIa. These aspartic acid residues apparently were important for factor XIIIa to expose the secondary cleavage site in the absence of calcium ions. Mutations at Asp476 and Asp479 probably induce a conformational change that renders the Lys513-

\[ \text{FXIII A} \begin{array}{c} \text{I} \text{V} \text{T} \text{K} \text{Q} \text{I} \text{G} \text{G} \text{D} \text{G} \text{M} \text{D} \text{T} \text{T} \\ \text{hTGk} \text{5} \text{T} \text{G} \text{K} \text{5} \text{3} \text{5} \text{5} \text{N} \text{M} \text{R} \text{E} \text{D} \text{I} \text{Y} \text{L} \\ \text{rTGk} \text{4} \text{2} \text{6} \text{E} \text{R} \text{E} \text{D} \text{I} \text{T} \text{H} \text{T} \\ \text{hTG} \text{4} \text{2} \text{6} \text{E} \text{R} \text{E} \text{D} \text{I} \text{T} \text{H} \text{T} \\ \text{bTG} \text{4} \text{2} \text{6} \text{E} \text{R} \text{E} \text{D} \text{I} \text{H} \text{T} \\ \text{mTG} \text{4} \text{2} \text{6} \text{E} \text{R} \text{E} \text{D} \text{I} \text{T} \text{H} \\ \text{pgTG} \text{4} \text{2} \text{6} \text{E} \text{R} \text{E} \text{D} \text{I} \text{T} \text{H} \text{T} \\ \text{2} \text{4} \text{4} \text{E} \text{R} \text{E} \text{D} \text{I} \text{T} \text{H} \text{T} \\ \text{Tg} \text{4} \text{2} \text{6} \text{E} \text{R} \text{E} \text{D} \text{I} \text{T} \text{H} \\ \text{TGe} \text{4} \text{2} \text{6} \text{E} \text{R} \text{E} \text{D} \text{I} \text{T} \text{H} \text{T} \end{array} \]
Ser^{514} site cryptic to thrombin. Alternatively, since thrombin has an anion-binding site that regulates substrate recognition, mutations at Asp^{476} and Asp^{479} to alanine could shield these residues from functioning as anion-binding sites that allow thrombin to align with its substrate (29).

Kinetic analysis of the single, double, and triple aspartic acid mutants demonstrated that alanine substitutions did not significantly affect the affinity of the enzyme for the primary amine (5-(biotinamido)pentylamine) and glutamine substrates, suggesting that the mutations had no effect on recognition of the glutamine and primary amine substrates. The affinity for the primary amine and glutamine substrates was not significantly changed in any of the aspartic acid mutants, demonstrating that there was not a global defect in the protein folding.

Factor XIIIa prefers to initially catalyze dimers in the γ-chains, whereas the tissue transglutaminase preferentially cross-links the α-chains (30–35). Results from analyzing the cross-linking pattern of these mutants suggest that modifying the glutamic (Glu^{485} and Glu^{490}) and aspartic acid (Asp^{472}, Asp^{476}, and Asp^{479}) residues did not alter the preference for the γ-chain site. The results from analyzing the fibrin binding properties of the putative calcium mutants document that the fibrin binding properties can be preserved despite losing ~85% of the FXIIIa activity. Previously, we established that expression of FXIIIa activity is not a requirement for fibrin binding since the inactive deletion mutant (ΔY481) could bind to fibrin (19).

In conclusion, this study provides new evidence that glutamic acids 485 and 490 play an important role in regulating the formation of the calcium-dependent thioester bond with glutamine substrates. Glutamic acids 485 and 490 are also critical in participating in the conformational change induced by calcium ions for the protection of the secondary thrombin cleavage site at Lys^{513}. Ser^{514}. Aspartic acids 476 and 479 are also important in regulating the interaction of the glutamine substrate with the catalytic triad, and their absence produces a protease-resistant conformation in the absence of calcium ions. Additional studies are needed to locate the portion of the molecule that binds the glutamine substrates. This study illustrates that detailed biochemical analysis of mutant molecules is needed to fully appreciate the significance of structures detected by x-ray crystallography.

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Addendum—Ikura et al. (41) described mutagenesis studies on conserved anionic regions of guinea pig liver transglutaminase. One of the mutants, TGM2 (E454Q,E448Q,E449Q,E450Q,E452Q), described in their study contains the calcium-binding ligands Glu^{445} and Glu^{450} since they correspond to Glu^{485} and Glu^{490}, respectively, of the factor XIII A-chain reported in this study. The results described in their study are consistent with this study.