Mapping of Factor XIII Solvent Accessibility as a Function of Activation State Using Chemical Modification Methods†

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ABSTRACT: The transglutaminase Factor XIII (FXIII) catalyzes the formation of covalent cross-links between adjacent noncovalently associated fibrin chains in blood coagulation. The resulting covalently cross-linked hard clot is much more mechanically stable and resistant to proteolytic degradation. FXIII is activated by the serine protease thrombin in the presence of calcium ions. Protein modification experiments involving the labeling of cysteine and lysine side chains of the enzyme were performed before and after activation of the enzyme in an effort to gain further insight into structural changes occurring during the activation of FXIII. The experiments revealed differences in the labeling patterns of nonactivated and activated FXIII. These differences result from the exposure or sequestration of specific cysteine or lysine residues when the enzyme is activated, either physiologically with thrombin or nonproteolytically by exposure to calcium. Of note is the acetylation of Lys 73 and Lys 221 upon activation. Both of these residues lie within possible substrate recognition regions of FXIII. The active site Cys 314 is consistently alkylated in the activated enzyme, as is Cys 409, located near the dimer interface. Within the β-barrel 2 domain of FXIII, Cys 695 becomes alkylated in activated FXIII. Within the same domain, an acetylated Lys (677 or 678), which is observed in the zymogen, cannot be found in the activated enzyme. The results provide a more extensive view of FXIII activation than has been previously available.

In blood coagulation, the structural protein fibrinogen is converted to fibrin through the action of the serine protease thrombin. Thrombin cleaves the fibrinopeptides A and B from the Aα and Bβ chains of fibrinogen, respectively. This reveals "knobs" in the central E domain of fibrin which can noncovalently associate with complementary "holes" in the terminal D domains of other fibrin chains. The result is a loose fibrin network, or soft clot. Thrombin also serves to activate coagulation Factor XIII in the presence of calcium. Factor XIII catalyzes the formation of isopeptide bonds between specific glutamine and lysine residues of fibrin, resulting in a covalently cross-linked fibrin network, or hard clot. The action of FXIII[1] increases the rigidity and mechanical stability of a blood clot (reviewed in refs *1* and *2*). In addition to acting on fibrin, other substrates for FXIII include α_2-antiplasmin (*3*) and plasminogen activator inhibitor (PAI) (*4*), both of which are incorporated into the hard clot and make it more resistant to proteolytic degradation by plasmin.

Factor XIII is one of a class of enzymes known as *transglutaminases*. The transglutaminases of known structure are all very similar to one another, containing predominantly β-sheet architecture and possessing nearly identical domain structures (*5*, *6*). Figure 1A displays an X-ray crystal structure of a single a-subunit of FXIII. The presence of calcium appears to be a universal requirement for the activation of transglutaminases. The presence of calcium alone is sufficient to induce transglutaminase activity in some isoforms of the enzyme, while others must first be subjected to proteolysis (*6*, *7*).

Factor XIII is unique among the transglutaminases in its possession of an *activation peptide*, a 37 amino acid N-terminal extension that is cleaved by thrombin in the physiological activation of FXIII. Cleavage of the activation peptide alone is not sufficient to activate FXIII; at least 1 mM calcium must also be present. Plasma FXIII, which is composed of two catalytic a-subunits and two regulatory b-subunits (a_2b_2), requires calcium to promote dissociation of the b-subunits. The function of calcium is less well understood in the activation of placental or platelet FXIII (generally referred to as *cellular FXIII*, a_2), which lacks the regulatory b-subunits (*2*, *5*) (Figure 1B). FXIII a_2 and FXIII a_2b_2 can be activated nonproteolytically in the presence of high concentrations of calcium (*8*, *9*). FXIII a_2 may also acquire activity in the presence of low concentrations of calcium and high concentrations (150 mM to 1 M) of monovalent cations, such as sodium or potassium. This nonproteolytic activation is abolished by addition of stoichiometric amounts of the regulatory b-subunit (*9*).

The enigma of FXIII's activation is deepened by the fact that X-ray crystal structures of activated and nonactivated FXIII show no significant differences (*10*). It is widely believed that a conformational change in the enzyme must occur to allow access to the enzyme's active site, which is well buried. The ability to label the catalytic cysteine residue 314 with iodoacetamide only when the enzyme is activated

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[1] Abbreviations: FXIII (FXIII a_2), recombinant human placental factor XIII; HDX, hydrogen/deuterium exchange; MALDI-TOF MS, matrix-assisted laser desorption−ionization time-of-flight mass spectrometry; NEM, *N*-ethylmaleimide; NMM, *N*-methylmaleimide.

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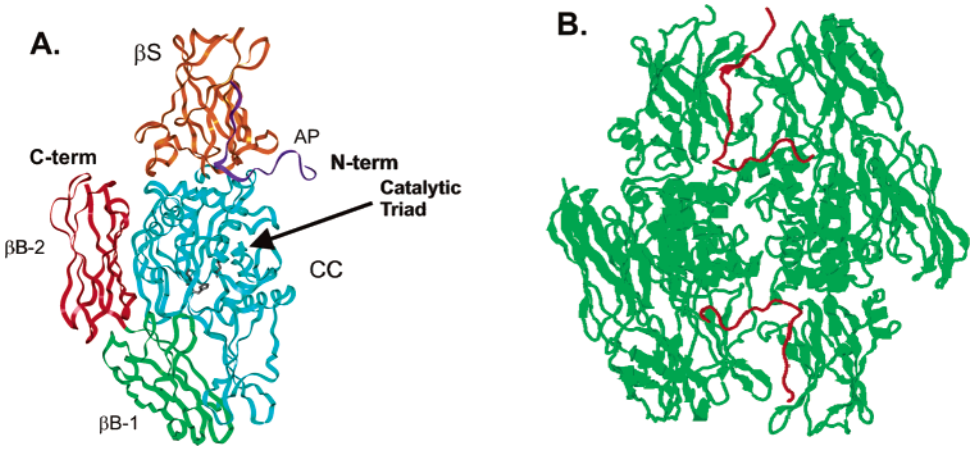

FIGURE 1: (A) The a-subunit of Factor XIII. Domains are color-coded for ease of identification: purple, activation peptide (AP); orange, β-sandwich (βS); cyan, catalytic core (CC); green, β-barrel 1 (βB-1); red, β-barrel 2 (βB-2). Residues of the catalytic triad are represented as dark sticks within the core. Dimerization of the enzyme occurs along a surface comprised of the β-sandwich and catalytic core domains. (B) Cartoon of the cellular FXIII a_2 dimer. The activation peptide of each a-subunit is depicted in red. (PDB code 1FIE).

has historically been viewed as evidence of this conformational change (*11*, *12*). These observations have motivated us to seek and implement solution-based techniques to study the structure and dynamics of activated FXIII.

The activation of FXIII a_2 was previously examined in both the thrombin-cleaved and nonproteolytically activated forms. The role of the continued presence of calcium following activation was also investigated by examining FXIII activated by thrombin in the presence or absence of 1 mM calcium (*13*). The initial study in this area utilized hydrogen/deuterium exchange (HDX) followed by MALDI-TOF mass spectrometry (*14*, *15*). HDX experiments on these activated forms of FXIII revealed subtle changes in structure that occur upon activation. It is believed that changes of this magnitude would be difficult to detect using X-ray crystallography (*13*). Decreases in degree of deuteration were located in regions of the β-sandwich and β-barrel 1 domains. These decreases depend strongly upon the presence of calcium. Increases in deuteration upon activation of the enzyme occurred within a region of the catalytic core previously implicated as a possible substrate recognition region, as well as within the activation peptide segment (*13*).

We sought to obtain more information about FXIII activation using a method complementary to the HDX experiments. Several different protein modification methods provide the ability to probe regions of the enzyme previously unexplored by the HDX experiments. These methods consist of labeling specific amino acid residues becoming more solvent exposed, in contrast to the larger areas of protein probed through HDX.

Two such methods were exploited to gain further information on FXIII activation: cysteine modification by alkyl maleimides (*16*) and lysine modification using acetic anhydride (*17*, *18*). FXIII contains 9 native cysteine residues and 38 native lysine residues that are well distributed through various regions of the enzyme. The reactivity of individual cysteine sulfhydryls and lysine amino groups is highly dependent upon the degree to which they are exposed to the bulk solvent. Modified cysteine or lysine residues could be easily identified by searching for peaks in the mass spectra that differed from their unmodified counterparts by the molecular mass of the labeling agent used. The native cysteine and lysine residues of FXIII occupy a variety of environments within the protein. This enables us to monitor an extensive area of the protein for structural changes upon activation. The approach provides a much broader description of the events associated with FXIII activation.

MATERIALS AND METHODS

Proteins and Chemical Reagents. Recombinant human Factor XIII a_2 (FXIII a_2) was a generous gift from Dr. Paul D. Bishop of Zymogenetics, Inc. (Seattle, WA) (*19*). D-Phe-Pro-Arg chloromethylketone dihydrochloride (PPACK) was purchased from Calbiochem. Calcium chloride dihydrate was purchased from Mallinckrodt (Paris, KY). Trifluoroacetic acid (TFA) was purchased from Burdick & Jackson (Muskegon, MI). Sequencing-grade trypsin was purchased from Roche Diagnostics (Mannheim, Germany). All other reagents were obtained from Sigma-Aldrich Chemicals.

FXIII stock solutions were prepared by dissolving the lyophilized enzyme in 18 MΩ deionized water (DI water). Concentration was checked by absorbance at 280 nm using an extinction coefficient of 1.49 mL/(mg·cm). The stock solution was divided into aliquots of 50−200 μL and stored in plastic microfuge tubes at −70 °C.

Activation of FXIII a_2 by Calcium or Thrombin. Nonproteolytic activation was achieved by combining equal volumes FXIII stock solution (37.5 μM), 20 mM borate buffer, pH 8.3, and 150 mM calcium chloride. The final concentration of FXIII in this mixture was 12.5 μM. The activation mixture was incubated at 37 °C for 2 h and vortexed periodically. Activity was verified by an assay based on the commercially available Berichrom XIII assay (*20*) (containing no additional thrombin). The activated enzyme was then buffer exchanged into 20 mM borate buffer, pH 8.3 (for alkylmaleimide labeling experiments), by dialysis (10 kDa molecular mass cutoff) or ultrafiltration (50 kDa molecular mass cutoff). Correct concentration was verified by Abs_{280}.

FXIII was also activated by thrombin. Bovine thrombin was activated and purified from bovine plasma barium sulfate eluate (Sigma) according to the method of Trumbo and Maurer (*21*). FXIII stock solution (50 μL) was combined with $CaCl_2$ (7.5 μL at 10 mM), bovine thrombin (2 μL at 2 μM), and 20 mM borate buffer (140.5 μL, pH 8.3). Bovine thrombin has a very high degree of sequence homology to

its human counterpart (*22*−*24*) and has been successfully employed as a substitute for human thrombin, as both enzymes perform the same proteolytic function in FXIII activation. Thrombin proteolysis of FXIII is often carried out in TANP buffer [100 mM Tris−acetate, 150 mM NaCl, 0.1% poly(ethylene glycol)]. However, as it is possible to achieve FXIII activation at high concentrations of monovalent cations (Na^+ for instance), the use of sodium was avoided in the present study where the focus was activation of FXIII by thrombin and calcium. The mixture of FXIII, thrombin, and calcium chloride was incubated at 37 °C for 12 min. Thrombin was inhibited by addition of D-Phe-Pro-Arg chloromethylketone (PPACK) (2 μL at 0.01 mg/mL) in DI water and incubated at 37 °C for an additional 15 min. The activated enzyme was then buffer exchanged and handled identically to the calcium-activated FXIII as detailed above.

Labeling Cysteine Residues with Alkylmaleimides. Stock solutions of 300 mM *N*-methylmaleimide (NMM) or *N*-ethylmaleimide (NEM) were prepared in 50% acetonitrile and DI water. FXIII (25 μL at 37.5 μM) in 20 mM borate was combined with additional 20 mM borate buffer (12.5 μL, pH 8.3). Cysteine alkylation was initiated by addition of NMM or NEM stock solution (2.5 μL). The mixture was maintained at room temperature and vortexed periodically. Aliquots of 7.5 μL were removed from the reaction mixture at intervals of 5, 10, 30, and 60 min. Alkylation was quenched by adding β-mercaptoethanol (1 μL at 1 M) in DI water to each aliquot, which was maintained at room temperature for at least 5 min. Each labeling experiment was repeated at least five times.

Maleimide-labeled FXIII was subjected to proteolysis with trypsin. Five microliters of quenched protein solution was diluted to 25 μL with 100 mM ammonium bicarbonate buffer, pH 8.0. Trypsin solution (2 μL, 1 mg/mL in 0.1% TFA) was then added. The mixture was incubated at 37 °C for 4 h. Trypsin proteolysis was quenched by addition of a 5% TFA solution (50 μL).

Acetylation of Lysine Residues Using Acetic Anhydride. At least seven acetylation experiments were performed on each state of the enzyme. Stock solutions of 0.6−2 M acetic anhydride were prepared in dimethylformamide (DMF). FXIII stock solution (25 μL) was diluted to 95 μL using 10 mM ammonium bicarbonate buffer, pH 8.0. Acetylation was initiated by addition of 5 μL of acetic anhydride in DMF. The mixture was maintained at room temperature and vortexed periodically. Aliquots of 25 μL were removed from the reaction mixture at intervals of 5, 10, 30, and 60 min. Acetylation was quenched by the addition of 1.5 μL of 1% TFA. Quenched aliquots were immediately frozen at −70 °C.

Residual buffer components, TFA, acetic anhydride, and byproduct acetic acid were removed by lyophilizing the quenched mixtures to dryness. Alternatively, the samples were treated by drying in a Speed-Vac concentrator device (Savant). The dry, modified protein was then reconstituted in 25 μL of 100 mM ammonium bicarbonate and proteolyzed by addition of 1 μL of trypsin (1 mg/mL in 0.1% TFA). The proteolysis mixture was incubated at 37 °C for 4 h and quenched by addition of 50 μL of 5% TFA.

Iodoacetamide Modification of FXIII. The labeling protocol is based on a method described by Curtis et al. (*25*). Stock solutions of 100 μM iodoacetamide were prepared in DI water. FXIII (25 μL at 40 μM) was treated with a slight molar excess of iodoacetamide (10 μL of the stock solution) in the absence and presence of 100 mM calcium chloride. Borate (20 mM) buffer solutions at pH values of 2, 8.3, and 9 were employed. The mixtures were incubated in the dark at room temperature for 30, 60, and 180 min. An aliquot of 30 μL was withdrawn at each time and quenched with 1.5 μL of 1 M HCl. The reaction mixture was then treated with a C18 Zip-Tip (Microcon) to remove the protein from excess iodoacetamide and eluted into 5 μL of 50% acetonitrile and 5 μL of 60% acetonitrile, both in 0.1% aqueous TFA. The solutions were combined and evaporated to dryness in the Speed-Vac device. Dry samples were reconstituted in 5 μL of 100 mM ammonium bicarbonate (pH 7.4). Trypsin (1 μL, 1 mg/mL in 0.1% aqueous TFA) was added and the mixture was incubated at 37 °C for 4 h. Tryptic digestion was quenched by addition of 12 μL of 5% aqueous TFA.

MALDI-TOF Mass Spectrometry. Acid-quenched proteolytic digests from NMM/NEM labeling or acetylation experiments were prepared in the same manner. Each time point digest (75 μL) was treated with a C18 Zip-Tip. The Zip-Tips were each washed with 0.1% TFA and their contents eluted into 5 μL of α-cyano-4-hydroxycinnamic acid matrix solution (10 mg/mL α-CHCA in 1:1:1 0.1% TFA/ethanol/acetonitrile). Matrix solutions containing tryptic peptides were deposited in 1 μL drops on a stainless steel MALDI target and dried under moderate vacuum using the Speed-Vac device. For detection of peaks greater than *m*/*z* 3000, the matrix solution was substituted with a solution containing 8 mg/mL ferulic acid in 50% acetonitrile in 0.1% aqueous TFA.

Spectra were acquired on a Voyager DE-Pro mass spectrometer (Applied Biosystems) in reflector mode. The mass range employed was *m*/*z* 500−5200. A total of 256 shots per spectrum were collected. Three spectra were acquired from each sample (each from a separate sample spot).

Data Analysis. All spectra acquired from modified proteins were carefully compared to spectra of an unmodified tryptic digest of FXIII. Two-point calibration was performed on each spectrum using masses provided by a theoretical tryptic digest (using the MS-Digest tool of Protein Prospector, http://prospector.ucsf.edu) (*26*). Peptide mass peaks were subsequently identified using the same theoretical digest. NMM- and NEM-labeled peptide masses were generated by adding *m*/*z* 111.1 and *m*/*z* 125.1, respectively, to the theoretical mass of each single-cysteine-containing tryptic peptide. The maleimide-labeled peptides were subsequently identified from the spectra using these masses.

Acetylated lysine-containing peptide molecular masses were generated by MS-Digest. Up to two missed cleavages were specified in the software resulting in a list of peptide masses for acetylated lysines. Acetylated peptides had masses differing from their nonacetylated counterparts by integer multiples of *m*/*z* 42. Detection of a peak in a minimum of 55% of experiments was considered sufficient evidence of its appearance. However, most acetyl-lysine adducts were located in 70−100% of experiments. The possibility of obtaining peaks representing one or two missed cleavage peptides, containing two or sometimes three lysine residues that could all potentially become acetylated, sometimes aided in the identification of specific acetylated lysine residues.

FIGURE 2: Sequence coverage of tryptic digest. Lysine and arginine residues are underlined. Portions of the sequence observed only when using αCHCA as a matrix are in italics. Sequence information obtained only when ferulic acid is used as a matrix are shown in the bold type. In bold italic are portions of the protein sequence seen using either αCHCA or ferulic acid. Strikethrough residues represent additional coverage obtained only when one or more lysine residues became acetylated.

Table 1: Cysteine Alkylation Experimental Results: Masses of Single-Cysteine-Containing Peptides and Their Predicted Alkylmaleimide Adduct Masses

unmodified mass (*m*/*z*)	peptide	Cys	adduct masses (predicted) +NMM (*m*/*z*)	adduct masses (predicted) +NEM (*m*/*z*)	activation state of FXIII (adducts observed?)[a] nonact.	activation state of FXIII (adducts observed?)[a] act. (Ca or IIa)
519.2959	152−156	152	630.3959	644.3959	no	uncertain
3004.3193	175−199	188	3115.4190	3129.4190	yes	yes
2498.1002	224−244	238	2609.2000	2623.2000	no	no
1907.9267	311−326	314	2019.0270	2033.0270	no	yes
729.4976	327−333	327	840.5076	854.5076	no	no
4902.1194	367−408	374	5013.2190	5027.2190	n/d[b]	n/d
973.5135	409−418	409	1084.6140	1098.6140	no	yes
3068.4974	419−445	423	3179.5970	3193.5970	n/d	n/d
2665.3052	682−703	695	2776.4050	2790.4050	no	yes

[a] Indicates the activation state in which NMM and NEM adducts were observed. NEM and NMM behaved identically. Nonact. refers to nonactivated FXIII. Act. (Ca or IIa) refers to FXIII activated by either exposure to calcium or thrombin cleavage. Both forms of activation produced identical results. [b] n/d: peptides not observed.

RESULTS

Improvement in Sequence Coverage Using Trypsin Proteolysis. Tryptic digests of FXIII generated a large number of peptides that appeared as very well resolved signals in the MALDI-TOF mass spectrum. Most could be unambiguously identified and spanned 66% of the total FXIII sequence (Figure 2). Other sequences, detected only when the enzyme was acetylated, elevated the total sequence coverage to 75%. This represents a significant improvement over the 45% coverage obtained from the peptic digest in the previous HDX study (*13*).

Cysteine Alkylation Using Alkylmaleimides. In preparation for alkylmaleimide labeling of FXIII, digests were examined in the mass range *m*/*z* 500−5200 to first characterize each of the nine single-cysteine-containing peptides (see Table 1 for the theoretical molecular mass of each). Note that these are all zero missed-cleavage peptides (i.e., the sequences contain no intervening lysine or arginine residues). Peptides of *m*/*z* <3000 were easily detected and were observed in all mass spectra acquired from tryptic digests of FXIII. They represented six of the nine native cysteine residues (C152, C238, C314, C327, C409, and C695). The remaining three cysteines residues Cys 188, Cys 374, and Cys 423, contained within peptides of *m*/*z* >3000 (Table 1), were seldom or not at all observed. The matrix material ferulic acid permitted observation of Cys 188 but not Cys 374 or Cys 423. Ferulic acid is often cited as a matrix used for the detection of oligonucleotides by MALDI-TOF MS (*27*, *28*), but it was employed in this context on the basis of the method of Apuy et al. (*16*) for the detection of peptides.

The alkylation state of seven of the nine native cysteine residues could be accurately tracked experimentally: C152, C188, C238, C314, C327, C409, and C695. It was also determined that no components of the tryptic digest of

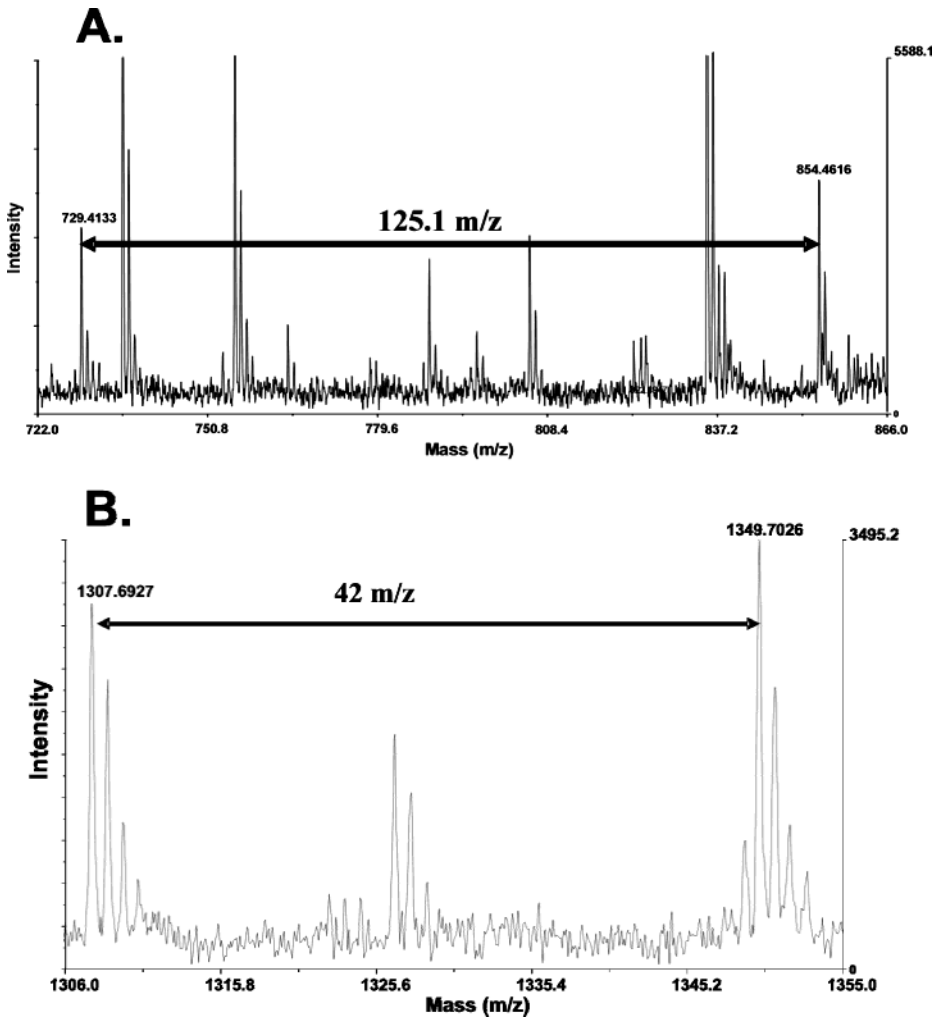

FIGURE 3: (A) Detail of a mass spectrum acquired from the tryptic digest of FXIII treated with NEM. Highlighted is the *m*/*z* 125.1 difference between the peak representing single-cysteine-containing peptide 729.4976 (327−333, Cys 327) and its NEM-modified counterpart, 854.5076. (B) Detail of a mass spectrum acquired from tryptic digest of FXIII treated with acetic anhydride. Highlighted is the *m*/*z* 42 difference between the lysine-containing peptide represented by 1307.6927 and its acetylated counterpart, 1349.7005.

unmodified FXIII overlapped with the isotopic envelopes of the predicted alkylation products. Figure 3A is a mass spectrum displaying a single-cysteine-containing peptide and its NEM adduct which are *m*/*z* 125.1 different from one another.

Alkylmaleimides NMM and NEM were added to nonactivated, calcium-activated, and thrombin-activated FXIII a_2 at 800-fold molar excess. Quenching was accomplished by addition of a large excess of β-mercaptoethanol, which served to react with any free labeling agent remaining in the reaction mixture. Treatment of FXIII with dithiothreitol (DTT) prior to alkylation had no effect on the pattern of cysteine modification (data not shown).

Treatment of nonactivated FXIII with either alkylmaleimide resulted in alkylation of only Cys 188, as judged by MALDI-TOF mass spectra from subsequent tryptic digests. Upon calcium or thrombin activation of the enzyme, NMM and NEM treatment resulted in the modification of several cysteine residues (Table 1). NMM and NEM adducts were repeatedly identified in peptides containing Cys 188, the catalytic Cys 314 (which typically appeared after just 5 min of alkylation), Cys 409, also within the catalytic core, and Cys 695, located in the β-barrel 2 domain. These cysteine residues were observed to be modified by either NMM or NEM, regardless of the way in which the enzyme was activated. Cys 188 adducts were observed in digests of nonactivated and activated FXIII when ferulic acid was used as a matrix.

Lysine Acetylation Using Acetic Anhydride. Lysine acetylation experiments were performed in much the same way as the cysteine alkylation study. The mass of a peptide containing a single acetylated lysine residue is exactly *m*/*z* 42 greater than its unmodified counterpart (see Figure 3B). Control experiments involving tryptic digests of nonacetylated FXIII determined the number of overlaps between tryptic peptides and possible acetylated products.

Nonactivated, calcium-activated, and thrombin-activated FXIII samples were treated with stock solutions of acetic anhydride in DMF. DMF was chosen as a solvent as it is nonprotic and should not catalyze the decomposition of acetic anhydride to acetic acid. The stock solutions were found to be fully miscible with aqueous FXIII solutions. Concentrations higher than 600 mM acetic anhydride (3000-fold molar excess over lysine) resulted in overacetylation of FXIII, and no significant difference in acetylation patterns could be determined between activated and nonactivated FXIII's (data not shown). Therefore, 600 mM acetic anhydride in DMF was employed for the duration of the acetylation experiments.

The results of these experiments were used to build a functional map of FXIII in which solvent accessibility of each of the 38 native lysine residues can be characterized in each state of the protein (Table 2). Each lysine residue is classified as acetylated in nonactivated FXIII, in calcium-activated FXIII, or in thrombin-activated FXIII. Some lysine residues are acetylated in multiple states, as indicated in Table 2. Acetylated lysines observed in all three states of the protein can be characterized as fully accessible to acetic anhydride at all times. These include K54, K133, K257, K482, K534, K583, K584, K621, K623, K635, K(677 or 678), and K704.

A doubly acetylated peptide containing Lys 677 and Lys 678 was only detectable in the nonactivated state of the enzyme. When the enzyme is activated, a peak representing a singly acetylated peptide containing Lys 677 and Lys 678 is found. By contrast, Lys 221 appears to be acetylated only when the enzyme is thrombin or calcium activated. It is located within a peptide segment of FXIII, 220−230, that was observed to undergo increased deuteration in the previous HDX experiments (*13*). Acetylation of Lys 73 and Lys 462 or Lys 467 occurs only when FXIII is activated by 50 mM calcium chloride. These residues are located in the *β*-sandwich and catalytic core domains of the enzyme, respectively. Lys 156 becomes acetylated only when the enzyme is activated by thrombin. It is notable that Lys 73 and Lys 156 are nearly adjacent to one another in the structure of FXIII.

In some cases, not enough information was available to make an accurate determination of the activation state in which a particular lysine became acetylated. It may also result from the inability to determine the acetylation state of adjacent lysine residues within the same peptide. These are classified in the row labeled "uncertain" (K68, K418, K503, K504, and K513). Blank columns in Table 2 denote lysine residues for which no acetylated adducts were observed. Use of ferulic acid as a matrix did not aid in the detection of these adducts.

DISCUSSION

Lysine Acetylation and Cysteine Alkylation Procedures. The goal of the current study was to obtain further information concerning the activation of cellular FXIII using solution-based techniques. Previous studies of FXIII by X-ray crystallography reveal no significant difference between the structures of the zymogen and the activated enzyme, whether thrombin cleaved or saturated with calcium. Our focus on solution-based techniques eliminates the possibility of losing subtle conformational changes to crystal packing effects. Cysteine modification was selected on the basis of prior successes in applying this technique to other proteins (*16*, *29*, *30*). The 38 lysine residues present in FXIII, widely distributed among the domains of the enzyme, prompted the choice of lysine acetylation. Patterns of lysine acetylation in activated and unactivated FXIII permit us to build a low-resolution map of the protein and quickly identify elements that may or may not be experiencing structural change upon activation of the enzyme. The use of tryptic proteolysis following modification afforded a greater degree of sequence coverage (up to 75%) than our previous HDX experiments [45% by peptic digest (*13*)], providing insight into areas of

Table 2: Lysine Acetylation Results: Lysine Residues of FXIII Presented by Domain and Acetylated Products Observed as a Function of Enzyme Activation State

	lysine residues																																	
	β-sandwich										catalytic core													*β*-barrel 1								*β*-barrel 2			
activation state of FXIII	54[a]	61[b]	68	**73**[c]	113	129	133	151	**156**	199	**221**	257	269	363	366	418	445 and 446	**462** or **467**	482	503 and 504	513	531	534	565	569	570	583 and 584	621	623	635	657	677 or 678[e]	**677** or **678**[e]	704
nonactivated	x						x					x							x				x				x	x	x	x		x	x	x
Ca act.	x			**x**			x				**x**	x						**x**	x				x				x	x	x	x		x		x
IIa act.	x						x		**x**		**x**	x							x				x				x	x	x	x		x		x
uncertain[d]			x													x				x	x													

[a] Acetylated products were observed in the nonactivated and both activated states of factor XIII. The lysine residue is characterized as always exposed. [b] Peptides containing acetylated lysine were never observed in the case of some residues (blank columns). [c] Residue numbers in bold indicate acetylated lysines detected only in one or two states of FXIII, as detailed in the text. [d] In some cases, not enough information was available to make an accurate determination of the activation state in which a particular lysine became acetylated. These are classified in the row labeled uncertain. [e] There is evidence for the modification of both Lys 677 and Lys 678 in the nonactivated state of the enzyme. However, upon activation, only one lysine of this pair appears to be modified. Whether it is Lys 677 or Lys 678 is unknown at this time.

FXIII that were not observed before, such as the dimer interface and the catalytic core. Furthermore, protein modification targets single amino acid residues. It is possible to immediately obtain information about the exposure of a particular cysteine or lysine residue within FXIII. The results correlate well with those of prior studies of FXIII activation, demonstrating the power of this technique. The implementation of these labeling techniques has yielded new information concerning conformational changes that occur upon the activation of FXIII.

Residues That Become Modified in both Activated and Nonactivated FXIII. The experimental results located several lysine-containing peptides that acquired an acetyl modification regardless of the activation state of the protein. These regions of FXIII are characterized as being always solvent accessible (see Table 2). The lysines may be part of surface-exposed areas on FXIII that do not participate in conformational changes upon activation. Alternatively, these lysines could be located in regions that undergo a change in conformation, while the lysines' susceptibility to acetylation is either maintained or even further enhanced. An additional issue to consider is the time scale for chemical modification effects. Chemical modification is typically a slower process than, for example, amide hydrogen exchange. The longer time regimes required for achieving acetylation could lead to modifications not picked up by the faster HDX process. Regardless of what regulates the propensity of these lysines toward acetylation, it is clear that certain regions of non-activated FXIII remain solvent exposed following activation.

Lysines that were always solvent accessible include lysines 54, 133, 257, 482, 534, 583, 584, 621, 623, 635, 677 or 678, and 704. Most of them reside in the predominantly β-strand architecture of the β-sandwich domain (K54, K133) and the β-barrel 1 and 2 domains [K534, K621, K623, K635, K(677 or 678), K704]. Lys 257 and Lys 482 appear to occupy surface positions within the catalytic core (refer to Figure 1A for domain structure). The X-ray crystal structure of FXIII (PDB code 1FIE) (*10*) shows each of these residues with its side chain oriented outward toward the bulk solvent, possibly explaining their reactivity to acetic anhydride regardless of the activation state of the enzyme.

Cys 188 is the only cysteine residue to become alkylated before the enzyme is activated (Table 1). The alkylated Cys 188 containing peptide is also detected following activation of FXIII. An examination of the X-ray crystal structure of FXIII (*10*) shows this residue to be in a relatively exposed location between the β-sandwich domain and the catalytic core (Figure 4B). Modification of this sulfhydryl regardless of the activation state of FXIII therefore does not seem unreasonable.

Table 2 shows that several acetylated lysine residues could not be accounted for in any of the experiments. The absence of an acetylated derivative for a particular lysine-containing peptide may, however, not be sufficient justification to classify the lysine as always inaccessible to solvent. It is possible that acetylation of residues such as Lys 363 occurs and that the modified product is simply not ionized, or its ionization is suppressed by an unknown mechanism. The overlapping of lysine and cysteine modification data may provide further insight in one case. Acetylated peptides containing Lys 151 were not detected. NEM or NMM modification of the neighboring Cys 152 was also not observed. The similarity of the two results might allow the classification of these residues as permanently protected from bulk solvent.

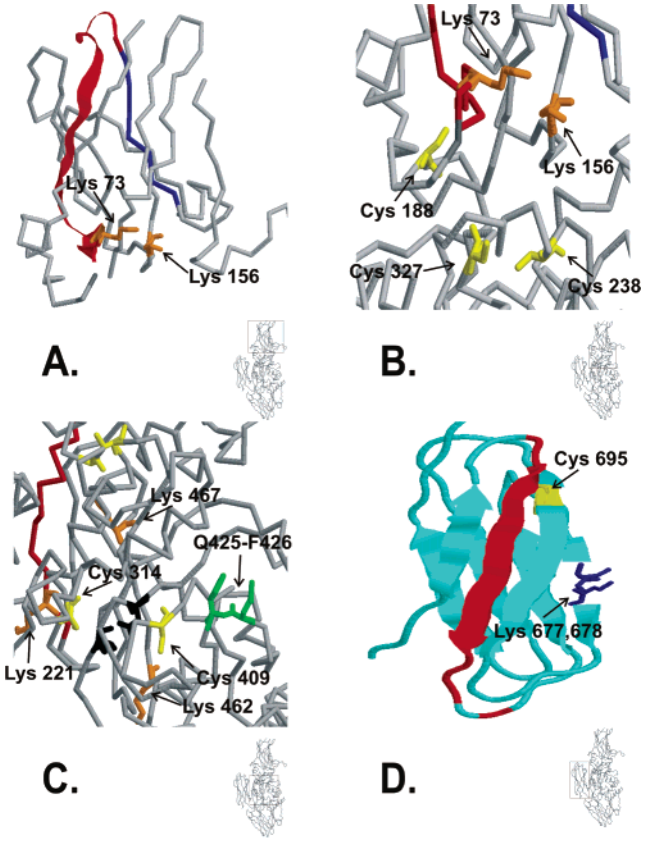

FIGURE 4: (A) β-Sandwich domain of FXIII. Peptide 4 (72−97) is depicted as a red ribbon; 98−104, the region becoming protected from HDX upon activation, is in blue. Lysines are portrayed as orange sticks. (B) Detailed view of the β-sandwich−catalytic core interface. Cysteines are portrayed as yellow sticks. (C) Detailed view of the catalytic core. Near the top of the panel are the cysteines described in (B). The red trace is peptide 220−230, showing increased deuteration upon activation in HDX. Lysines are in orange. Cysteines are in yellow. Cys 314 (yellow), His 373 (black), and Asp 396 (black) complete the catalytic triad. The non-prolyl *cis*-peptide bond Q425−F426 is in green. The β-barrel domains have been deleted from this view for clarity. (D) Cartoon of β-barrel 2: in red, 643−658, comprising strand 2; in yellow, Cys 695, located in adjacent strand 5; in blue, Lys 677 and Lys 678.

Acetylated lysine-containing peptides were detected in some cases that could not be properly classified as always accessible in one or more states of the enzyme. These residues were classified as uncertain, as seen in Table 2. Employing this classification scheme, it is possible to account for 25 of the 38 native lysine residues.

β-Sandwich Domain: Lys 73, Lys 156, and a Link to Peptide 4. Acetylation of Lys 73 within the β-sandwich domain was observed upon activation of the enzyme by calcium. A previous study by Achyuthan et al. (*31*) reports competitive inhibition of FXIII by peptides derived from the sequence of FXIII. Among the most potent of these inhibitors were the peptides they termed peptide 4 (residues 72−97) and peptide 7 (residues 190−230) (see Figure 4A,C). Because these peptides act as competitive inhibitors of FXIII, the corresponding regions of the enzyme are believed to play a role in glutamine-containing substrate recognition. Lys 73 is located at the N-terminus of this particular region of the enzyme. The increased exposure of lysine 73 to solvent upon

activation also coincides with the decreased deuteration of peptide 98−104 that was reported in our HDX study (*13*). Observations in the current experiment may offer an explanation for the decreased deuteration of the 98−104 peptide. It may be that this portion of the enzyme must become more protected from bulk solvent to allow increased exposure of the peptide 4 region.

Lys 156 becomes acetylated only when the enzyme is activated by thrombin. Referring to Figure 4A, it is clear that Lys 156 is almost adjacent to Lys 73, which is modified upon activation of FXIII by calcium. The Cα−Cα distance between the two residues is 9.7 Å as measured from the crystal structure 1FIE, and both side chains appear to be oriented in approximately the same direction. The exposure of Lys 156 might therefore be connected to the event resulting in the exposure of Lys 73. The proximity of the two side chains could also define this particular event as one that occurs regardless of the mode of activation.

Catalytic Core and β-Barrel 1 Domains. Many of the observed differences among the zymogen and activated forms of FXIII occur within the catalytic core domain. Most striking is the modification of Cys 409 located at the dimer interface of the a_2 dimer (refer to Figure 4C and Table 1). The increased reactivity, and therefore, solvent accessibility, of this side chain upon activation of the enzyme, appears to point to a conformational change propagating at the dimer interface. Improved solvent accessibility of Cys 409 correlates with the location of a non-prolyl *cis*-peptide bond between Gln 425 and Phe 426 (*32*), also depicted in Figure 4C. A second non-prolyl *cis*-peptide bond is located near the active site cysteine between Arg 310 and Tyr 311. Interestingly, these unusual *cis*-peptide bonds have also been identified in human transglutaminase 3 (*33*). It has been proposed that the conversion of one or both of these peptide bonds, from the *cis* form to the more energetically favorable *trans* form, could drive the sort of large-scale conformational change necessary to expose the enzyme's buried active site to substrates (*32*, *34*). The Q425−F426 *cis*-peptide bond is stabilized through a water-mediated hydrogen bond between Gln 425−O and Ser 413−N and a second H-bond between Phe 426−N and Pro 411−O. A third *cis*-peptide bond exists between Gly 410 and Pro 411 (*32*). The increased exposure of Cys 409 may occur as a direct result of the breakdown of this hydrogen-bonding network upon activation of FXIII, preceding the proposed conversion of the Q425−F426 peptide to the *trans* form. The alkylation of nearby Cys 423 would have been a more positive indicator of this event; however, the Cys 423 containing peptide could not be detected in either the unmodified or alkylated forms.

The catalytic triad of FXIII is in a region of the enzyme previously unexamined by HDX due to a lack of sequence coverage by pepsin proteolysis within this region. Cys 314 becomes more reactive regardless of the means of activation when both NMM and NEM are used as labeling agents. Alkylation of Cys 314 is in agreement with the observed iodoacetamide modification of Cys 314 upon activation of the enzyme (*11*, *12*). Titration by iodoacetamide (particularly ^{14}C labeled) has long been used to measure total transglutaminase activity; it provides a means to assay the number of accessible active sites available in a preparation of activated FXIII (*11*, *12*, *25*, *35*). Results from both alkylmaleimide and iodoacetamide titrations may be in line with the findings of X-ray crystallography, which suggest no large-scale conformational change in the activated enzyme prior to substrate binding (*8*, *32*, *36*). Previous HDX experiments demonstrate conformational changes occurring upon activation that are subtle in magnitude (*13*). Such subtle changes may be sufficient to admit small molecule substrates such as iodoacetamide or alkylmaleimides to the active site while excluding peptide or protein substrates.

Our previous report on HDX experiments performed on activated states of FXIII detailed the increasing solvent exposure of a peptide spanning residues 220−230 following activation of the enzyme. This segment belongs to the previously mentioned peptide 7 (190−230) region of the enzyme, which, along with peptide 4, is implicated as a possible substrate recognition region (*31*). Lysine acetylation has revealed that, following thrombin and calcium activation of FXIII, Lys 221 within this peptide segment becomes modified (Figure 4C). Thus, the acetylation experiment has allowed us to identify Lys 221 as a specific residue within 220−230 that is becoming exposed upon activation.

Acetylation of Lys 462 or Lys 467 occurs upon activation of the enzyme by calcium (Figure 4C). Lys 462 is located near the interface of the catalytic core with the β-barrel 1 domain of FXIII. The increasing reactivity of Lys 467 to acetic anhydride upon activation seems more likely, however. The X-ray crystal structure of FXIII shows that Lys 462 occupies a position that is expected to be exposed at all times and, therefore, reactive to acetic anhydride in both the zymogen and activated states. Increased exposure of the side chain of Lys 467 upon activation could be a consequence of the increased exposure of the peptide 7 region. Lys 467 and Arg 223, located within peptide 7, lie 10 Å apart. The distance between Lys 467 and Ile 212, also within peptide 7 but in the adjacent β-strand, is shorter still, at 7.5 Å.

β-Barrel 2: Links to an Unusual Inhibitor's Binding Site. Events occurring in β-barrel 2 were previously unobserved by HDX once again due to a lack of sequence coverage by the peptic digest within this domain (see Figure 4D). Cys 695 becomes more solvent exposed upon activation of the enzyme. Cys 695 was not labeled at all when the enzyme was in the nonactivated state. A peptide containing Lys 677 and 678 (663−679) was found to be singly acetylated in both the zymogen and activated forms of the enzyme. A second peptide (663−682) also containing Lys 677 and Lys 678 was detected as a doubly acetylated peptide but in the nonactivated form of FXIII only. These data suggest that one of the two lysines is readily acetylated in both the zymogen and activated enzyme. The other lysine is modified solely in nonactivated FXIII. We cannot positively identify which of the two, 677 or 678, is unmodified in the activated enzyme. However, the fact that a doubly acetylated peptide is observed for the 663−682 sequence in the nonactivated state suggests that one of these lysine residues may be unavailable to react with acetic anhydride after the enzyme is activated.

Involvement of β-barrel 2 in activation is difficult to explain. A previous study investigated the case of a monoclonal antibody 5A2 directed against fibrinogen Aα (529−539), which was found to be cross-reactive with recombinant FXIII (*37*). Subsequent kinetic tests characterized 5A2 as an uncompetitive inhibitor of FXIII. The antibody exhibited enhanced binding to activated FXIII in the presence of

glutamine-containing substrate, leading Mitkevich et al. to hypothesize that binding of FXIII to glutamine-containing substrate is responsible for a conformational change in the enzyme. ELISA studies and sequence homology identified the binding epitope of 5A2 as FXIII residues 643−658, within the β-barrel 2 domain (*37*).

Our own observations may have significance when seen in the context of Mitkevich et al. Although binding of 5A2 to FXIII is enhanced in the presence of a glutamine-containing substrate, 5A2 was shown to bind unactivated FXIII to some extent, and the binding of 5A2 is significantly enhanced by activation of the enzyme (*37*). These characteristics may point to increased exposure of the 643−658 peptide segment upon activation. Cys 695 is located in the β-strand adjacent to β-strand 646−658 (Figure 4D), which might explain its increased solvent exposure and reactivity. Lysines 677 and 678 are both located in the β-strand adjacent to the strand containing Cys 695. The sequestration of one of these lysines may follow the exposure of Cys 695 through some as yet unknown mechanism.

Direct evidence for increased exposure of residues 643−658 would offer more support for this proposal. However, the only lysine residues located in the 643−658 region occur at positions 635 and 657. Lys 635 is indicated as always accessible to acetic anhydride. Peptides containing acetylated Lys 657 were never detected.

Iodoacetamide's Specificity for Cys 314 Relative to N-Alkylmaleimides. Iodoacetamide's use as a tool for quantifying FXIII active sites implies that Cys 314 is the only reactive cysteine available in activated FXIII. Alkylmaleimide modification, however, is clearly not exclusive to Cys 314 but is indeed more widespread. The source of iodoacetamide's specificity likely arises from environmental conditions exclusive to the fully formed catalytic triad of FXIII. The catalytic triad of FXIII bears striking similarity to the active sites of cysteine proteases (*2*, *5*, *6*, *38*). It has been demonstrated that the ion pairing between an active site histidine and the reactive cysteine sulfhydryl side chain results in enhanced nucleophilicity and, therefore, reactivity of the active site cysteine in the cysteine protease papain (*39*). pH-dependent kinetic studies as well as site-directed mutagenesis experiments on FXIII have suggested a similar tight ion pairing between Cys 314 and His 373 (*40*−*42*). The microenvironment of an amino acid side chain exerts considerable influence over its pK_a. The effect is by no means exclusive to thiol-based proteases or transpeptidases but has been observed in a wide variety of enzymes such as lysozyme (*43*) and acetoacetate decarboxylase (*44*).

Our chemical modification experiments with alkylmaleimides or iodoacetamide were run in buffers of pH 8.3. Given that Cys 314 was the only cysteine residue to become iodoacetamide modified, it would appear that the pK_a of the Cys 314 sulfhydryl [normally reported as 8.3 for the free amino acid (*45*)] has been decreased to a value lower than 8.3. An earlier report by Curtis et al. (*35*) examined the pH dependence of [^{14}C]iodoacetamide incorporation by FXIII. No evidence of isotope incorporation was observed below pH 6. Maximal reactivity was recorded between pH 7 and pH 8.5, but no pH values above 8.5 were tested. Our own results from variable pH experiments indicate widespread labeling of FXIII cysteines by iodoacetamide at pH above 9 and no labeling of cysteine at pH 2. Curtis et al. report the pK_a of Cys 314 as 6 (*35*), confirming our hypothesis that the catalytic cysteine exists in a lower pH microenvironment that can be readily targeted by iodoacetamide.

N-Alkylmaleimides are typically noted as being specific for cysteine sulfhydryl groups at acidic pH (*45*). However, both NMM and NEM have been found to perform satisfactorily at near-neutral pH both in this study and in previous studies using bacterial luciferase (*17*) and a zinc-finger transcription factor (*29*). The widespread labeling of FXIII at pH 8.3 and below with NEM or NMM implies a reduced dependence on sulfhydryl deprotonation for cysteine's reactivity with these compounds. In contrast, cysteine reactivity with iodoacetamide appears to require full deprotonation of the sulfhydryl side chain, as evidenced by the widespread reactivity of cysteines within FXIII at pH $>$8.3 and the exclusive targeting of Cys 314 by iodoacetamide at pH $<$8.3.

SUMMARY

Cysteine alkylation and lysine acetylation methods have provided new and useful insights into the activation of Factor XIII, expanding upon the information previously obtained by HDX studies (*13*). When superimposed upon an X-ray crystallograph of FXIII, the residues becoming modified upon activation of the enzyme appear to occur in clusters or in conjunction with structural features identified or hypothesized in previous studies. This provides insights into the dynamics of FXIII activation and the structure of the activated form previously unavailable.

Lysines 73 and 156 within the β-sandwich domain exhibit increased exposure to solvent in the calcium- and thrombin-activated states of FXIII, respectively. The location of Lys 73 coincides with a possible substrate recognition region, peptide 4 (residues 72−97) (*31*). The proximity of these two lysine residues is interesting, indicating that the same event may be occurring regardless of the mode of activation employed (Figure 4B). The results may indicate increased solvent exposure at the β-sandwich/catalytic core interface following activation of FXIII.

The increased reactivity of lysine 221 to acetic anhydride coincides with its location within peptide 7 (residues 190−230), another possible substrate recognition region of FXIII (*31*). A portion of this segment, 220−230, underwent increased H/D exchange in previous experiments (*13*). The acetylation of lysine 467 upon activation may be related to proximity between this residue and peptide 7. Alkylation of Cys 314 upon activation signals exposure of the well-buried FXIII active site to bulk solvent, a finding previously established by iodoacetamide labeling (Figure 4C). Concomitant labeling of Cys 409, at the dimer interface of the enzyme, may be related to the breakdown of a hydrogen bond network stabilizing an unusual non-prolyl *cis*-peptide bond linking Q425−F426 (*32*) (Figure 4C).

The most surprising find is possibly the change of exposure in residues located within the β-barrel 2 domain of FXIII. Cys 695 becomes alkylated upon activation of FXIII. Residues Lys 677 and Lys 678 can both be acetylated in the nonactivated state of the enzyme; however, one of these lysines becomes inaccessible to acetic anhydride when the enzyme is activated. No clear connection to the activation of FXIII exists for these events, although the position of these

residues suggests that the events may be linked to a finding previously published by Mitkevich et al. (*37*).

Protein modification techniques have proven themselves a valuable tool for the continued investigation of Factor XIII activation in a solution environment, a task that was begun in earlier hydrogen/deuterium exchange experiments. The current work has permitted the observation of changes in FXIII structure in regions separate from those examined previously. Although cysteine alkylation and lysine acetylation as employed in the current study are nonquantitative, the extent of modification reported here is likely of the same subtle nature as that observed by HDX. The different events recorded are proposed to foreshadow larger changes that may occur in activated FXIII upon binding of a glutamine-containing substrate.

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