Identification of Respective Lysine Donor and Glutamine Acceptor Sites Involved in Factor XIIIa-catalyzed Fibrin α Chain Cross-linking

Received for publication, August 25, 2011, and in revised form, October 25, 2011 Published, JBC Papers in Press, October 26, 2011, DOI 10.1074/jbc.M111.297119

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**Background:** Factor XIIIa catalyzes e-(γ-glutamyl)-lysyl bonds between glutamine and lysine residues on fibrin α and γ chains.

**Results:** Site-specific determinations of intermolecular glutamine-lysine pairs on fibrin α chains were accomplished using modern mass spectrometry methods.

**Conclusion:** Nine specific cross-links between four glutamine and five lysine residues on fibrin α chains were identified.

**Significance:** The findings advance the current understanding of molecular packing in fibrin.

Factor XIIIa-catalyzed e-(γ-glutamyl)-lysyl bonds between glutamine and lysine residues on fibrin α and γ chains stabilize the fibrin clot and protect it from mechanical and proteolytic damage. The cross-linking of γ chains is known to involve the reciprocal linkages between Gln398 and Lys406. In α chains, however, the respective lysine and glutamine partners remain largely unknown. Traditional biochemical approaches have only identified the possible lysine donor and glutamine acceptor sites but have failed to define the respective relationships between them. Here, a differential mass spectrometry method was implemented to characterize cross-linked α chain peptides originating from native fibrin. Tryptic digests of fibrin that underwent differential cross-linking conditions were analyzed by high resolution Fourier transform mass spectrometry. Differential intensities associated with monoisotopic masses of cross-linked peptides were selected for further characterization. A fit-for-purpose algorithm was developed to assign cross-linked peptide pairs of fibrin α chains to the monoisotopic masses relying on accurate mass measurement as the primary criterion for identification. Equipped with hypothesized sequences, tandem mass spectrometry was then used to confirm the identities of the cross-linked peptides. In addition to the reciprocal cross-links between Gln398 and Lys406 on the γ chains of fibrin (the positive control of the study), nine specific cross-links (Gln223–Lys508, Gln223–Lys539, Gln237–Lys418, Gln237–Lys508, Gln237–Lys539, Gln237–Lys556, Gln366–Lys539, Gln563–Lys539, and Gln563–Lys601) on the α chains of fibrin were newly identified. These findings provide novel structural details with respect to the α chain cross-linking compared with earlier efforts.

Fibrinogen is a glycoprotein in human blood plasma at a concentration of ~2.5 g/liter and is essential for hemostasis. It is composed of three pairs of polypeptides chains (α, β, γ)2 that are organized in a symmetrical dimeric fashion linked together by 29 disulfide bonds. In the hemostatic process, fibrin formation is initiated by the cleavage of fibrinopeptides by thrombin, converting fibrinogen to the fibrin monomer (α, β, γ)2. Through an orderly sequence of macromolecular assembly steps, fibrin monomers aggregate and form fibrin bundles (2, 3). The resulting fibrin network is further stabilized by covalent ligation or cross-linking of specific amino acids by a transglutaminase, factor XIIIa. The cross-linking is done via a transamidation reaction that forms an e-(γ-glutamyl)-lysyl isopeptide bridge between the deprotonated lysine donor residue and the acceptor glutamine residue (4). Although it is well established that these factor XIIIa-catalyzed inter-chain isopeptide bonds stabilize fibrin and protect the clot from mechanical and proteolytic damage, considerably less is known on the finite structural features of the cross-links. Of the three fibrinogen component chains, α and γ chains are factor XIIIa substrates with the β chain inert to factor XIIIa catalyzed cross-linking. The cross-links on the γ chains of fibrin, the reciprocal e-(γ-glutamyl)-lysyl isopeptide bonds between Gln398 and Lys406 near the carboxyl terminus (5), are the only intermolecular cross-links with the precise locations of respective Gln and Lys residues determined. To date, detailed structural features of the cross-links on the α chains of fibrin, which is also a substrate of factor XIIIa and actively involved in forming the fibrin clot, remain largely unknown. The precise locations of partnering Gln and Lys residues involved in cross-linking on the α chains would add to our current understanding of the molecular packing of fibrin as well as provide molecular moieties for quantitative assessment of fibrin cross-linking. Previous attempts at elucidating the positions of Gln and Lys residues involved in α chain cross-linking yielded insightful, yet incomplete, results because of the limitations of study designs. These efforts studied cross-linking using recombinant fragments of fibrinogen α chain or employed lysine labeling systems to probe donor sites on fibrin (6–11). The former strategy

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2 The numbering of the fibrinogen sequence in this study is based on the processed mature product. To align the sequences of the mature forms with the sequences in Swiss-Prot database (accession numbers P02671 and P02679 for fibrinogen αα and γ chain, respectively), shift 19 residues for the αα chain and 26 residues for the γ chain.
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Although these previous studies speculated the regions of the Edman degradation or mass spectrometry (MS) methods (11), the identities of cross-linked α chain lysine residues cross-linked to the peptide probes, rather than to α chain glutamine residues, were revealed by peptide sequencing with either Edman degradation or mass spectrometry (MS) methods (11). Anti-peptide antibodies recognizing the peptide-decorated products were then used to enrich cross-linked peptides, and the identities of cross-linked α chain lysine residues cross-linked to the peptide probes, rather than to α chain glutamine residues, were revealed by peptide sequencing with either Edman degradation or mass spectrometry (MS) methods (11). Although these previous studies speculated the regions of the α chains where residues are the most receptive to cross-linking peptide probes, they failed to generate the specific pairing information between participating Gln and Lys residues. In addition, the excess of the peptide probes in the cross-linking reaction might have altered the dynamics of fibrin polymerization and the structure of fibrin fibrils and led to false-positive results.

Structural characterization of the cross-linked fibrin fibril clotted under native conditions leads to the precise localization of Gln–Lys pairs involved in the cross-linking by utilizing modern MS technology. Increasingly regarded as one of the most versatile instrument platforms, MS has been used routinely to identify and confirm sequences of proteins, to quantify peptides and proteins, to characterize PTMs, to study protein structure and folding, to probe protein/protein interactions, and to study protein functions (12–16). In studying the primary structure and PTMs of proteins, two complementary protein MS methods, “bottom-up” and “top-down,” are commonly practiced. In typical bottom-up methods, proteins are converted to peptides by enzymatic digestion, and the resulting peptides are sequenced using tandem mass spectrometry (MS/MS) followed by database search. In typical top-down methods, proteins are analyzed directly by compatible MS/MS techniques without being converted to peptides (17). Neither approach by itself, however, is amenable to mapping inter-chain isopeptide cross-links on fibrin. The molecular mass of fibrinogen is ~340 kDa, which is close to the limit of the top-down approach (18). The molecular mass of cross-linked fibrin is infinite, and its gel-like state renders direct ionization of the molecule impossible, making the top-down approach unfeasible. The solubility and ionization efficiency of the analytes would not be concerns in the bottom-up approach after fibrin is converted to peptides. However, the complexity of the fibrin digest would undermine the bottom-up method’s utility in finding cross-linked peptides. Because of generally lower ion intensity of the cross-linked peptides compared with that of linear ones (which are dominant in the digest mixture) coupled with the intrinsic informatics challenge in sequencing the cross-linked peptides (19–22), an unmodified bottom-up shotgun sequencing strategy without differentiation between cross-linked peptides and linear ones would have produced a high number of false positives and yielded little information on the true cross-links.

In this study, a differential mass spectrometry (dMS) strategy is demonstrated to be effective in characterizing cross-links on the α chains of fibrin. This undertaking takes advantage of the “label-free” dMS approach, which is a commonly used comparative method in proteomics studies for finding differentially expressed proteins/peptides under different conditions (23–25). In the design of this study, by analogy, differential clotting conditions with regard to the extent of cross-linking were established for fibrin, and the cross-linked fibrin peptides were “differentially expressed” as a result. Their MS signatures, mass-to-charge ratios (m/z), were readily distinguished from the linear peptides in the background. A fit-for-purpose algorithm was implemented to assign sequences to cross-linked peptides, and MS/MS was used to confirm the identities of the cross-linked peptides. In aggregate, nine e-(γ-glutamyl)-lysyl isopeptide bonds formed between four glutamine and five lysine residues on the α chains of fibrin were unequivocally identified.

EXPERIMENTAL PROCEDURES

Materials—Human fibrinogen, purified from plasma, was purchased from EMD Biosciences (Darmstadt, Germany). Human α-thrombin and factor XIIa were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Sequencing grade trypsin was purchased from Promega (Madison, WI). Inorganic reagents (sodium hydroxide, guanidinium chloride solution, ammonia bicarbonate, and calcium chloride) were purchased from Sigma. Iodoacetic acid, acetic acid, and formic acid were purchased from Fluka. Dithiothreitol was purchased from Fluka. The solvents, HPLC-grade water and acetonitrile, were purchased from Honeywell Burdick & Jackson (Morristown, NJ).

In Vitro Clotting of Fibrinogen under Different Conditions—Human fibrinogen, at concentrations 1 and 2 mg/ml, was clotted in vitro with modified procedures from Mosesson et al. (26). Briefly, human α-thrombin (final concentration 1 unit/ml) and factor XIIa (final concentration 50 units/ml) were added to fibrinogen solution (1 and 2 mg/ml concentration) in 0.1 M NaCl, 20 mM Hepes, pH 7.5 buffer. In clotting conditions that promoted cross-linking, CaCl₂ was added to the final concentration of 10 mM, while in the clotting conditions that prohibited cross-linking, water was added in place of CaCl₂ solution. All fibrinogen solutions were then incubated at 37 °C for 24 h, and fibrin gels formed in all clotting conditions.

Conversion of Fibrin Clots to Tryptic Digests—A denaturant, 8.0 M guanidinium chloride buffered with 20 mM Tris-HCl, pH 8.0, was mixed with fibrin solutions at a 3 to 1 ratio in volume. A reducing agent, dithiothreitol, was subsequently added at the final concentration of 10 mM. The denatured fibrin solution was incubated at 37 °C until the opaque fibrin gels were re-solubilized. An alkylating agent, iodoacetic acid neutralized in equal molar base concentration (NaOH), was then added to the denatured fibrin solution at the final concentration of 25 mM. The alkylation reaction was carried out at room temperature for 30

3 The abbreviations used are: PTM, post-translational modification; CID, collision-induced dissociation; FTICR, Fourier transform ion cyclotron resonance; FT, Fourier transform; z, charge state of an ion; RT, liquid chromatography retention time; dMS, differential mass spectrometry; ANOVA, analysis of variance.
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RESULTS

Outline of Strategy—This study identified cross-linked pairs of Gln and Lys residues on the $\alpha$ chains of fibrin through sequencing the cross-linked peptides. The strategy is outlined in Fig. 1. A dMS analysis on tryptic digests of fibrin clotted under noncross-linking and cross-linking conditions resulted in a list of candidate precursor ions of cross-linked peptides. Only peptides that exhibited a difference in signal intensity between the two conditions were subjected to sequencing. This simplification/pre-selection process allowed a fit-for-purpose algorithm to take advantage of the high mass accuracy measurement afforded by the FTMS instrument as the primary criterion in assigning sequences to cross-linked peptide pairs. Tandem mass spectrometry, which included both high resolution CID MS/MS analyses, provided a secondary confirmation of identified cross-linked peptide pairs. It was critical that the analysis was inclusive of true positives, i.e., the cross-linked peptides, and exclusive of interfering peptides that led to intensity differences because of artifacts. To ensure the sensitivity and selectivity of the dMS analysis, the two experimental conditions in comparison had to be carefully balanced to minimize artificial differences that could lead to false positives. This prerequisite was achieved by exploiting the requirement of $\mathrm{Ca}^{2+}$ as a co-factor in factor XIIIa-catalyzed fibrin cross-linking.

Fibrinogen under Differential Clotting Conditions—Human fibrinogen was clotted under in vitro conditions either promoted or prohibited inter-chain cross-linking. In the condition that promoted cross-linking, both factor XIIIa and $\mathrm{Ca}^{2+}$ were

min in the dark. The denaturant and reduction-alkylation reagents were removed from the mixtures by buffer exchange against 50 mM (NH$_4$)$_2$CO$_3$ at pH 8.3 with a 5-kDa molecular mass cutoff spin filter (Millipore, Billerica, MA). Trypsin of 1% weight equivalence of the denatured, reduced, and alkylated fibrin was then added to the mixtures with incubation at 37 °C overnight. The resulting fibrin digests were desalted with C18 cartridges (3-ml SepPac$^{\text{TM}}$, Waters). After being lyophilized to dryness, the desalted peptide mixtures were resuspended in 0.1 M acetic acid in H$_2$O for high resolution LC/MS analysis.

High Resolution LC/MS Profiling on Tryptic Digests of Clotted Fibrin—Peptide mixtures of digested fibrin under different conditions were analyzed by a reverse phase nano-HPLC coupled to a LTQ-FTICR hybrid mass spectrometer (LTQ-FT Ultra, ThermoFisher). Re-solubilized fibrin digests were injected with an auto-sampler (Series 1100, Agilent, Santa Clara, CA) onto a nano-LC column packed with BioBasic C18 media (5 cm × 75 μm, New Objective, Woburn, MA). A micro-flow HPLC pump (Agilent Series 1100) delivered a binary gradient increasing from 0% hydrophobic phase (0.1 mM acetic acid in acetonitrile) to 50% hydrophobic phase at a rate of 1%/min while maintaining the flow rate at 1 μl/min. Peptides eluting from the nano-LC column were introduced into the mass spectrometer by electrospray ionization using a 3-kV needle voltage, heated metal capillary temperature of 270 °C, and tube lens voltage at 80 V. Ion injection times into linear ion trap were adjusted by the instrument automatic gain control (1 × 10$^7$ arbitrary unit setting) with a maximum accumulation time not to exceed 3 s. Ions were passed to the FTICR cell, and full scan spectra with mass-to-charge ratio (m/z) from 300 to 2,000 were acquired approximately every 3 s. While the full scan MS spectra were acquired in the FTICR cell at an instrument-solving power of 50,000, data-dependent MS/MS scans of the six most intense precursor ions in the preceding full MS scan were collected in the linear ion trap. The MS/MS scans did not affect the throughput of the full scan MS analysis while providing complementary information that was used to evaluate the quality of the tryptic digests.

Database Search by Mascot$^{\text{TM}}$—MS/MS spectra collected in the profiling analysis, although not used to yield sequence information on cross-linked peptides, were surveyed to confirm the quality of trypsin digestion. The spectra were searched using the Mascot$^{\text{TM}}$ search engine (Matrix Sciences, version 2.1) for peptide identifications against the human protein database (downloaded from NCBI in May 2007) composed of sequences from Swiss-Prot and RefSeq) containing 46,859 Homo sapiens sequences (27). The parent mass tolerance was set to 5 ppm, and MS/MS mass tolerance was 0.5 Da. The enzyme was specified as trypsin with up to two missed cleavages. Carboxymethylation of cysteine was searched as a fixed modification, and amino-terminal pyro-Glu and oxidation of methionine were searched as variable modifications. Identified sequences with peptide scores greater than 40 were automatically accepted as positive matches. Sequences with scores between 30 and 40 were manually inspected, and low quality matches (poor MS/MS quality, wrong charge state assignment, and major fragment ions not assigned) were discarded. No identification with a peptide score lower than 30 was accepted.
present in the clotting solution. In the condition that prohibited cross-linking, only factor XIIIa was added to the clotting solution. Because the only difference in the composition of the clotting solutions between the two conditions was Ca\(^{2+}\), which was readily removed in the desalting step following enzymatic digestion and prior to LC/MS analysis, this design maximized the possibility of finding cross-linked fibrinogen peptides by eliminating other irrelevant differences in the abundance of peptides between the two conditions. There are alternative approaches to create the difference in cross-linking between the two clotting conditions, such as withholding factor XIIIa from the noncross-linking condition or using factor XIII (the inactive zymogen for factor XIIIa) in the noncross-linking condition and factor XIIIa in the cross-linking condition. However, these methods would have introduced artificial differences in the abundance of peptides besides those arising from cross-linking.

In addition to tightly controlled clotting conditions to minimize false positives, the concentration of clotted fibrinogen was also incorporated as a factor to further improve the selectivity of the analysis. Specifically, fibrinogen was clotted in technical triplicates at 1 and 2 mg/ml concentrations in both noncross-linking and cross-linking conditions. After overnight incubation at 37 °C, the clotting solution turned to gel under all four conditions. The gels formed under the noncross-linking conditions were immediately re-solubilized upon treatment with denaturant (guanidinium chloride, 6.0 M final concentration); the gels formed under the cross-linking conditions, however,
took much longer to be re-solubilized in denaturant and only in the presence of dithiothreitol.

High Resolution LC/MS Analysis of Fibrin Tryptic Digests—Tryptic digests of fibrin clots were analyzed with a high resolution FTMS instrument. The MS method was optimized for the throughput of full MS scans because precursor ion intensities were compared in the dMS analysis. The low resolution MS/MS spectra of the fibrin digests were searched against a human protein database, and the results served as a survey to evaluate the quality of tryptic digestion. Overall, 337 peptides from fibrin were identified with sequence coverage for α, β, and γ chains being 80, 85, and 80%, respectively. The identified peptides were evenly distributed throughout the fibrin chains. This observation, combined with the relatively high sequence coverage, suggested the following: 1) the digestion of the clotted fibrin by trypsin was complete; 2) the resultant peptide mixture, although complex, was readily quantified with the high resolution LC/MS platform. The profiles of the LC/MS data, as judged by the base peak ion chromatograms, were consistent in both clotting conditions without discernable differences when examined manually (Fig. 2). The LC/MS data were further processed with Elucidator®, a mass spectrometric data analysis suite that aligns raw data, extracts m/z, RT, and intensity data for comparative analysis.

Differential Mass Spectrometry Analysis of Fibrin Digests under Different Clotting Conditions—Elucidator® extracted 10,244 isotope clusters (referred to as peaks from here on) with distinct m/z within a restricted RT window from the raw LC/MS data files for statistical analysis (see “Experimental Procedures” for details). Two separate ANOVA analyses were performed to find peaks with statistically significant differences (ion intensity ratio between groups >100, p < 0.01) in ion intensity between noncross-linking and cross-linking conditions of fibrinogen clotted at 1 and 2 mg/ml. The ANOVA analyses resulted in 167 and 275 peaks differentiating the noncross-linking condition from the cross-linking condition at 1 and 2 mg/ml concentrations, respectively. The differentiating peaks that overlapped the two comparisons, 31 in total, were considered candidate precursor ions of cross-linked peptides. After peaks from different charge states of the same monoisotopic masses were combined, 25 unique monoisotopic masses were generated as the final results of the differential analysis and masses were combined, 25 unique monoisotopic masses were derived from the differential analysis. For example, in identifying the cross-linked MADEAGSEADHEGTHSTKR and QFTSSTSYNR (underlined Q and K denote residues responsible for cross-linking) peptide pairs, the calculated molecular partner peptide that provides the Lys donor in cross-linking must have the donor lysine as the tryptic missed cleavage site, and the other partner peptide needs to contain at least one glutamine. In practice, an in silico digestion of fibrin α chain generated a list of tryptic peptides with 0 and 1 missed cleavage sites. Tryptic peptides containing Lys at the missed cleavage sites were selected as possible sequences that provide Lys donors in cross-linking. The peptides containing Gln were selected as possible sequences that provide corresponding Gln acceptors in cross-linking. A table composed of calculated masses of in silico cross-linked tryptic peptides was constructed based on all possible pairing situations between Lys donor and Gln acceptor containing tryptic peptides. For fibrin α chain, calculated masses of 2,376 in silico cross-linked peptides were used to assign sequences for the 25 monoisotopic masses derived from the differential analysis. For example, in identifying the cross-linked MADEAGSEADHEGTHSTKR and QFTSSTSYNR (underlined Q and K denote residues responsible for cross-linking) peptide pairs, the calculated molecular
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The e-(γ-glutamyl)-lysyl bonds between specific Gln and Lys residues were identified through sequencing the cross-linked tryptic peptides. In total, nine cross-linked peptides of fibrin α chains were identified, and four Gln acceptor and five Lys donor sites were involved. All nine cross-linked peptides have Δm < 2 ppm between measured and calculated mass. The identities of all nine peptides were confirmed with tandem mass spectrometry.

| Cross-linked Peptides | Measured Mass (Da) | Calculated Mass (Da) | Δm (ppm) |
|-----------------------|--------------------|----------------------|----------|
| GSESGIFTNTKESSSHHPGIAEFPSR | 3200.3689 | 3200.369 | 0.04 |
| GSESGIFTNTKESSSHHPGIAEFPSR | 3344.6182 | 3344.6137 | 1.3 |
| GSESGIFTNTKESSSHHPGIAEFPSR | 3930.8051 | 3830.8034 | -0.42 |
| HPDEAAFFDTASTGTGKFPGFFSPMLGEFVSETESR | 4425.0569 | 4425.0651 | 1.8 |
| HPDEAAFFDTASTGTGKFPGFFSPMLGEFVSETESR | 4883.2299 | 4883.2183 | -1.6 |
| MADEAGSEADHEGTHSTKR | 8714.8888 | 8714.8931 | 0.50 |

mass (3200.369) of the cross-linked peptide, the combined molecular mass of the Lys donor and Gln acceptor peptides minus the molecular mass of NH3, matches the measured molecular mass (3200.3689) with Δmass (Δm) being 0.04 ppm. Nine cross-linked peptides from fibrin α chains were thus postulated when their measured masses were matched with the masses of in silico cross-linked tryptic peptides (Table 1). The molecular mass of the identified cross-linked peptides ranged from ~1,900 to ~9,000 and the maximum Δm was 1.8 ppm. To confirm the fidelity of the sequencing algorithm and validate the sequences of the peptides, MS/MS spectra of the nine positively matched cross-linked pairs were acquired to verify the identities of the peptides.

Tandem Mass Spectrometry Confirming the Identities of Cross-linked Peptides—Unlike peptides with linear sequence, the cross-linked peptides normally have high charge states, often greater than +5, and generate fragment ions with high charge states as well (28). It was also observed that the MS/MS spectra of cross-linked peptides generally contain fewer fragments compared with those of linear peptides (29). The lack of efficiency in fragmentation coupled with the difficulty in determining the masses of fragment ions lead to higher false-positive identification rates for cross-linking peptides (30). In this study, because the primary structures of the cross-linked peptides were identified by a differential analysis with accurate mass measurement, the dependence on MS/MS spectra to deduce sequence information was greatly relaxed. In their role as confirmatory evidence, high resolution CID MS/MS spectra were acquired on LTQ-FTMS for all nine cross-linked peptides. When a peptide was identified with more than one charge state, the precursor ion of the charge state with less co-eluting, interfering ions was selected for MS/MS analysis. In the analysis, the precursor ions were isolated in the linear ion trap and fragmented to generate b and y ions, which were subsequently measured with high mass accuracy after being transmitted into the FTICR cell. Eight of the nine cross-linked peptides yielded useful high resolution CID MS/MS spectra and were used to manually verify the identities of the peptides. Examples of manual verification of sequences of cross-linked peptides are shown in Fig. 4. Fig. 4A illustrates fragment ion assignment for the cross-linked peptide of QFTSTSYNR and MADEAGSEADHEGTHSTKR. The precursor ion 641.0813 [M + 5H]5+ was isolated and fragmented by CID in the ion trap, and fragment ions were measured in the FTICR cell. Two b ions, 597.7584 (z = 4) and 762.9918 (z = 3), and four y ions, 452.2247 (z = 1), 539.2567 (z = 1), 727.3333 (z = 1), and 768.3383 (z = 4), were matched with calculated value with Δm less than 10 ppm. Three water loss peaks derived from y ion 768.3383 (z = 4) were also observed in the high resolution CID spectra (zoomed in m/z range of Fig. 4A). Fig. 4B shows fragment ion assignment for the cross-linked peptide composed of QFTSTSYNR and GSESGIFTNTKESSSHHPGIAEFPSR. The precursor ion 656.1414 [M + 6H]6+ was selected to generate high resolution CID spectra. Two b ions, 857.3914 (z = 4) and 894.1522 (z = 4), and six y ions 359.2033 (z = 1), 452.2242 (z = 1), 506.2724 (z = 1), 539.2565 (z = 1), 635.3131 (z = 1), and 819.3872 (z = 2), were matched. Similarly, Fig. 4C illustrates fragment ion assignment for the cross-linked ALTDMPQMR and HPDEAAFFDTASTGKTFPGFFSPMLGEFVSETESR, where the precursor ion 797.6532 [M + 5H]5+ was fragmented and six b ions, 956.4611 (z = 3), 1211.2114 (z = 3), 1248.9023 (z = 3), 1310.9269 (z = 3), 1359.9485 (z = 3), and 1434.6603 (z = 2), and four y ions, 492.2403 (z = 1), 621.284 (z = 1), 708.3145 (z = 1), 807.3813 (z = 1), were assigned. Overall, ~50% of m/z in high resolution CID spectra were matched with b and y ions with Δm less than 10 ppm. Only one cross-linked peptide (GSESGIFTNTKESSSHHPGIAEFPSR and GSESGIFTNTKESSSHHPGIAEFPSR and GSAGHWTESSVSSTG-}

FIGURE 3. Results of differential mass spectrometry analysis. A, candidate precursor ions of cross-linked fibrinogen peptides. High resolution full scan MS spectra (on the right) and ion intensities of precursor ions (bar graphs on the left, in triplicates) of cross-linked peptides are plotted for each experimental condition. The bar graphs and the mass spectra of the precursor ions are color coded as follows: magenta, −Ca2+ 1 mg/ml fibrinogen concentration; blue, −Ca2+ 2 mg/ml fibrinogen concentration; green, +Ca2+ 1 mg/ml fibrinogen concentration; yellow, +Ca2+ 2 mg/ml fibrinogen concentration. All candidate precursor ions of cross-linked peptides share the following characteristics: 1) absent in noncross-linking conditions (−Ca2+) and present in cross-linking conditions (+Ca2+); 2) in cross-linking conditions, ion intensity correlates with fibrinogen concentration. 8 examples of precursor ions of noncross-linked fibrin peptides. The peptide sequence for 1146.478 [M + 2H]2+ is FFTSHNGMQSTWDNDNK from the γ chain of fibrinogen. The peptide sequence for 793.421 [M + 2H]2+ is IDETVNSNPTNL from the β chain of fibrinogen. The ion intensities of these precursor ions correlate with fibrinogen concentration but do not differentiate cross-linking conditions from noncross-linking conditions.

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FIGURE 4. Examples of high resolution MS/MS analysis confirming the identities of cross-linked peptides. A, LTQ-FTICR CID spectrum of precursor ion 641.0813 [M + 5H]$^+$ confirming the cross-link between QFTSSTSYNR and MADEAGSEADHEGTHSTKR. B, LTQ-FTICR CID spectrum of precursor ion 656.1414 [M + 6H]$^+$ confirming the cross-link between QFTSSTSYNR and GSESGIFTNTKSSSHHPGIAEPFSR. C, LTQ-FTICR CID spectrum of precursor ion 977.6532 [M + 5H]$^+$ confirming the cross-link between ALTDMPQMR and HPDEAAFFDTASTGTFPGFSMPMLGFVSETESR. The assigned $m/z$ peaks (labeled with arrows) have $\Delta m < 10$ ppm between the measured and the calculated values of $b$ and $y$ ions.
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FIGURE 4 — continued

B)  

C)  

FIGURE 4 — continued
0.3 Da for all four y ions. The partial sequence confirmation by low resolution CID coupled with the high mass accuracy measurement of the parent ion confirmed the identity of the cross-linked peptide.

**Locations of Inter-chain ϵ-(γ-Glutamyl)-Lysyl Cross-links on Fibrin α Chains**—Fig. 6 illustrates precise locations of the Gln and Lys residues involved in the cross-linking on fibrin α chains. Four glutamine (Gln223, Gln239, Gln366, and Gln563) and five lysine (Lys418, Lys508, Lys539, Lys556, and Lys601) residues were involved in nine inter-chain cross-links on fibrin α chains. Detailed one-to-one pairings between involved Gln and Lys residues were illustrated by lines connecting them. The numbering of the amino acid residues was according to the mature form of fibrinogen chain, and only part of the sequence, residues 221–625, was displayed in Fig. 6. Gln237 and Lys539 were found to be the most involved in cross-linking, forming ϵ-(γ-glutamyl)-lysyl bonds with four different lysine (Lys418, Lys508, Lys539, and Lys556) and four glutamine (Gln223, Gln237, Gln366, and Gln563) residues, respectively.

**DISCUSSION**

In hemostasis, the fibrin clot is stabilized by the formation of covalent bonds between specific Gln and Lys residues catalyzed by a plasma transglutaminase, factor XIIIa. Although many aspects of fibrin structure and function have been elucidated (31, 32), the finite structural features of the cross-linking were not fully understood, especially on the α chains of fibrin. Although earlier attempts focusing on surveying chain Gln and Lys residues involved in the cross-linking provided critical insights on regions susceptible to cross-linking, they failed to establish the corresponding relationships between participating Gln and Lys residues. The lack of such knowledge hinders a precise understanding of the molecular packing in fibrin. This report describes a novel mass spectrometry-based approach intended at mapping the one-to-one relationships between cross-linked Gln and Lys residues. In this approach, the mass spectrometry signatures (m/z, RT) of the cross-linked fibrin peptides were distinguished from the background by a differential mass spectrometry workflow. In doing so, fibrin clots that
underwent cross-linking conditions were compared with fibrins clotted under noncross-linking conditions. The outcomes of the comparison, precursor ions of the cross-linked fibrin peptides, were sequenced with a fit-for-purpose algorithm. Positively identified, covalently bonded peptide pairs were then confirmed with tandem mass spectrometry. In addition to the positive control of the study, the reciprocal cross-links between Gln398 and Lys406 on the \( \text{H9253} \) chains of fibrin, nine specific cross-links between four Gln and five Lys residues were established on the \( \text{H9251} \) chains of fibrin. The findings provided more refined structural details on the \( \text{H9251} \) chain cross-linking compared with earlier efforts.

Several aspects of the study deserve comments. First, unlike previous efforts that used recombinant fibrinogen fragments or peptide labeling systems to study the cross-linking on fibrin, the current effort studied human fibrinogen purified from plasma, clotted \( \text{in vitro} \) under native conditions similar to those used in structural studies on fibrin fibers. In the structural studies, the fiber-like structure of fibrin was confirmed with electron microscopy. Therefore, the cross-links elucidated in this study are more reflective of the structural features involved in the \( \text{in vivo} \) clotting process than in earlier reports. Second, in contrast to earlier efforts, the cross-linked peptides in this study were never physically purified or separated from the digested peptide mixtures. Instead, this approach took advantage of the high resolving power of FTMS and sophisticated informatics tool for MS data reduction to isolate \( \text{in silico} \) the MS signals of the cross-linked peptides from the background. The streamlined automated workflow has higher throughput with yet fewer false positives compared with complex biochemical procedures that involve either affinity enrichment with anti-peptide antibodies or multidimensional HPLC separation. Third, this study overcame the difficulty in sequencing cross-linked peptides by MS/MS by placing it as a secondary, confirmatory method. The fit-for-purpose sequence assignment algorithm relied primarily on the match between calculated and measured molecular mass of the cross-linked peptides. Equipped with candidate sequences, CID MS/MS was then used to confirm the identities of the cross-linked sequences with manual verification of the fragment ion spectra. This sequence assignment method, although proven efficient in this experimental design, would have had limited success if it were used directly to identify cross-linked peptides (or even linear ones) from fibrin digests prior to data reduction by dMS. The prerequisite for this fit-for-purpose algorithm was a list of accurate masses of purported cross-linked peptides, which was the outcome of the dMS experiment.

Twenty five precursor ions of candidate cross-linked peptides were generated as the results of the dMS experiment, and 10 were assigned sequences of cross-linked peptide pairs, one previous known \( \gamma \) chain cross-linked peptide and nine newly discovered cross-linked peptides from fibrin \( \alpha \) chains. A few explanations may account for the unassigned precursor ions. The human fibrinogen used in this study was purified directly from human plasma, and the impurity might have contributed to cross-linked structures besides those on fibrin. Because it is known that fibrinogen may bind to multiple plasma proteins such as fibronectin, fibulin, albumin, von Willebrand factor, and \( \text{H9251} \)2-antiplasmin (33–35), intermolecular covalent bonds might have formed between fibrinogen chains and bound proteins in this study. Indeed, peptides from fibronectin, fibulin, albumin, von Willebrand factor, and \( \alpha2 \)-antiplasmin were identified in the database search using the MS/MS data of the fibrin tryptic digests (data not shown). Factor XIIIa-catalyzed isoglu-
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tamyl lysine isopeptide bonds might have formed between fibrin and bound proteins. One example of such cross-linking is the cross-link between α2-antiplasmin (Gln41) and the α chain of fibrinogen (Lys303) (36). These cross-links, however, were not the focus of this study and were not investigated. Besides impurity, glycosylation on fibrinogen chains might have also contributed to the unassigned precursor ions. Because the current understanding on α chain glycosylation is limited to the positions of glycosylated Asn residues but with the structures of the glycans not known (37, 38), it was not possible to consider glycosylation as a PTM in the sequence assignment algorithm. Using endoglycosidases such as peptid:N-glycosidase F to remove the glycans from trypptic digests prior to the LC/MS analysis could potentially increase the number of identified cross-linked peptides. Other known PTMs on fibrinogen chains, such as phosphorylation and sulfation, were taken into consideration in sequencing; however, no additional cross-linked fibrin peptides were identified.

The results of this study confirmed earlier observations on regions of fibrin α chains that are most susceptible to cross-linking. Three glutamine acceptor sites (Gln223, Gln237, and Gln366) and four lysine donor sites (Lys308, Lys339, Lys566, and Lys601) identified in this study have been previously proposed as key participants in the α chain cross-linking (10, 11). These α chain cross-linking residues are located on the αC region of fibrin α chains (residues Aα221–610), a region that is composed of a flexible tether known as αC-connector (residues 221–391) and a compact globule known as αC domain (residues 392–610) (39). Crystallographic studies have failed to resolve the structure of the αC region (31, 32). Only part of the amino-terminal region (residues Aα425–503) of the αC domain has been revealed by NMR studies as a β-sheet consisting of two β-hairpins (40). Studies indicate that in fibrinogen two αC domains form a dimer through interaction with each other and the central region of the molecule. In polymeric fibrin, factor XIIa covalently cross-links αC regions from different fibrin molecules to produce the fibrin network (41–43). It is speculated that the Gln acceptors located near the amino terminus of fibrin αC regions (αC-connector) form cross-links with Lys donors located near the carboxyl terminus of αC regions (αC domain) from another fibrin molecule (44). This hypothesis agrees with the current understanding of the mechanism of α chain polymerization, where one α chain is believed to interact with at least two others on different fibrin molecules in a half-staggered fashion to longitudinally elongate fibrin protofibrils (45). The findings of this study mostly support this model as follows: three of the four Gln residues participating in the cross-linking are located in the αC-connector region, while all five Lys residues are in the αC domain. In addition to generating novel evidence for the α chain’s contribution to longitudinal aggregation of fibrin polymerization, the new discovery of a Gln acceptor site in the αC domain of the α chain, Gln563, also sheds light on the role of the α chain in enhancing lateral aggregation in fibrin polymerization. The formation of the inter-chain cross-links, Gln563 to Lys339 and Gln563 to Lys601, indicates aligned, not staggered, fibrin α chains contributing to the lateral aggregation of fibrin fibers because of the close vicinity of the participating residues in protein sequence.

The finding that some α chain Gln residues form isoglutamyl-lysyl isopeptide bonds with different Lys residues and vice versa highlights the structural heterogeneity affecting the cross-linking domain of the fibrin α chain (46, 47). Previous studies have shown that naturally occurring truncated forms of fibrin α chains retain certain cross-linking capabilities (48, 49). It is believed that the retention of cross-linking on degraded fibrin α chains balances the fibrinolytic process and maintains the stability of the fibrin network. This hypothesis predicates that individual α chain donor/acceptor site interacts with multiple acceptor/donor sites. The promiscuity of the cross-linking is necessary to counter the process of degradation and loss of sites for cross-linking. This intrinsic heterogeneity of cross-linking was proposed based on the observation of multiple Lys donor sites discovered from earlier studies (11), but it was never substantiated due to the lack of information on respective Gln acceptor sites. This study produced direct evidence for the claim through characterization of cross-linked α chain peptides originating from native fibrin. Gln237 and Lys601 were found to be the most actively involved in the intermolecular interaction with each forming three additional cross-links besides the isopeptide bond between each other. In addition to confirming the intrinsic heterogeneity of α chain cross-linking, these newly established cross-links can serve as molecular moieties in future studies on the dynamics of fibrin polymerization and the fibrinolytic process as they represent structural information at the molecular level.

Acknowledgments—I thank James P. Conway, Gregory J. Opiteck, Zhu Chen, Qing Yan, and Bernard K. Choi for critical reading of the manuscript and Ann Marie Norris from Merck Creative Services for help with figure preparation.

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J. Biol. Chem. 2011, 286:44952-44964.
doi: 10.1074/jbc.M111.297119 originally published online October 26, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.297119

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