Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1113–1117, February 1989
Biochemistry

Identification of covalently linked trimeric and tetrameric D domains in crosslinked fibrin*

(fibrinogen/plasma transglutaminase/factor XIII)

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Communicated by Russell F. Doolittle, September 19, 1988 (received for review July 6, 1988)

ABSTRACT **Following proteolytic conversion of fibrinogen to fibrin, clot assembly commences with formation of double-stranded fibrils that subsequently branch extensively in forming a three-dimensional network. Plasmin digests of fibrin clots that had first been covalently crosslinked by plasma transglutaminase (factor XIIIa) contained multimeric proteolytic fragments composed of crosslinked outer (D) domains of neighboring fibrin molecules. Two of these were larger than the well-known "D dimer" fragment and corresponded to D trimers and D tetramers, respectively. Whereas D dimers originate from crosslinked D domains at bimolecular junctions within two-stranded fibrils, D trimers and D tetramers evidently arise through crosslinking of contiguous D domains at trimolecular and tetramolecular junctions or at fibril branch points, respectively. Measurement of the widths of fibrils comprising trifunctional branches in thin fiber networks revealed tetramolecular branch points, which are formed by bifurcation of two double-stranded fibrils. In addition, another type of trifunctional structure, which we term the trimolecular branch point, was composed of three double-stranded fibrils. Crosslinking of D domains to form trimers may occur at this type of junction. These findings add to our understanding of the crosslinking arrangements that stabilize fibrin clot structure and the ways that fibrin molecules polymerize to form branches in the clot matrix.**

Following proteolytic conversion of fibrinogen to fibrin, polymer assembly commences with formation of double-stranded fibrils in which fibrin molecules, by virtue of noncovalent intermolecular interactions between outer (D) and central (E) domains (1–6), are arranged in a staggered overlapping manner (7–13) (Fig. 1). Subsequently, lateral fibril associations result in increased fiber thickness (11, 16–18), which is believed to account for interfibril connections and the trifunctional branching structures that comprise the three-dimensional matrix (11, 18–21). In the presence of plasma transglutaminase (factor XIIIa) and Ca^{2+}, fibrin molecules undergo covalent crosslinking by formation of ε-(γ-glutamyl)lysine [ε-(γ-Glu)Lys] isopeptide bonds (22, 23). Intermolecular crosslinking between D domains forms dimers (24), which occur as reciprocal bridges between a lysine at position 406 of one γ chain and a glutamine at position 398 or 399 of another (25–28). In addition, slower intermolecular crosslinking among α chains creates oligomers and larger α-chain polymers (29–31).

Plasmin digestion of crosslinked fibrin results in early release of crosslink-containing α-chain segments from core structures (32–36). Thus, their existence in fibrin does not contribute significantly to the structure of major intermediate or terminal plasmin core fragments. In contrast, the intermolecular ε-(γ-Glu)Lys γ-chain bonds result in degradation products unique to crosslinked fibrin, of which the bimolecular fragment, "D dimer," is the most abundant and best characterized (32, 37–39). Crosslinked core derivatives larger than D dimer are known to exist (36, 40–45), each of which is postulated (36, 43–45) to contain one or more E domains (e.g., DY, YY, DXD).

The presence of Ca^{2+} confers resistance against plasmin cleavage in the COOH-terminal region of the fibrin(ogen) γ chain (46). This results in retention of the COOH-terminal segment containing the crosslinking site and consequent preservation of D dimer fragments in advanced digests of crosslinked fibrin (47–49), whereas intermediate derivatives containing E domains are eventually all consumed.

In this study, we analyzed the components in plasmin digests of crosslinked fibrin and identified two previously unrecognized crosslinked fragments larger than D dimer, corresponding to D trimers and D tetramers. These crosslinked fragments evidently originate from trimolecular or tetramolecular junctions or fibril branch points within the fibrin network.

MATERIALS AND METHODS

Crosslinking and Plasmin Digestion of Fibrin. To purified human fibrinogen (2.5–3 mg/ml) in 0.15 M NaCl/10 mM Tris·HCl, pH 7.4, buffer was added plasma factor XIII (50) (2–5 Loewy units/ml), $CaCl_2$ (2–10 mM), and α-thrombin (1–2 units/ml), and the mixture was incubated at room temperature for 2–5 hr. The crosslinked clots were then subjected to syneresis, washed, suspended at 37°C in a 0.1 M Tris·HCl (pH 8.6) buffer containing EDTA (10 mM) or $CaCl_2$ (10 mM), and plasmin (51) (0.9–1.0 unit/ml). Aliquots of digest samples were added to an equal volume of an aprotinin solution (500 kallikrein inhibitor units/ml) for subsequent analysis.

Electrophoretic/Immunologic Procedures. Anti-D, anti-E, and anti-S carboxymethylated (S-CM) γ and $/\alpha$‡ chains were prepared in rabbits. NaDodSO$_4$/PAGE was carried out on cylindrical gels (56); crossed immunoelectrophoresis of such gels was conducted as described (57). Two-dimensional NaDodSO$_4$/PAGE was carried out on unreduced specimens in the first dimension (3 mm; 4.5% gels), and in the second

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Abbreviations: TEM, transmission electron microscopy; STEM, scanning TEM.

*This work was presented at the Eighth International Fibrinogen Workshop, June 13–15, 1988, Milwaukee, WI.

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‡Terminology for cleavage products of fibrin(ogen) includes the following: $(/\gamma)_2$, $(/\gamma)_3$, and $(/\gamma)_4$, crosslinked multimeric γ-chain fragments derived from γ dimers ($\gamma\gamma$), γ trimers, and γ tetramers, respectively; $/\gamma/$ and $/\gamma\cdot//\gamma$, fragments derived by asymmetric cleavage of a $(/\gamma)_2$ chain to yield a monomolecular fragment, $/\gamma/$, and a bimolecular fragment, $/\gamma\cdot//\gamma$, which retains the ε-(γ-Glu)Lys crosslink; $/\beta$-134–461, Bβ-chain fragment; $/\alpha/$, Aα-105–197; $/\alpha$, COOH-terminal Aα-chain fragments; A$\alpha/_4$, NH_2-terminal Aα-chain core fragment (≈58 kDa). Assignment of peptide sequences to derivative chains is based on the known sites of plasmin cleavage of fibrinogen (52–55), crosslinked fibrin (46), or upon sequencing data from this work.

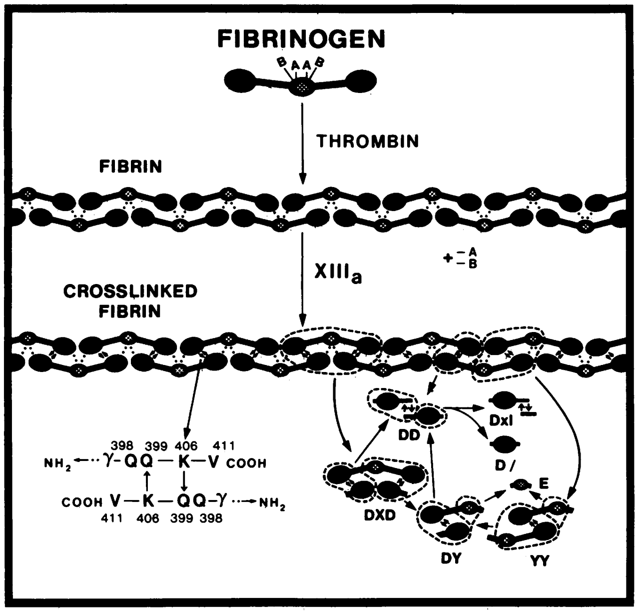

FIG. 1. Diagram illustrating how fibrin molecules assemble to form two-stranded fibrils after thrombin cleavage of fibrinopeptides A (FPA) and B (FPB) from the central (E) domain of fibrinogen. Cleavage of FPA alone (α-fibrin) or FPB alone (β-fibrin) results in the same type of fibril assembly (14). Crosslinking by factor XIIIa results in formation of lysine (K) donor to glutamine (Q) acceptor ε-(γ-Glu)Lys bridges between γ chains from neighboring D domains. These are represented by pairs of arrows between D domains and in greater detail for the predominant γ-chain species in fibrinogen, γ_A (γ-1–411 Val) (15). Plasmin cleavage (dashed lines) results in a family of crosslinked core fragments containing the D dimer (DD) structure. The terminology (and sizes) of digest components containing D (95 kDa) and/or E (50 kDa) domains includes X (240 kDa), derivative of fibrin(ogen) containing the E and both D domains; Y (145 kDa), fragment composed of one D and one E domain; D_{x1} ($\approx$97 kDa) and D/ ($\approx$80 kDa), complementary D fragments derived from asymmetric cleavage of D dimer (190 kDa).

dimension after reduction with 10% 2-mercaptoethanol into a 1.5-mm-thick gradient slab gel (5–23% polyacrylamide) containing Laemmli resolving buffer (58). Immunoblotting was carried out on samples that had been separated by NaDodSO$_4$/PAGE on a 3% stacker/7% resolving slab gel (58), electroblotted to nitrocellulose paper, and probed with either ^{125}I-labeled rabbit anti-γ- or anti-α-chain IgG.

Amino Acid Sequence Analyses. A reduced (5% 2-mercaptoethanol) crosslinked fibrin digest was separated on 7.5% polyacrylamide gels (58) and electroblotted to a polyvinylidene difluoride membrane (59). Peptide-containing bands were excised and subjected directly to automated sequencing on a model 477A Applied Biosystems pulsed liquid-phase sequencer with a model 120A on-line phenylthiohydantoin amino acid analyzer.

Fragment Preparation and Electron Microscopy. An early monomeric D fragment (D_1) was isolated from an intermediate plasmin digest of fibrinogen (60). A D trimer/D tetramer-enriched fraction was prepared from a digest of a crosslinked clot by selective pooling of fractions that had been rechromatographed on Sepharose 4B-CL in 50 mM Tris·HCl/1.0 M NaCl, pH 7.5, buffer. D dimer-containing fractions were pooled and rechromatographed to homogeneity.

For transmission electron microscopy (TEM), samples were dialyzed against 0.15 M ammonium acetate/30% (vol/vol) glycerol solution, pH 7, diluted to $\approx$30 μg/ml, sprayed onto mica discs, and rotary shadowed with platinum-carbon. For scanning TEM (STEM), samples were diluted to 5–10 μg/ml in 0.15 M NaCl/10 mM Hepes, pH 7, buffer, applied to a thin carbon film, and freeze-dried (60).

Samples of α,β-fibrin for critical point drying were prepared from fibrinogen (50 μg/ml in 10 mM Tris·HCl/0.15 M NaCl/5 mM EDTA, pH 7.4, buffer) after thrombin addition (2 units/ml) and overnight incubation at room temperature. β-Fibrin was processed after overnight incubation at 14 ± 1°C in a 10 mM Tris·HCl/0.15 M NaCl, pH 7.4, buffer, to which copperhead venom procoagulant enzyme (61), 0.13 unit/ml, had been added.

TEM was carried out in a Philips 400 electron microscope at 60–80 kV. High-resolution STEM (62) was performed at the Brookhaven Biotechnology Resource using a 40-kV probe. Mass measurements on STEM images were performed off-line using a circle program (63).

RESULTS

Electrophoretic analysis of intermediate plasmin digests of crosslinked fibrin in the presence of Ca^{2+} yielded a characteristic band pattern (Fig. 2*A*) that included two prominent components of 300 kDa (range, 290–310 kDa) and 365 kDa (range, 350–380 kDa), respectively, which were larger than the well-characterized DY fragment (240 kDa). Other major components included the D dimer band, smaller bands corresponding to monomeric forms of fragment D (D_{x1} and D/), plus a fragment E band. At an advanced phase of digestion (Fig. 2*B*), the DY component had been consumed, whereas the 300-kDa (D trimer) and 365-kDa (D tetramer)

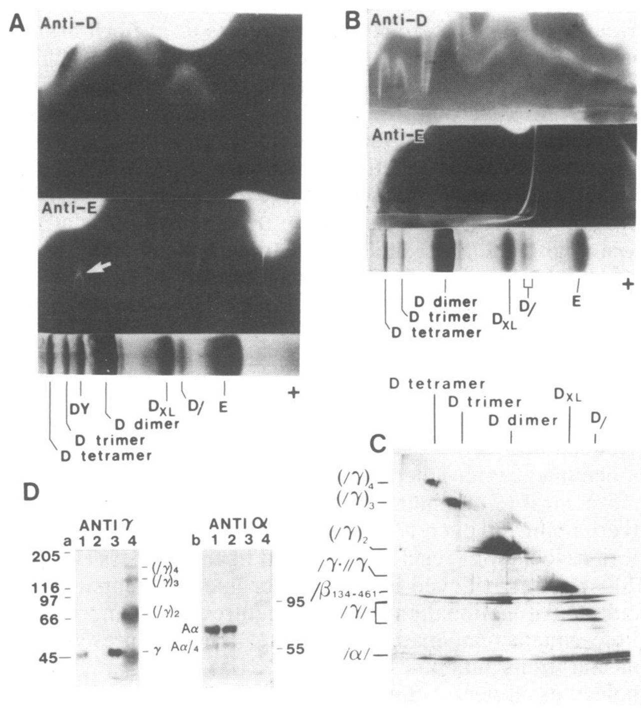

FIG. 2. Analyses of plasmin digests of crosslinked fibrin. (*A* and *B*) NaDodSO$_4$/PAGE (56) of nonreduced digest samples on 5% gels (horizontal positions) stained with Coomassie blue; crossed immunoelectrophoresis of intermediate (3 hr; *A*) and advanced (18 hr; *B*) digest samples into agarose gels containing monospecific anti-D or anti-E antisera. The band pattern of the gel slice subjected to crossed electrophoresis is illustrated by substituting a stained gel of an identical sample. The irregular white areas at the sides and top of each agarose gel represent the NaDodSO$_4$/Lubrol PX complex that forms in this system. (*C*) Silver-stained two-dimensional NaDodSO$_4$/PAGE of a pooled column fraction from an advanced digest of crosslinked fibrin that had been chromatographed on Sepharose 4B-CL to remove smaller components (e.g., fragment E). (*D*) Immunoblotting of reduced (2-mercaptoethanol) or S-CM samples: reduced fibrinogen fraction I-2, 15 μg (lane 1); S-CM Aα chain, 5 μg (lane 2); S-CM γ chain, 5 μg (lane 3); reduced advanced plasmin digest of crosslinked fibrin, 200 μg (lane 4). Molecular mass markers (kDa) are indicated at the left.

components, as well as D dimer, remained resistant to further hydrolysis. Densitometric analyses indicated that the putative D trimer and D tetramer components represented 1.7% ± 0.9% and 1.5% ± 0.7%, respectively, of the D-containing fragments, whereas dimeric and monomeric forms of fragment D accounted for 85% ± 9% and 12% ± 4.8%, respectively. The same pattern was observed in plasmin digests of crosslinked fibrin in which factor XIIIa had been inactivated after crosslinking by treatment with *N*-ethylmaleimide or 4 M urea, and in a crosslinked fibrin digest prepared from fibrinogen containing ≈50% γ' chains (γ-1–427 Leu) (64, 65). In contrast to the plasmin resistance of the D dimer, D trimer, and D tetramer components in the Ca^{2+}-containing digest, at an advanced phase of an EDTA-containing digest, only monomeric forms of fragment D (≈80 kDa) and smaller digest components remained (data not shown).

Crossed immunoelectrophoresis of the intermediate Ca^{2+}-containing digest sample against anti-D antiserum (Fig. 2*A*) revealed a precipitin arc extending from the gel origin to the region of the E band. Distinct precipitin arcs against anti-E were observed only in the region corresponding to the DY band (arrow) and fragment E. Similarly, a precipitin arc against each of the D-containing fragments was clearly revealed in the advanced digest sample (Fig. 2*B*), whereas only the E fragment formed a precipitin arc against anti-E.

The peptides comprising these digest components were investigated by two-dimensional electrophoretic analysis of an advanced digest (Fig. 2*C*). All D-containing fragments yielded a 42-kDa Bβ-chain remnant, /β-134–461, and a 12-kDa Aα-chain remnant, /α/. D dimer yielded the expected γ dimer remnant, $(/\gamma)_2$ (78 kDa). This band was absent from D trimer and D tetramer and was replaced in them by bands having apparent molecular masses of 132 kDa (n = 9; range, 123–150 kDa), $(/\gamma)_3$, and 147 kDa (n = 9; range, 133–160 kDa), $(/\gamma)_4$, respectively.

Immunoblotting established that only peptides of γ-chain origin comprised the $(/\gamma)_3$ and $(/\gamma)_4$ band positions (Fig. 2*D*). Anti-γ-chain antibodies reacted with several components in a plasmin digest of crosslinked fibrin (lane 4), ranging from $(/\gamma)_4$ to derivatives the size of γ chains or smaller (i.e., $/\gamma\cdot//\gamma$ and $/\gamma/$). The anti-α-chain antibody reacted strongly with Aα chains (lanes 1 and 2) but not with γ chains, $(/\gamma)_2$, $(/\gamma)_3$, or $(/\gamma)_4$ chains (lanes 3 and 4). In addition, three weak reactions were observed in the plasmin digest sample (lane 4) in a band that was cathodal to the $(/\gamma)_2$ position (≈95 kDa), in a band migrating near the position of the intact γ chain (≈55 kDa), and in a band migrating near the tracking dye front. This finding, plus related studies (data not shown) in which we have identified bands larger than γ dimer reacting with both anti-α- and anti-γ-chain antibodies, suggests that small populations of crosslinked fibrin α and γ chains exist.

Amino acid sequence analysis was carried out on reduced components from a crosslinked fibrin digest. The $(/\gamma)_4$ (13 steps), $(/\gamma)_3$ (19 steps), and $(/\gamma)_2$ (20 steps) peptides yielded two amino acids in about equal amounts through the first 11 steps and at most steps thereafter. In each case, the pattern corresponded to γ-chain sequences beginning at γ-86 and γ-89, respectively. The /β-134–461 peptide yielded a single sequence (20 steps) corresponding exactly to that of the Bβ chain beginning at position 134.

TEM images of rotary shadowed fragment D_1 (Fig. 3*B*) revealed particulate forms having the same size and shape as the outer domains of fibrinogen molecules (Fig. 3*A*) from which they arise. D dimers (Fig. 3*C*) appeared as oblong structures about twice the length (≈20 nm) of D_1 fragments. Fractions enriched in D trimers and D tetramers (Fig. 3*D*) revealed unique structures reflecting the presence of these fragments. D trimer configurations were triangular (solid arrowheads in Fig. 3*D*; Fig. 3 *E–G*) or L-shaped (open arrowhead in Fig. 3*D*), suggesting that there were both open as well

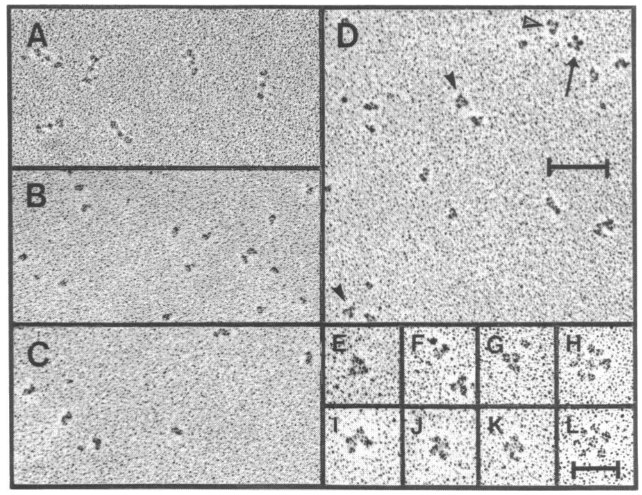

FIG. 3. TEM images of fibrinogen (*A*); fibrinogen fragment D_1 (*B*); D dimer (*C*); a D trimer/D tetramer-enriched preparation (*D*). (Bar = 100 nm.) A gallery is shown of trimeric forms (*E–G*), tetrameric forms (*I–K*), and aggregates (*H* and *L*). (Bar = 50 nm.)

as closed trimeric structures. Tetramers were composed of four nodular domains and tended to be square-shaped (Fig. 3 *J* and *K*), although they sometimes were composed of two dimeric components joined in only one place (arrow in Fig. 3*D*; Fig. 3*I*). Aggregates larger than trimeric and tetrameric structures were commonly seen (Fig. 3 *H* and *L*).

STEM mass analyses of a D trimer/D tetramer-enriched fraction (data not shown) indicated the presence of D fragments ranging from monomeric (≈100 kDa) to those with a mass >1300 kDa. The particle mass distribution fell into three major peaks between 150 and 475 kDa corresponding to D dimer (182 ± 22 kDa), D trimer (276 ± 25 kDa), and D tetramer (398 ± 37 kDa) and accounted for 63% of the total particles. Many trimeric and tetrameric forms had shapes corresponding to those identified in TEM images. About 25% of the objects had a mass between 500 and 1300 kDa, consistent with the aggregated structures observed in TEM images (Fig. 3); the remainder had masses <150 kDa. Mass analysis of D_1 and D dimer preparations showed a monotonic particle distribution clustering around 100 and 200 kDa, respectively. Only rarely (<5%) were larger particles found.

To identify matrix elements that might give rise to tri- and tetramolecular crosslinking arrangements, we analyzed critical point dried fine fibrin clots (Fig. 4), which consist of a branching network of thin twisting fibrils (66, 67). The narrowest of these are double-stranded (7–13) and have widths of 13.0 ± 2.0 nm (n = 38; range, 8.3–17.5 nm). Two types of trifunctional junctions were identified on the basis of fibril width measurements. The first is well known and consists of a four-stranded fibril 21.8 ± 2.4 nm wide (n = 39; range, 17–28 nm) that is formed from, or bifurcated into, two 13-nm thin fibrils (arrowheads)—the tetramolecular branch point. The second type, which we term the trimolecular branch point, has not previously been described, and is composed of three 13-nm thin fibrils (arrows). The molecular basis for this structure is considered further below.

DISCUSSION

The foregoing data indicate the existence of two heretofore unidentified fibrin degradation products, D trimer and D tetramer, derived from fibrin molecules crosslinked by trimeric and tetrameric γ chains, respectively. The evidence supporting this conclusion includes (*i*) NaDodSO$_4$/PAGE and crossed immunoelectrophoresis of digests of crosslinked fibrin demonstrated two plasmin-resistant D-containing core fragments of ≈300 and ≈365 kDa, respectively; (*ii*) two-

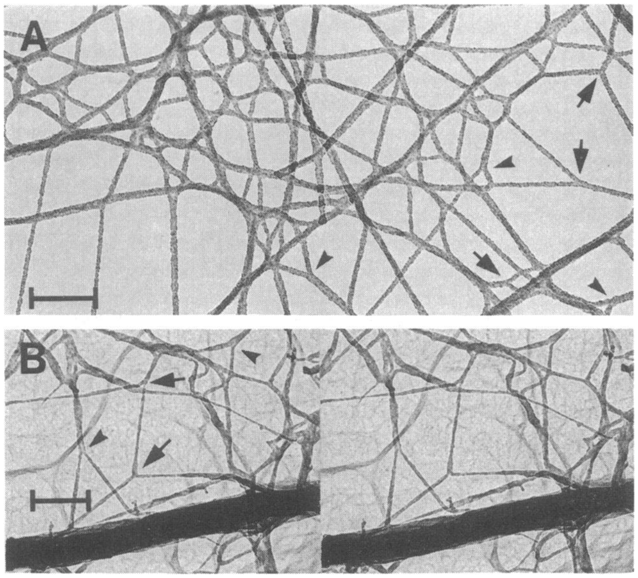

FIG. 4. TEM images of critical point dried fibrin specimens. (*A*) α,β-Fibrin. (*B*) Stereo images of β-fibrin. The axial twisting of these fibrils is often evident, particularly when viewed in stereo (*B*), and probably accounts for the observed intrafiber variations in fibril diameters. Trifunctional junctions at which all contributing branches are double-stranded (the trimolecular branch point) are indicated by arrows; those at which one of three branches is four-stranded (the tetramolecular branch point) are indicated by arrowheads. (Bar = 200 nm.)

dimensional gel electrophoresis plus immunoblots of reduced digest samples showed the presence of trimeric and tetrameric γ-chain remnants; (*iii*) amino acid sequencing of $(/\gamma)_3$ and $(/\gamma)_4$ remnants revealed only γ-chain sequences; (*iv*) TEM images and STEM mass analyses revealed structures corresponding in shape and size to trimeric and tetrameric forms of fragment D. Their existence is inconsistent with the assumption that every crosslinked degradation product larger than D dimer must contain one or more covalently linked E domains (36, 43–45) and raises the question as to whether certain E-containing digest components, especially those that are about the same size as D trimers or D tetramers (e.g., YY, 290 kDa; XY, 385 kDa), have been overestimated or, under certain conditions, misidentified.

The discovery of trimeric and tetrameric γ-chain remnants, and the corresponding forms of crosslinked D fragments, indicates that ε-(γ-Glu)Lys bridging of these chains differs from the reciprocal bridging pattern that characterizes D dimers (Fig. 1). Since there is only one donor lysine residue at γ-406 (25, 26), we assume that trimeric and tetrameric crosslinked structures form by utilization of that same residue. Four bond arrangements can account for these structures (Fig. 5). Closed tripartite or tetrapartite loops of ε-(γ-Glu)Lys bridges utilizing all γ-406 lysine donors would result in structures Ia or IIa, respectively. Trimers and tetramers would also result if only two of three (Ib) or three of four (IIb) γ-406 lysine donors were utilized, and would create an open arrangement, such as we have visualized (Fig. 3). It also seems possible that open trimeric or tetrameric structures utilizing all γ-406 lysine donors could result from bridging at two adjacent glutamine acceptors on one γ chain, but this is unlikely since concomitant utilization of adjacent glutamine residues in model substrates does not take place (68).

Given the staggered overlapping arrangement of fibrin units comprising a fibril and the imputed γ-trimer and γ-tetramer crosslinking arrangements, it appears that the process of concatenation of dimeric crosslinked junctions in the assembled fibrin fiber is consistent with crosswise (i.e., trans) positioning of ε-(γ-Glu)Lys bridges. In this arrangement, formation of interfibril crosslinked tetrameric structures permits trans bridges to stay in register throughout, as is the case for a crosslinked trimolecular branch structure. The trans orientation corresponds to the arrangement in assembled fibrin proposed by Selmayr *et al.* (69, 70). It is not in accord with the conclusion extrapolated from observations

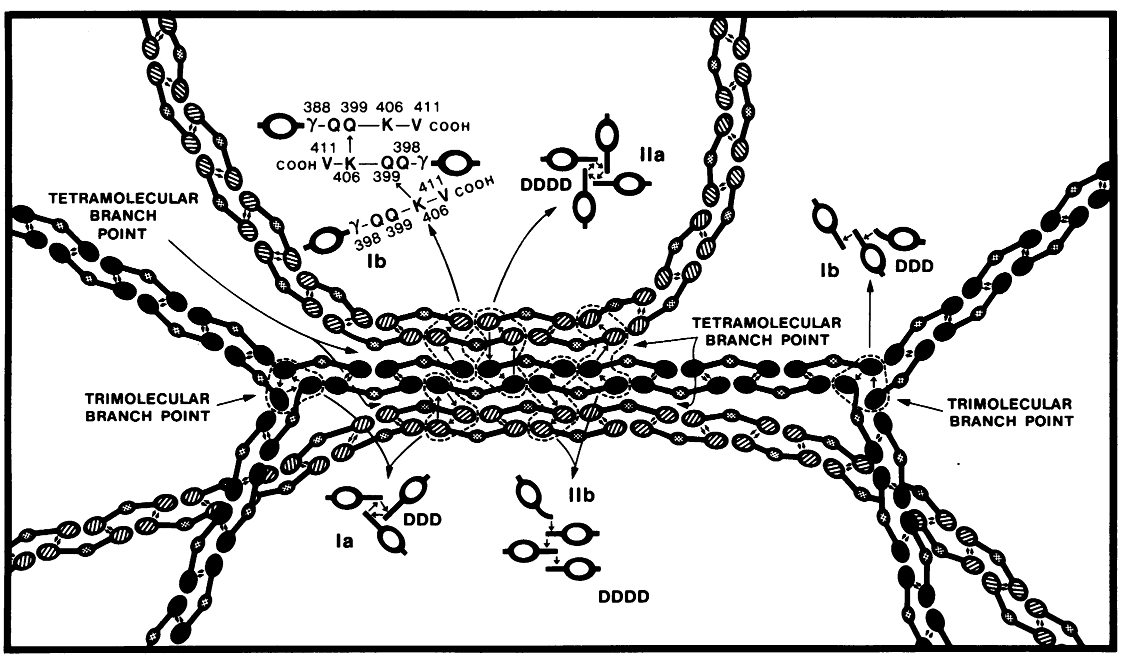

FIG. 5. Diagram showing the proposed spatial and crosslinking arrangements of fibrils forming trifunctional branch points. D domains from fibrils forming trimolecular branches are represented by solid ellipsoids; the others are hatched. The fibrils are drawn in a single plane for ease of presentation of ε-(γ-Glu)Lys bond positions, which are indicated by arrows between D domains. Trimolecular or tetramolecular γ-chain crosslinking among neighboring D domains can take place at branch points or through interfibril bridging (dashed lines) and also are illustrated in terms of the ε-(γ-Glu)Lys bond arrangements of multimeric D fragments that are generated from these structures. The lysine (K) donor and potential glutamine (Q) acceptor γ-chain residues on γ chains that participate in forming a type Ib crosslinked D trimer are shown in detail.

on crosslinked dimers of fibrinogen (71)—namely, that such bonds are oriented longitudinally within each strand of two-stranded fibrils in a so-called "DD long" contact (19–21, 71). The trans configuration appears likely to be a function of constraints imposed within assembled fibers by the complementary noncovalent associations between E and D domains that govern the assembly process (1–6). In the absence of these constraints (e.g., fibrinogen dimers), more extended bond orientations become possible and perhaps even likely.

By analysis of fibrin fibril widths, we have identified two different types of trifunctional branches. The first of these has been proposed as the sole basis for network branching (11, 18–21) and consists of a pair of double-stranded fibrils that become laterally associated to form a four-stranded fibril (Fig. 5)—the tetramolecular branch point. The second type, the trimolecular branch point, is composed of three double-stranded fibrils. This newly discovered structure evidently forms through occupancy of an E (or D) domain polymerization site within a bimolecular fibril by the corresponding complementary domain from an extraneous fibrin molecule (Fig. 5). Further interactions between D and E domains of other molecules serve to complete the structure. The presence of an additional fibrin molecule at such a junction makes it possible to form trimolecular γ-chain crosslinks without disturbing the symmetry or register of reciprocal trans-oriented dimeric bridges and suggests that at least some trimeric γ-chain structures form at trimolecular branch points, with the remainder originating at sites of lateral fiber associations. With respect to crosslinked tetramolecular structures, however, it cannot be deduced as yet whether any such structures originate at branch points.

We thank Ellen Feller-Goldfarb and Diane Bartley for technical support, Joyce Mitchell for graphics services, Joy Reeve for secretarial support, and William Semrad for photographic services. This project was supported by National Institutes of Health Program Project Grant HL-28444, National Institutes of Health Grant RR-01777 to the Brookhaven STEM National Biomedical Resource Facility, and National Institutes of Health Grant RR-03326 to the Protein–Nucleic Acid Shared Facility at the Medical College of Wisconsin.

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