Fibrinogen Assembly and Crosslinking on a Fibrin Fragment E Template

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Summary

There is an ongoing controversy concerning whether crosslinked γ chains in fibrin are oriented “transversely” between fibril strands or “end-to-end” along fibril strands. From the latter viewpoint, Veklich et al. [Proc Natl Acad Sci (USA) 95: 1438, 1998] observed that fibrinogen fibrils that had been assembled on a fibrin fragment E template, crosslinked with factor XIIIa, and then dissociated in acetic acid solution, were aligned end-to-end. This led to the conclusion that crosslinked γ chains in fibrin under physiological conditions were also aligned end-to-end. To assess its validity we studied the assembly and organization of fibrinogen molecules on a des AB-fibrin fragment E (E-des AB) or a des A-fibrin fragment E (E-des A) template.

We evaluated the roles of E polymerization sites E₆ and E₇, and D association sites γ₉₃, Da, Db, β₇, and αC in this process. E₆, Da interactions caused fibrinogen: E “DED” complexes to form, and markedly enhanced the γ chain crosslinking rates of fibrinogen or des αC-fibrinogen. Fibrinogen crosslinking without added fibrin E was slower, and that of des αC-fibrinogen was still slower. These events showed

that although αC domains promote fibrinogen fibril assembly and crosslinking, they contribute little to increasing the E₆, Da-dependent crosslinking rate. Electron microscopic (STEM) images of E-des AB and fibrinogen plus factor XIIIa showed single-, double-, and multi-stranded fibrils with interstrand DED complexes aligned side-to-side. This alignment was due to β₆:β₇ contacts resulting from D subdomain rearrangements initiated by the E₆, Db interactions, and also occurred in mixtures of des αC-fibrinogen with E-des AB.

In contrast, a mixture of fibrinogen and E-des A plus XIIIa revealed double-stranded fibrils with interstrand DED complexes in a half-staggered arrangement, an alignment that we attribute to crosslinking of γ₉₃ sites bridging between fibrils strands. These and other features of E-des A-based fibrinogen fibrils, including interstrand γ chain bridges and early and extensive lateral fibril strand associations concomitant with accelerated γ chain crosslinking, indicate that crosslinking of fibrin fibril strands takes place preferentially on transversely positioned γ chains.

Introduction

Thrombin-mediated cleavage of FPA from the central D domain of fibrinogen exposes a polymerization site, E₆ (1, 2, 3) which subsequently combines with a complementary site (Da) in the D domain of neighboring molecules (4, 5, 6) resulting in twisting double-stranded fibrin fibrils, with constituent units aligned in a staggered overlapping arrangement (7, 8, 9). Release of FPB by thrombin exposes another polymerization site, E₇ (10), that interacts with a Db site in the β chain segment of the D domain (Fig. 1) (6, 10, 11). The E₆, Db interaction, though not absolutely required for lateral fibril and fiber associations, nevertheless contributes to lateral association by inducing rearrangements in the β₂ region of the D domain that permit intermolecular β₆:β₇ contacts to occur (12). Another domain, termed ‘αC’, originates at α₉₃, not far from where it emerges from the D domain, and it terminates at α₉₃₉ₙ (13). Fibrin clots formed from fibrinogen molecules lacking 100 or more C-terminal αC domain residues (such as plasma fractions I-6 or I-9) (14, 15) display a prolonged thrombin time, develop reduced turbidity, and produce thinner fibers (16, 17, 18), the last observations suggesting that αC domains participate in lateral fibril associations (19). The Da:E₆₆₃₉₃ interaction greatly facilitates anti-parallel C-terminal alignment of intermolecular γ chain pairs, thereby accelerating the rate of XIIIa-mediated crosslinking (20, 21, 22). The location of these γ chain pairs within a crosslinked fibrin polymer has been controversial for more than twenty years. Evidence summarizing this situation was presented in 2000 at the XVI International Fibrinogen Workshop in Leiden and is now in print (23). In brief, we believe that crosslinked γ
chain pairs in assembled fibrin or fibrinogen polymers are positioned ‘transversely’ between D domains on opposing strands of a double-stranded fibrin or fibrinogen fibril as depicted in (1).

In this scheme each fibrinogen molecule is drawn as two white oval D domains connected to a circular black E domain. C-terminal γ chains protrude inwardly from each fibril strand and their interstrand covalent connections are drawn. The opposite view on the location of cross-linked γ chains involves the belief that crosslinked γ chain regions in fibrin are positioned outwardly, crossing the distal ends of linearly aligned molecules in an end-to-end arrangement as illustrated in (2).

The most recent experiments providing evidence for end-to-end crosslinking in fibrin came from electron microscopic images of fibrinogen fibrils that had been assembled as DED complexes on a fibrin fragment E template to form non-covalent DED complexes, and then crosslinked with factor XIIIa (24). Under physiological buffer conditions, single-stranded fibrils were commonly observed and lateral fibril associations were noted as well. In other experiments, crosslinked fibrinogen fibrils that had initially been incubated with fibrin fragment E under physiological conditions, and then dissociated with acetic acid, were examined. Owing to the residual covalent γ chain crosslinks, end-to-end aligned single-stranded fibrils were found. These observations led to the extended conclusion that end-to-end γ chain crosslinking must also exist in native fibrin fibrils. In a companion study, Lorand et al. (25) observed that XIIIa-mediated γ chain crosslinking of fibrinogen was increased in the presence of a bivalent GPRP ligand that mimicked the bilateral valency of the fibrin E fragment. From this result they also inferred that increased crosslinking had resulted from enhanced end-to-end γ chain alignment and crosslinking, no doubt relying upon the conclusions drawn by Veklich et al. (24).

Our present experiments on the assembly and crosslinking of fibrinogen on a fibrin fragment E template were conceived after we had read the Veklich et al. report, and accordingly we designed experi-

Fig. 1  The assembly and crosslinking of fibrinogen molecules on a fibrin fragment E template. The basis for the structural model of fibrinogen including its major domains, association sites and intermolecular interactions has recently been summarized (23). The recent contribution by Yang et al. based upon crystal structures (12). has clarified the role of the Eβ polymerization site in promoting rearrangement of the βC subdomain, and this information has been incorporated into our model. The preferential orientation of C-terminal regions of γ chains is toward the E domain (proximal), as shown, but this region is known to be flexible and can be found in more distal regions of the fibrinogen molecule (39). We have not attempted to draw the “end-to-end” crosslinking mechanism as an alternative, mainly because it does not fit well with our observations.
ments to address questions that had been raised by their study. We believe that our results provide new and relevant observations on the fibril assembly process, per se, as well as offering a sound basis for modeling fibril assembly, lateral fibril association, and factor XIII-mediated crosslinking.

Materials and Methods

Preparation of fibrinogen and factor XIII. Fibrinogen 1 and des α-C-fibrinogen: Human fibrinogen fraction I-2 was isolated from normal citrated plasma by glycine precipitation (16) and then separated into fibrinogens 1 and 2 by anion exchange chromatography (26). Des α-C-fibrinogen was produced from fibrinogen 1 by limited digestion with plasmin (15) followed by purification of the α-C-domainless fibrinogen component, which we now term des α-C-fibrinogen (aka, fraction I-9D). Each of these molecules lacked ~390 C-terminal Aα chain residues (27, 28). Factor XIII was purified from human plasma (29) and had a specific crosslinking activity of 2200 Loewy units/mg. The XIII level in normal human plasma ranges from 85 to 120 Loewy units/ml.

Preparation of hementin-derived fragment E, des A-fragment E, and des AB-fragment E. Anterior glands from the giant Amazon leech, Haementaria ghilianii were a most generous gift from Dr. Andrei Budzynski of Temple University, Philadelphia, PA. The hementin was obtained by extracting 35 mg dried material twice with cold 20 mM Hepes, 10 mM CaCl2, pH 7.8 buffer, and then centrifuged to remove particulate matter (31). The pooled extracts had an estimated concentration of 2.3 mg/ml (assumed A280, 10.0). Fibrinogen 1 (8.5 mg/ml) in Hepes buffer, pH 7.8 containing 0.02 % (w/v) sodium azide, was digested by adding 0.25 mg of the extract per 100 mg fibrinogen, and incubating at 37°C for 96 h. As assessed by SDS-PAGE of non-reduced samples on 8-25% Phastgles (Pharmacia Biotechnology, Piscataway, NJ), molecular weight fragments >120 kDa had been degraded by this time, and the enzymatic process was terminated by addition of EDTA (12 mM, final) plus O-phenanthroline (1 mM, final), which inhibits the enzyme. The specimen was then dialyzed against 5 mM PO4-Tris buffer, pH 8.4, heated at 56°C for 3 min to precipitate D-containing fragments, quickly cooled in an ice bath, and the precipitate removed by centrifugation. The fragment E-enriched supernatant solution was then subjected to ion exchange chromatography on a 2.5 × 20 cm DE-52 (Whatman) column using a programmable FPLC chromatography system (Pharmacia Biotechnology, Piscataway, NJ). Elution buffers were the same as previously described (26), and were: Buffer A, 5 mM PO4-Tris, pH 8.4; Buffer B, 500 mM PO4-Tris, pH 4.1. The DE-52 column was equilibrated with buffer A, and sample then applied in a 95% buffer A, 5% buffer B solution. Following initial wash-off, a 400 ml linear gradient from 5-50% buffer B was imposed. Following this, fragment E was eluted isocratically in 6 ml fractions with a 30% buffer A, 0% buffer B solution. E-containing fractions were pooled on the basis of SDS-PAGE analyses, “vacuum-centrifuged” in a Speed Vac device (Savant, Piscataway, NJ), and then dialyzed against PBS (0.1 M NaCl, 20 mM phosphate, pH 7.4). The final fragment E concentration was 3 mg/ml (A280, 10.2) (32). Confirmation of the intact E fragment fibrinopeptide content was obtained by treating aliquots with trypsin (27) or thrombin to release FPA or FPB, or both, and evaluating subunit composition by reduced SDS-PAGE on high-density Phastgles. The fragment E molecular weight was estimated to be ~70 kDa based upon its migration in unreduced SDS-PAGE Phastgles. The molecular weight calculated from its amino acid sequence and carbohydrate content (33, 34) was 74 kDa. From this value, the molecular weight of E-des A was calculated to be 71 kDa, and that of E-des AB, 69 kDa.

Crosslinking conditions for electrophoresis experiments. Factor XIII at a concentration of 1000 u/ml in 0.1 M NaCl, 20 mM Hepes, pH 7 buffer (HBS) was activated by adding human α-thrombin to a concentration of 5 u/ml and then incubating at 37°C for 30 min. Thrombin was then inactivated with a tenfold excess of hirudin (50 u/ml, final concentration). Fibrinogen assembly and crosslinking experiments were carried out in HBS containing 10 mM CaCl2 at a fibrinogen concentration of 1.5 mg/ml (4.4 μM) and E-des A or E-des AB at a ~10% molar excess with respect to fibrinogen. XIIIa was added last at a final concentration of 100 u/ml as timing was initiated. For electrophoretic analyses, reactions were terminated by adding an equal volume of 5% SDS, 10 mM Tris, 1 mM EDTA, 10% β-mercaptoethanol, pH 8 solution. Samples were then subjected to electrophoresis on 8 to 25% gradient gels in a Phast gel apparatus (Pharmacia/LKB). For densitometry, Coomassie blue-stained gels were scanned with an AlphaImager CCD camera and then analyzed with AlphaEase image processing and analysis software (Alpha Innotech Corp., San Leandro, CA). The degree of γ chain crosslinking was determined from the ratio of γ dimers to the total γ chain population, γD/γ. The degree of Aα chain crosslinking to form α polymers was determined from densitometric scans of the Aα chain position, using the Bβ chain band for normalization.

Specimen processing for STEM and mass measurements. Samples for STEM analyses were assembled under the same conditions described for electrophoresis experiments, up to the sample processing step. For STEM measurements, timed samples were rapidly diluted in HBS to a final concentration of 3 to 5 μg/ml, loaded onto ultrathin carbon films by injecting 3 μl into a 3 μl droplet of sample buffer on the grid surface, and allowing an attachment time of 1 min before exchanging the fluid on the grid surface with multiple wicking/washing with 150 mM ammonium acetate, pH 7. The grid was then snap frozen in degassed liquid nitrogen, freeze-dried, and transferred to the STEM microscope stage under vacuum. Specimens were imaged at the Brookhaven STEM Biotechnology Resource Facility by using a 40-kv probe focused at 0.25 nm, usually at a magnification of 125,000 (512 nm full scale width) at an electron dose of ~2.5 × 106 e⁻/nm², and stored digitally as a raster of 512 × 512 pixels. Background filtering of digitized STEM images was optimized for contrast and brightness offset by an image processing program (Adobe Photoshop). Mass measurements on STEM images were based on electron scattering measurements and were performed off-line as described (35, 36, 37).

Results

We used hementin-derived fibrinogen fragment E for fibrinogen assembly experiments. This fragment is intact as far as fibrinopeptide-containing sequences are concerned (Fig. 1), and this unique feature provided us with a useful experimental design advantage over using plasmin-derived E fragments such as were used by Veklich et al. (24), since it permitted us to precisely compare E fragments with both E des A and E des AB at a concentration of 100 u/ml as timing was initiated. For electrophoretic analyses, reactions were terminated by adding an equal volume of 5% SDS, 10 mM Tris, 1 mM EDTA, 10% β-mercaptoethanol, pH 8 solution. Samples were then subjected to electrophoresis on 8 to 25% gradient gels in a Phast gel apparatus (Pharmacia/LKB). For densitometry, Coomassie blue-stained gels were scanned with an AlphaImager CCD camera and then analyzed with AlphaEase image processing and analysis software (Alpha Innotech Corp., San Leandro, CA). The degree of γ chain crosslinking was determined from the ratio of γ dimers to the total γ chain population, γD/γ. The degree of Aα chain crosslinking to form α polymers was determined from densitometric scans of the Aα chain position, using the Bβ chain band for normalization.

Crosslinking of fibrinogen or des α-C-fibrinogen on a fibrin fragment E template. As assessed by SDS-PAGE analyses of γ chain dimer formation, XIIIa-containing mixtures of fibrinogen or des α-C-fibrinogen with E-des AB or E-des A became crosslinked much more rapidly than did fibrinogen alone, or des α-C-fibrinogen alone, which was even slower than fibrinogen (Fig. 2). The enhanced E-dependent fibril assembly and crosslinking rates were attributable to the presence of fibrin E fragments, but were nevertheless slower and less complete than that of fibrin, which became 100% dimerized within 5 min. In contrast to the fibrin crosslinking rate, the initial rapid crosslinking rates of fibrinogen-fragment E mixtures slowed and leveled off before reaching 80% completion. We believe that this effect is related to cotextual ‘fixation’ of fiber network architecture or to formation of single-strand-looped loops (cf., Fig. 3), or both, which effectively limit fibril-fibril and

* The results shown in Figure 2 were carried out as a single experimental set. Fibrinogen crosslinking experiments on fibrin E templates were repeated 4 to 6 times, and there were no statistically significant differences in the initial crosslinking rates among the various conditions.
strand-strand interactions. The relatively rapid initial crosslinking rate of fibrinogen compared with des αC-fibrinogen suggests that αC domains enhance lateral association of fibril strands, and thereby the γ chain crosslinking rate. Such observations fit well with other data indicating that fibrinogen αC domains participate in lateral fibrin fibril associations (16, 17, 18, 19), thus enhancing fibrinogen γ chain crosslinking (38).

The initial Aα chain crosslinking rate was about the same under all conditions (panel B) and always was less than that of the γ chains. In this respect, we did not observe the reversal in the sequence of initial chain crosslinking (i.e., Aα > γ) reported by Veklich et al. (24). However, in the “fibrinogen-alone” system, the level of Aα chain crosslinking eventually exceeded that of the γ chains.

We also compared the fibrinogen/E-des AB and the fibrinogen/E-des A crosslinking systems by SDS-PAGE analyses of timed samples on non-reduced as well as disulfide-reduced gels, with respect to their rates of γ chain crosslinking and the size of the crosslinked fibrinogen polymers (data not shown). The crosslinking rates and the sizes of the crosslinked fibrinogen polymers formed were indistinguishable from one another during the entire period of sampling and included rapid and progressive formation of very large crosslinked fibrinogen polymers. In other words, there were no unusual or unexpected γ chain pairings (e.g., “closed” crosslinked fibrinogen tetramers** in these systems other than the concatenary crosslinking arrangement that is expected to take place during assembly of crosslinked fibrinogen fibrils (20). This observation was useful for correctly modeling the γ chain crosslinking pattern in these systems (vide infra).

**This type of crosslinking arrangement might be expected to occur if γ chains became crosslinked directly across side-to-side oriented DED complexes, rather than in the expected concatenated pattern that is illustrated in figure 1 (3).
tin-derived fibrin E fragment that was non-covalently complexed with two fibrinogen D domains (DED), as diagrammed in Fig. 1. The molecular composition of DED was verified by STEM mass measurements under each condition, and indicated a mass of 271 ± 15 kDa (n, 31) for E-des AB-containing DED complexes (computed mass, 269 kDa), and 267 ± 22 kDa (n, 23) for E-des A-containing DED complexes (computed mass, 271 kDa). Double- or multi-stranded fibrils were found commonly (panel A-fields 1, 3, 4; panel B-fields 3, 4) and DED complexes between fibril strands were usually aligned side-to-side (brackets), thus retaining the major 45 nm periodicity conferred by the constituent fibrinogen molecules***. Side-to-side alignment of DED complexes is attributable to $E_{\gamma}:D_b$ interactions triggering $B_{\gamma}c$ subdomain rearrangements that in turn promote intermolecular contacts between $B_c$ domains [see Fig. 1, (2) or (3)]. The putative $B_c:B_c$ contacts were often identifiable as proteinaceous or filamentous bridges at the proximal ends of DED complexes (“$B_c:B_c$”). Filamentous bridges between the middle portions of DED complexes most likely reflect $\gamma$ chain interactions (“$\gamma-\gamma$”)**.*

Images of crosslinking fibrinogen/E-des A mixtures revealed relatively fewer single-stranded fibrils (Fig. 4, panels A and B) compared to the fibrinogen/E-des AB system, but in their place were double-stranded fibrils (some marked “DS”), multi-stranded fibrils or fibrillar aggregates (Panel A-fields 1, 2; panel B-field 4). Fibrillar structures seemed to be better organized in the 10 min sample (panel B) compared to the 5 min sample (panel A), consistent with the degree of crosslinking that had taken place. Filaments bridging between strands of these fibrils were commonly observed and in this crosslinking system reflected inter-strand $\gamma$ chain bridging (“$\gamma-\gamma$” and unlabeled arrows). The inter-strand alignment of laterally associated E-des A-based fibrils differed markedly from that observed in the E-des AB system, in that DED complexes in double-stranded fibrils were in a half-staggered arrangement, yielding a major period of 22.5 nm as diagrammed in Figs. 1, (5) or (6). Our interpretation of the half-staggered double-stranded arrangement is when only $E_{\alpha}$ sites are available on E-des A for interaction with $D_{\alpha}$ sites, fibrinogen $X_{\alpha\alpha}$ sites bridge between fibril strands and preferentially orient opposing strands in a half-staggered arrangement. These interacting sites become crosslinked by factor XIIIa as depicted in Fig. 1 (6). The same intermolecular arrangement and 22.5 nm periodicity occurs with fibrinogen fibrils (20).

STEM images of crosslinking des $\alpha\alpha$-fibrinogen/E-des AB mixtures with factor XIIIa revealed single-stranded fibrils ("SS") that were indistinguishable from the single-stranded structures formed with intact fibrinogen (Fig. 5, panel A). Double-stranded polymers tended to be shorter and less-well organized than those formed from mixtures containing intact fibrinogen, and often formed difficult-to-interpret aggregates (panel A, fields 1, 3). Nevertheless, DED complexes comprising these fibrils were often associated side-to-side (brackets).

To assess the contribution of factor XIIIa-mediated crosslinking to fibrinogen fibril assembly, we studied fibrin E-fibrinogen mixtures without added factor XIIIa (Fig. 5, panel B; Fig. 6). Images of fibrinogen/E-des AB mixtures revealed the same DED-based fibrillar structures as had been found in the presence of XIIIa (Fig. 6, panel A). Single-stranded fibrinogen fibrils (SS) were found commonly, as well as poorly defined aggregates (arrowheads) and multi-stranded fibrillar aggregates (double arrowhead, field 2). Double-stranded structures, per se, were uncommon, suggesting that $\gamma$ chain crosslinking by XIIIa (cf., Fig. 3) helps to “maintain” double-strandedness over multi-stranded fibrils or aggregates. Nevertheless, there were recognizable side-to-side DED alignments (brackets). Occasional filamentous structures (“$\gamma-\gamma$”) bridging between DED domains were observed, and probably represented...
interacting γ chains. Unassociated fibrinogen molecules and E fragments were also found, as in the other systems.

Images of fibrinogen/E-des A mixtures without XIIIa (Fig. 6, panel B) formed DED-based structures that tended to be shorter than in other assembly systems (e.g., panel B, field 2), plus numerous ill-defined aggregates (arrowheads) and occasional filamentous bridges between DED complexes (“γ-γ”). Double-stranded fibrils, per se, in these mixtures were not well delineated, suggesting that γ$_{XL}$ site crosslinking plays a prominent role in forming double-stranded fibrils in this system.

Des αC-fibrinogen-E-des AB mixtures lacking XIIIa (Fig. 5, panel B), formed similar structures to those observed in the crosslinking mixture (Fig. 5, panel A) including single-stranded DED-based fibrils plus loosely organized and ill-defined aggregates (arrowheads). Side-to-side oriented DED complexes were difficult to find. This suggests that although DED assembly involving des αC-fibrinogen takes place readily on the fibrin E template, αC domains play a constructive role in orienting the fibril strands, even when E$_B$ sites are available to promote lateral association.
Discussion

Our studies provide new observations on the assembly and crosslinking of fibrinogen molecules on a fibrin E template, including information on the roles played by E\(_A\) and E\(_B\) sites, \(\gamma\) chains, \(\alpha\)C domains, \(\beta\) domains, and factor XIIIa in this process. The overall results provide strong evidence for “transverse” \(\gamma\) chain positioning and crosslinking between fibrinogen fibril strands, adding to an existing body of experiments that seem to us to be persuasive in their own right (23). The present observations and considerations that contribute to this conclusion are the following: 1) Fibrin fragment E is required for enhancing XIIIa-mediated crosslinking of fibrinogen or des \(\alpha\)- fibrinogen, and \(\gamma\) chain crosslinking is increased to virtually the same extent in either case, thus indicating that \(\alpha\)C domains, per se, contribute little to this process. However, since crosslinking of des \(\alpha\)-fibrinogen alone is much slower than that of fibrinogen itself, \(\alpha\)C domains do indeed promote fibrinogen fibril assembly and crosslinking, thus confirming observations made in previous studies on fibrinogen crosslinking (20, 38). 2) Fibrinogen or des \(\alpha\)-fibrinogen assembly and crosslinking on a fibrin E template is accompanied by accelerated crosslinking and concomitant lateral strand and fibril associations. Even without benefit of further details, this type of event favors transverse \(\gamma\) chain crosslinking, though not to the exclusion of at least some degree of end-to-end crosslinking. 3) The arrangement of laterally associated fibril strands depends upon whether E\(_B\) sites are available in addition to E\(_A\) sites, and when both sites are available, opposing DED complexes become oriented side-to-side. This is evidently due to \(\beta\)-\(\beta\)-contacts that are promoted as a result of E\(_D\):Db interactions (12). In this system, filamentous bridging across fibril strands is attributable either to \(\beta\)-\(\beta\)-contacts or to bridging by \(\gamma\) chain \(\gamma\)\(_{XL}\) sites, and a morphological distinction between them can often be made. Bridges due to \(\beta\)-\(\beta\)-contacts are situated in the more N-terminal regions (i.e., proximal) of side-to-side oriented DED complexes, whereas filamentous bridging in the middle portion of side-to-side oriented DED complexes is most likely attributable to \(\gamma\) chain bridging. The fact that in this system there exist two contact mechanisms for forming laterally associated fibril strands, seems to be consistent with either an end-to-end or transverse crosslinking mechanism. 4) \(\gamma\)\(_{XL}\) sites comprise an independent self-association and singular crosslinking site that contributes importantly to the lateral strand association and half-staggered alignment of crosslinked fibrinogen fibril strands (20, 39). By that same token, the half-staggered alignment of fibril strands that we observed in the fibrinogen/E-des A system almost certainly results from “transverse” interstrand \(\gamma\)\(_{XL}\) interactions and crosslinking. Filamentous connections that bridge between the DED complexes of fibril strands in this system provide additional evidence for the location of \(\gamma\)\(_{XL}\) sites between fibril strands. Taken together, these findings constitute a persuasive argument for transverse positioning of \(\gamma\)\(_{XL}\) sites between fibrinogen fibril strands.

Our conclusions regarding the location of crosslinked \(\gamma\) chains in fibrinogen fibrils are distinctly different from those drawn by our colleagues in Pennsylvania (24). We think it is valuable to examine the details behind their argument that crosslinked \(\gamma\) chains in fibrin are situated in end-to-end positions along fibril strands. This interpretation was mainly based upon the appearance of factor XIIIa-crosslinked fibrinogen fibrils that had been dissociated in solutions containing 30% glycerol under non-physiological conditions, i.e., in acetic acid solutions. The fibrinogen molecules in these single-stranded fibrils were aligned end-to-end, as were the crosslinked \(\gamma\) chains that connected them. Although this much is indisputable, the further extrapolation that the alignment in acetic acid solutions predicts the arrangement in native fibrin fibrils is inconsistent with existing data (22). For one example, in related studies on the structure of crosslinked D-fibrin-D complexes we showed that under physiological conditions, crosslinked \(\gamma\) chains in D-fibrin-D complexes were transversely oriented with respect to the arrangement of D domains on the fibrin template (41). This folded arrangement had resulted from non-covalent Da:E\(_A\) interactions. However, in the presence of acetic acid, D-fibrin-D complexes became unfolded, and the covalently linked D strands came to reside at the ends of fibrin molecules in end-to-end alignment with respect to their crosslinked \(\gamma\) chains.

Secondly, in our current experiments we’ve shown that DED-driven assembly and crosslinking of fibrinogen fibrils formed on E-des AB or E-des A is always accompanied by extensive lateral strand and fibril associations. Since DED assembly takes place without crosslinking (Figs. 5 and 6), the mere demonstration that fibrinogen molecules become assembled on a fibrin E template, cannot alone predict where the \(\gamma\) chains are situated. In our view, a valid argument for end-to-end crosslinking of \(\gamma\) chains in the fibrin fragment E assembly system under physiological buffer conditions would require that all DED-based fibrils remain single-stranded throughout the period of factor XIIIa crosslinking. Simply stated, this does not occur.

In this connection, it is worth noting that Veklich et al. reported a tendency for lateral fibril aggregation, but they apparently did not apply this observation in rendering their interpretations. Since they sampled their mixtures for microscopy well after \(\gamma\) chain crosslinking had been completed (12 to 24 h), we speculate that they would not have observed the extensive lateral fibril association and aggregation that is evident at much earlier times. At their late sampling time, the crosslinking mixture would probably have become largely depleted of observable multi-stranded fibrils and aggregates due to extensive self-association or precipitation, or both.

In summary, when E\(_B\) and E\(_A\) sites are available in the fibrin E fragment (E-des AB), DED complexes in fibrinogen fibril strands become aligned side-to-side. This observation is consistent with transverse crosslinking, but does not exclude end-to-end crosslinking. However, when only E\(_A\) sites are available in fibrin fragment E (E-des A), DED complexes become aligned in a staggered overlapping arrangement owing to preferential orientation by \(\gamma\)\(_{XL}\) sites [Fig. 1, (5), (6)]. In this case, only transverse \(\gamma\) chain crosslinking can account for the formation of such structures. We further believe that it is valid to extend this logic as a general model for fibrin fibril \(\gamma\) chain crosslinking.

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References

5. Pratt KP, Côté HCF, Chung DW, Stenkamp RE, Davie EW. The primary fibrin polymerization pocket: three-dimensional structure of a 30-kDa C-terminal γ chain fragment complexed with the peptide gly-pro-arg-pro. Proc Natl Acad Sci USA 1997; 94: 7176-81.


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