Covalent structure of single-stranded fibrin oligomers cross-linked by FXIIIa

Mark A. Rosenfeld[*], Vera B. Leonova, Alexander N. Shchegolikhin, Anna V. Bychkova, Elizaveta A. Kostanova, Marina I. Biryukova

N. M. Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, 4, Kosygina Str., 119334 Moscow, Russia

ABSTRACT

FXIIIa-mediated isopeptide $\gamma-\gamma$ bonds are produced between γ polypeptide chains of adjacent monomeric fibrin. Despite the use of the different methodological approaches there are apparently conflicting ideas regarding the orientation of $\gamma-\gamma$ bonds. To identify the orientation of these bonds a novel approach has been applied. It was based on self-assembly of soluble cross-linked fibrin protofibrils ongoing in the urea solution of moderate concentrations followed by dissociation of protofibrils in the conditions of increasing urea concentration. The oligomers were composed of monomeric desA fibrin molecules created by cleavage of the fibrinopeptides A from fibrinogen molecules with thrombin-like enzyme, reptilase. The results of elastic and dynamic light scattering coupled with analytical ultracentrifugation indicated an emergence of the double-stranded rod-like fibrin protofibrils. For the first time, the protofibrils are proved to exhibit an ability to dissociate under increasing urea concentration to yield single-stranded structures. Since no accumulation of α polymers has been found the covalent structure of soluble single-stranded fibrin oligomers is entirely brought about by $\gamma-\gamma$ bonds. The results of this study provide an extra evidence to support the model of the longitudinal $\gamma-\gamma$ bonds that form between the γ chains end-to-end within the same strand of a protofibril.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

Under the action of thrombin on fibrinogen the fibrinopeptides A and B are removed from NH_2-termini of the polypeptide Aα and Bβ chains creating exposure of the binding sites, called knobs 'A' and 'B', in the E region of monomeric fibrin molecules (monomeric desAB fibrin). Reptilase, thrombin-like enzyme, is capable of cleaving only fibrinopeptides A to convert fibrinogen into monomeric desA fibrin. The sites knobs 'A' and 'B' are complementary to initially open the sites holes 'a' and 'b' that are localized within the COOH-terminal parts of the γ and β polypeptide chains of the D region [1]. The knobs 'A'-holes 'a' interactions are the driving force of fibrin polymerization [2,3] while the knobs 'B'-holes 'b' interaction is mainly responsible for increasing the lateral association of protofibrils [4]. Fibrin assembly commences with the formation of structurally rigid rod-like double-stranded twisting protofibrils [5,6] in which monomeric molecules are arranged in a staggered,

overlapping manner. The end-to-end alignment of monomers in each protofibril strand abuts the γ chains of two adjacent molecules to form the D:D interface. Lateral protofibril association gives rise to formation of fibrils and fibers, which subsequently branch and represent a basis for three-dimensional structure of fibrin scaffold.

The activated plasma fibrin-stabilizing factor (FXIIIa) exhibits a capability to yield cross-links between both the γ and α chains of fibrin resulting in emergence of $\gamma-\gamma$ dimers and α polymers [7–9]. Despite the use of the different methodological approaches there have been fundamental disagreements regarding the geometry of the carboxyl-terminal regions of cross-linked isopeptide $\gamma-\gamma$ dimers in double-stranded protofibrils. One group of researchers adheres to the longitudinal orientation of isopeptide $\gamma-\gamma$ bonds formed between the γ chains of two monomeric fibrin molecules contacting end-to-end within the same strand of a protofibril [10–13], whereas another supposes the transverse orientation of these bonds formed between the γ chains belonging to monomeric fibrin molecules from the different strands of a protofibril, i.e. located side-to-side [14–17]. To distinguish between these two models and to clarify the orientation of $\gamma-\gamma$ bonds we used property of FXIIIa to retain enzymatic activity in the medium of urea

* Corresponding author. Fax: +7 4991374101.
  E-mail address: markrosenfeld@rambler.ru (M.A. Rosenfeld).

with moderate concentration [18]. In the aforementioned article the self-assembly of soluble double-stranded unlinked and cross-linked desA fibrin oligomers nascent from monomeric desA fibrin has been thoroughly studied in the environment of urea with moderate concentrations. Unfortunately, our efforts in trying to divide double-stranded protofibrils into single-stranded fibrin oligomers by increasing urea concentration up to 1.80 M have failed. That study has not provided any information about the geometry of γ−γ bonds. The present study is a continuation and development of the previous investigation. Since the dissociation of double-stranded cross-linked fibrin protofibrils into single-stranded oligomers could be a strong argument testifying to the longitudinal orientation of γ−γ bonds we undertake a new attempt for separating double-stranded protofibrils by means of significantly altering conditions of the experiments.

2. Material and methods

Human blood plasma was obtained from the Moscow Central Station of Blood Transfusion. Fibrinogen, α-thrombin (EC 3.4.21.5), p-nitroanilide-H-D-phenylalanyl-pipecolyl-arginine (S-2238), 1,4-aminophenylmercuric acetate were purchased from Sigma-−Aldrich (USA). Hirudin and reptilase were obtained from MP Biomedicals (USA). Affi-Gel 15 was purchased from Bio-Rad (USA). DEAE-ToyoPearl M650 was purchased from Tosoh Bioscience (Germany). Chemicals for SDS/PAGE were provided by Pharmacia (Uppsala, Sweden). All solutions were prepared with Milli-Q water.

2.1. Isolation of pFXIII

pFXIII (EC 2.3.2.13) was obtained from human blood plasma by ion-exchange chromatography on DEAE-ToyPearl M650 as described earlier [19]. Further purification of the protein was performed by affinity chromatography [20] using a mercury-containing sorbent, Affi-Gel 15 with 1,4-aminophenylmercuric acetate as the reactive group. pFXIII was activated with human α-thrombin (EC 3.4.21.5) [18]. To inactivate the enzyme activity of thrombin, hirudin was added to thrombin-activated FXIIIa prior to incubation with fibrin monomer as reported elsewhere [21]. A full thrombin inactivation was tested with the chromogenic substrate S-2238 [22].

2.2. The procedure of obtaining soluble fibrin oligomers

Monomeric desA fibrin dissolved at 1.60 M urea solution was prepared with reptilase using a method described in detail earlier [21]. Self-assembly of soluble cross-linked desA fibrin oligomers was initiated by lowering the urea concentration from the initial value of 1.60 M to 1.45 and 1.25 M levels followed by adding 10 μl (6.4 U) of FXIIIa solution. The final concentration of monomeric fibrin molecules in all samples was equal to 0.50 mg/ml. The duration of cross-linking reaction for all samples was 12 h. The dissociation capability of desA fibrin oligomers prepared at 1.25 M urea was evaluated after increasing urea concentration up to 4.20 M by adding 5.50 M urea solution. Concentration of 1.25 M urea was taken because the largest amount of cross-linked fibrin oligomers is generated in these conditions as compared to those formed at 1.60 and 1.45 M urea.

2.3. PAGE of the fibrin polypeptide chains

The enzymatic covalent cross-linking of desA fibrin was stopped by using 7 M urea and 2% sodium dodecyl sulfate (SDS) solutions. The existence of the cross-linked polypeptide chains formed under the action of FXIIIa was confirmed by electrophoresis (SDS-PAGE) of reduced samples on 7.5% SDS-polyacrylamide gel in the presence of 1% β-mercaptoethanol. The gels were stained by Coomassie brilliant blue R-250. Densitometric scanning of Coomassie blue-stained gels was carried out on a densitometer HYRYS-2 (Sebia, France).

2.4. Optical absorbance measurements

The concentrations of fibrinogen and of soluble fibrin oligomers in the samples were measured spectrophotometrically on SF-2000 (Russia) spectrophotometer. The mass extinction coefficient for the fibrinogen in 0.05 M Tris−HCl/0.15 M NaCl was taken to be 1.56 at 280 nm [21]. The mass extinction coefficient values for soluble fibrin oligomers and for fibrinogen molecules were assumed to be the same at equal urea concentrations.

2.5. Elastic light scattering measurements

The weight-average molecular weight, M_w, and form-factor, $P(\theta)$, of fibrin oligomers were evaluated by elastic light scattering using a Malvern spectrometer (England) as described in detail previously [21]. The refractive index increment of fibrinogen was determined using a KMX-16 differential refractometer (Chromatix, USA). The dn/dc values for the soluble fibrin oligomers were considered to be equal to those of fibrinogen molecules at the same urea concentration [21]. Before measurements, all protein solutions were carefully purified by ultrafiltration.

2.6. Dynamic light scattering measurements

The translational diffusion coefficient values, D_z, for the different samples of fibrin oligomers were measured with aid of dynamic light scattering (DLS) on Zetasizer Nano-S instrument (Malvern, England) [18]. All protein solutions, as in the case of elastic light scattering measurements, were dust-free. The measurement was divided into 30 runs. In accordance with the software, the runs that contained the poorest data were automatically rejected while the remaining runs were analyzed and used in the final measurement calculation.

2.7. Sedimentation velocity measurements

Sedimentation velocity analytical ultracentrifugation studies were carried out using a Beckman model E analytical centrifuge (Austria) equipped with an optical system, photoelectric scanner, monochromator and a computer for collecting data on-line [18]. The samples of fibrin solutions (400 μl) with various urea concentrations in 0.05 M Tris/0.15 M NaCl buffer in the presence of 5 mM $CaCl_2$ were loaded into the 12 mm optical pathlength double-sector cells and placed into the An-F Ti rotor. The experiments were performed at the rotor rotation rate of 48,000 rpm at 20 °C. To avoid possible errors in the comparable assessment of the macromolecular size-and-shape distributions by sedimentation velocity, all test samples were subjected to ultracentrifugation contemporaneously in one run. Concentration profiles and the movement of the sedimenting boundary in the cells were recorded by optical density measured at 280 nm. The obtained data were analyzed by continuous size distribution analysis using SEDFIT version 14.1 software [23] Sedimentation coefficients $s_{20,w}$ were corrected for the viscosity and density of the solvent, relative to that of water at 20 °C.

2.8. Statistical analysis

The measurements were done in triplicate. The statistical significance of differences between means was evaluated using Student's t-test.

3. Results

3.1. Enzymatic cross-linking of the soluble fibrin oligomers

In the presence of FXIIIa, the minor amount of γ–γ dimers is formed in soluble desA fibrin oligomers at 1.60 M urea (Fig. 1). The involvement of γ chains of desA fibrin oligomers into cross-linking is facilitated at lower urea concentration with γ–γ dimers accumulating at higher rates (Fig. 1, 3–4). However, the electrophoresis data reveal no appearance of α polymers in the samples.

3.2. Self-assembly and dissociation of the cross-linked desA fibrin oligomers

3.2.1. The distribution of the fibrin molecules by their hydrodynamic properties

Lowering the urea concentration down initiates the self-assembly of cross-linked desA fibrin oligomers. The analytical ultracentrifugation data demonstrate a bimodal distribution of the molecules by their sedimentation velocity throughout the range of urea concentrations (1.60–1.25 M) (Fig. 2). The slowly sedimenting fraction corresponds to monomeric desA fibrin, the amount of which diminished with lowering the urea concentration. The rapidly sedimenting fraction represents cross-linked high-molecular-weight products, the content of which increases at lowering the urea concentration. The dynamic light scattering method has also revealed the bimodal distribution of the molecules by translational diffusion coefficients (hystogramms are not shown).

The analytical ultracentrifugation data indicates that the bimodal distribution of macromolecules by sedimentation coefficients (as well as by translational diffusion coefficients) is retained upon raising urea concentration up to 4.20 M. Along with the slowly sedimenting fraction that still indicates the existence of monomeric desA fibrin, the rapidly sedimenting fraction differs from both monomeric desA fibrin and double-stranded oligomers (Fig. 2). The values of the sedimentation and translational diffusion coefficients for the cross-linked oligomers amounting to 11.55 ± 0.54 S and $(0.82 \pm 0.05) \cdot 10^{-7}$ cm^2/s are strongly different from those obtained for double-stranded protofibrils, testifying to the appearance of a new structure of fibrin oligomers. It is reasonable to suppose that the newly formed structures emerge due to dissociation of double-stranded protofibrils.

3.2.2. The elastic light scattering: double- and single-stranded cross-linked oligomers

The angular dependences of the elastic light scattering intensity for the different fibrin samples are shown in Fig. 3. Diminishing
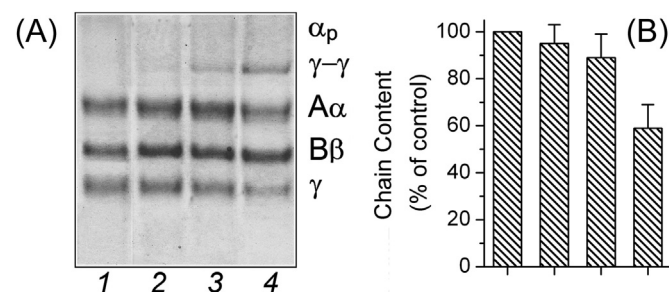
Fig. 2. Normalized sedimentation coefficient distribution c(s) for the different fibrin samples at urea concentrations: 1.60 M (1); 1.45 (2); 1.25 M (3) and 4.20 M (4).

urea concentration from 1.60 up to 1.25 M has led to an increase both in the light scattering intensity and the values of M_w determined from the intercept at zero angle of light scattering (Fig. 3).

As it has been shown earlier [18] the set of the analytical ultracentrifugation and elastic light scattering data enables one to characterize the molecular weight values and spatial organization of the cross-linked oligomers which are responsible for the rapidly sedimenting fraction. Since the weight-average molecular weight M_w of polydisperse ensemble of desA fibrin molecules is expressed

Fig. 1. PAGE of the polypeptide chains for the different samples of desA fibrin oligomers (A): monomeric desA fibrin (not-containing FXIIIa) in 1.60 M (1); desA fibrin oligomers cross-linked under the action of FXIIIa at urea concentrations of 1.60 M (2); 1.45 (3); and 1.25 M (4); Densitometric analysis of the γ chain content (B). Content of the γ chains in the sample 1 is taken as 100%.
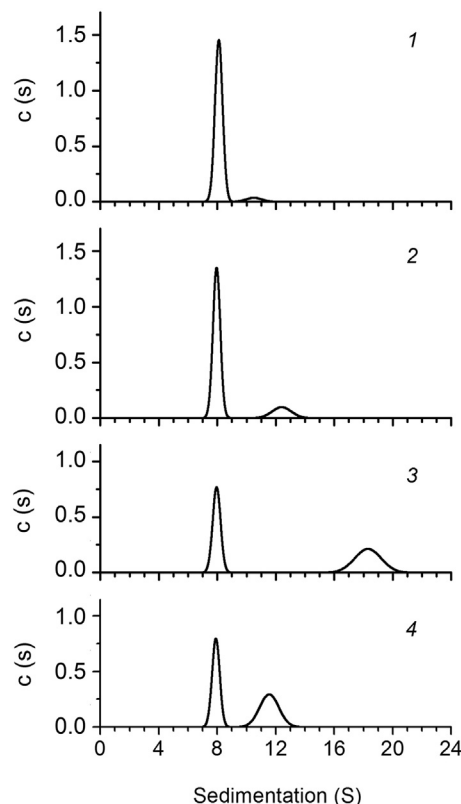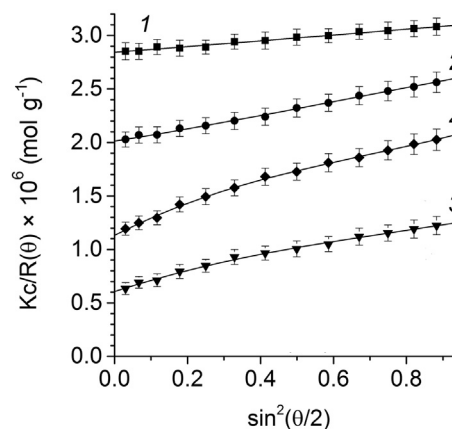
Fig. 3. The Zimm plots of the light scattering intensity for the different samples of desA fibrin molecules at the urea concentrations: 1 (3.60 ± 0.27; 8.35 ± 2.33) − 1.60 M; 2 (5.00 ± 0.72; 13.77 ± 2.12) − 1.45; 3 (17.50 ± 2.14; 32.85 ± 2.96) − 1.25 M; and 4 (9.47 ± 0.86; 15.70 ± 1.42) − 4.20 M. The M_w values calculated from the plots and the $M_{w,o}$ values for cross-linked oligomers, respectively are indicated in parentheses, in 10^5 g/mol. The additional explanations are given in the text.

as $M_w = M_m w_m + M_{w,o} w_o$, where M_m and $M_{w,o}$ are the values of the weight-average molecular weights for monomeric desA fibrin and its oligomers, w_m and w_o correspond to weight contributions of slowly and rapidly sedimenting fractions, it is possible to calculate the $M_{w,o}$ values of the cross-linked fibrin oligomers. The $M_{w,o}$ values grow with decreasing urea concentration reaching a maximum value equal to $(32.85 \pm 2.96) \cdot 10^5$ Da at 1.25 M urea. However, in elevating the urea concentration up to 4.20 M the $M_{w,o}$ value of cross-linked oligomers becomes equal to $(15.70 \pm 1.42) \cdot 10^5$ Da. Twofold falling down the $M_{w,o}$ value in comparison with that for double-stranded cross-linked protofibrils clearly demonstrates the fact that double-stranded cross-linked oligomers dissociate into single-stranded oligomers.

To confirm this fundamental result the experimental light scattering data were also assessed in the framework of the Casassa approximation [24]. The weight-average molecular weight per unit weight-average length, M_w/L_w, of the ensembles of rather long rod-like (or worm-like) macromolecules can be derived. The Casassa method is applicable in a range of angles and particle sizes provided the condition of qL > 3.8 [25], where q is the scattering wavevector being equal to $q = (4\pi n/\lambda)\sin(\theta/2)$. The experimental Casassa plots show a remarkable degree of linearity at high scattering angles for the samples obtained in 1.45, 1.25 and 4.20 M urea (Fig. 4). However, linearity is not maintained at small scattering angles due to significant amount of monomeric desA fibrin in the samples, as it follows from the hydrodynamic data (Fig. 2). Since $M_w/L_w = (M_m/L_m)w_m + (M_{w,o}/L_{w,o})w_o$, (where M_m/L_m and $M_{w,o}/L_{w,o}$ correspond to mass-length ratio for monomer and oligomer fractions, w_m and w_o are their weight contributions respectively), the value $M_{w,o}/L_{w,o}$ may be estimated. The resulting experimental data demonstrate that at both 1.45 and 1.25 M urea the $M_{w,o}/L_{w,o}$ values for cross-linked desA fibrin oligomers were equal to on the average $(1.27 \pm 0.24) \cdot 10^{11}$ g/(mol·cm) while the value of M_m/L_m for fibrin monomer molecules is known to be within $(0.64 \pm 0.04) \cdot 10^{11}$ g/(mol·cm) [25]. Accordingly, the $M_{w,o}/L_{w,o}$ value determined for cross-linked structures of desA fibrin oligomers supports formation of the structure for classic double-stranded fibrin protofibrils. It is in close agreement with the results published earlier [18]. At the same time, the $M_{w,o}/L_{w,o}$ value for the new fibrin structure formed in 4.20 M urea is equal to $(0.68 \pm 0.08) \cdot 10^{11}$ g/(mol·cm). By comparing this value with that for the cross-linked oligomers initially formed at 1.25 M urea it is possible to come to the important conclusion that double-stranded cross-linked fibrin protofibrils transform into the single-stranded structure.
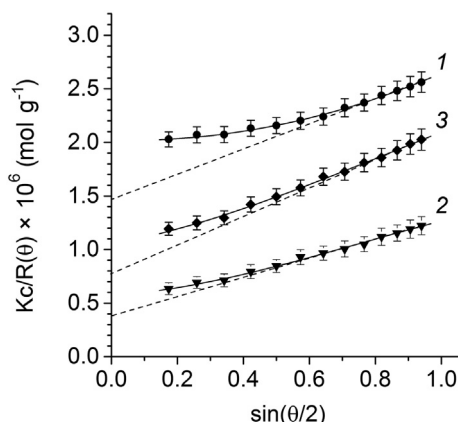

Fig. 4. The Casassa plots obtained for the samples of desA fibrin molecules at the different urea concentrations: *1* (0.74 ± 0.06) − 1.45 M; *2* (0.93 ± 0.06) − 1.25 M; *3* (0.63 ± 0.07) − 4.20 M. Values of M_w/L_w, in 10^{11} g/(mol·cm), are indicated in parentheses.

4. Discussion

The studies carried out clearly show that in the conditions of rising urea concentration up to 4.20 M cross-linked double-stranded desA protofibrils undergo the dissociation followed by the emergence of single-stranded fibrin oligomers. It is well known that the formation of unlinked double-stranded protofibrils nascent from monomeric desA fibrin molecules mainly proceeds through the non-covalent interaction between the D and E regions by means of the precise recognition of 'A' knobs and the complementary sites, holes 'a' [2,3]. Besides, an intermolecular interaction of the D regions of fibrin molecules contacting end-to-end occurs in the every strand of double-stranded protofibril [26]. It is important to note that at 1.60 M and higher the urea concentration, unlinked double-stranded protofibrils completely dissociate into monomeric desA fibrin [18]. The molecular mechanism of the dissociation seems to be obvious. The monomeric desA fibrin located at the ends of the strands are removed one after another due to the disruption of the non-covalent bonds, i.e. the bonds formed by both end-to-middle and end-to-end types. However, the similar mechanism of dissociation should fail in case of the emergence of cross-linked fibrin oligomers in which monomeric desA fibrin molecules are joined through the covalent bonds. Since no sign of the resultant α polymers was detected (Fig. 1), the covalent structure of soluble double-stranded protofibrils is entirely brought about by the involvement of the γ chains into cross-linking. The transversal linking of γ−γ bonds arising between two different strands of a protofibril should have completely prevented transformation of this protofibril from being dissociated into single-stranded oligomers. Opposite, in the event when isopeptide bonds are formed between the γ polypeptide chains of two desA fibrin molecules contacting end-to-end within the same strand of a fibrin protofibril, a network of all weak, non-covalent lateral bonds keeping two single-stranded cross-linked oligomers together could be destroyed. As a result, single-stranded oligomers would be detected in solution. As it follows from the results of our work, to destroy the bonds high urea concentration (4.20 M and higher) should be used. It is for this reason we have failed earlier to divide cross-linked double-stranded protofibrils by using only slight increase of urea concentration from 1.20 up to 1.80 M [18]. Besides, at 1.20 M urea protofibrils reached higher molecular weight and therefore could be less prone to dissociation compared to those obtained in the present study.

Thus, double-stranded cross-linked protofibrils are converted into the single-stranded cross-linked structures when the urea concentration has grown to 4.20 M. This result fully supports the model of the longitudinal orientation of γ−γ bonds [10−13] and is apparently in contradiction with the model of the transversal linking γ−γ bonds [14−17]. Most data evidences in favor of parallel orientation γ−γ dimers belonging to the polypeptide γ chains within the same strand of a protofibril. In this context, the structure of cross-linked fibrin products investigated with aids of electron microscopy was proved to be single-stranded [10]. It is worth noting that the soluble fragments of plasmin-digested cross-linked fibrin clots [27] and cross-linked single-stranded desA fibrin oligomers obtained in the present study by dissociation of double-stranded desA fibrin protofibril are structural homologs. In the both cases the single-stranded structures are identified but they are not counterparts. Being different extent of plasmin degradation, the cross-linked fibrin fragments were composed of the heterogeneous molecules that are differing in their structures from initial monomeric fibrin. Predominantly, fragments lack αC-domains since the carboxyl-terminal end of the Aα chain is cleaved first. Whereas, the cross-linked single-stranded desA fibrin oligomers are homo-oligomers that are comprised of identical monomeric desA fibrin. The longitudinal orientation of γ−γ bonds is supported by the D-

dimer fragment structure that shows the end-to-end relationship of the carboxyl-terminal γ chains [10,27]. The data on FXIIIa-mediated self-assembly of cross-linked fibrinogen homopolymers have also evidenced that the C-termini of the γ chains bind to each other in the end-to-end fashion [28]. The enzymatic cross-linking reaction at the γC sites of fibrinogen is shown to be immediately enhanced when fibrinogen molecules form linear assemblies through complex formation of fibrinogen D regions with the fibrin E fragments [29]. On the other hand, the studies of the structure of cross-linked products formed in mixtures of fibrin, plasmic fragment D, and factor XIIIa indicate that γ chain cross-links occur transversely in D:fibrin:D complexes [30]. One of the important arguments in favor of this model has served the fact that the transversal cross-linking of two γ chains of a protofibril should explain the ability of a cross-linked protofibril completely to recover its initial form after the nearly twofold mechanical elongating [31]. However, the mechanical properties of a cross-linked protofibril are also consistent with longitudinal cross-links because the distinct structural fibrinogen elements, first of all, the α-helical coiled-coil region that have to partially unfold upon fiber stretching [32,33]. The results of our study provide additional compelling evidence to support the model of the longitudinal γ−γ bonds. Such architecture of γ−γ dimers is the only possible one that cannot prevent double-stranded protofibrils from being divided into single-stranded oligomers.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

Acknowledgments

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.052.

References

[1] B. Blombäck, Fibrinogen and fibrin — proteins with complex roles in hemostasis and thrombosis, Thromb. Res. 83 (1996) 1–75, http://dx.doi.org/10.1016/0049-3848(96)00111-9. PMID: 8837305.

[2] J.W. Weisel, R.I. Litvinov, Mechanisms of fibrin polymerization and clinical implications, Blood 121 (2013) 1712–1719, http://dx.doi.org/10.1182/blood-2012-09-306639. PMID: 23305734.

[3] R.I. Litvinov, O.V. Gorkun, S.F. Owen, H. Shuman, J.W. Weisel, Polymerization of fibrin: specificity, strength, and stability of knob-hole interactions studied at the single-molecule level, Blood 106 (2005) 2944–2951, http://dx.doi.org/10.1182/blood-2005-05-2039. PMID: 15998829.

[4] E. Mihalyi, Clotting of bovine fibrinogen. Calcium binding to fibrin during clotting and its dependence on release of fibrinopeptide B, Biochemistry 27 (1988) 967–976, http://dx.doi.org/10.1021/bi00403a020. PMID: 3365373.

[5] M. Krakow, G.F. Enders, B.M. Siegel, H.A. Scheraga, An electron microscopic investigation of the polymerization of bovine fibrin monomer, Mol. Biol. 71 (1972) 95–103. PMID: 4564302.

[6] R.C. Williams, Morphology of fibrinogen monomers and of fibrin protofibrils, Ann. N Y Acad. Sci. 408 (1983) 180–193, http://dx.doi.org/10.1111/j.1749-6632.1983.tb23244.x. PMID: 6575683.

[7] R. Chen, R.F. Doolittle, γ−γ cross-linking sites in human and bovine fibrin, Biochemistry 10 (1971) 4486–4491. PMID: 5168975.

[8] P.A. McKee, P. Mattock, R.L. Hill, Subunit structure of human fibrinogen, soluble fibrin, and cross-linked insoluble fibrin, Proc. Natl. Acad. Sci. U S A 66 (1970) 738–744. PMID: 5269236.

[9] V.R. Richardson, P. Cordell, K.F. Standeven, A.M. Carter, Substrates of Factor XIII-A: roles in thrombosis and wound healing, Clin. Sci. 124 (2013) 123–137, http://dx.doi.org/10.1042/CS20120233.

[10] J.W. Weisel, C.W. Francis, C. Nagaswani, V.J. Marder, Determination of the topology of factor XIIIa-induced fibrin gamma-chain cross-links by electron microscopy of ligated fragments, J. Biol. Chem. 268 (1993) 26618–26624. PMID: 7902838.

[11] Y. Veklich, E.K. Ang, L. Lorand, J.W. Weisel, The complementary aggregation sites of fibrin investigated through examination of polymers of fibrinogen with fragment E, Proc. Natl. Acad. Sci. U S A 95 (1998) 1438–1442. PMID: 9465033.

[12] R.F.J. Doolittle, X-ray crystallographic studies on fibrinogen and fibrin, Thromb. Haemost. 1 (2003) 1559–1565. PMID: 12871291.

[13] J.W. Weisel, Cross-linked gamma-chains in fibrin fibrils bridge transversely between strands: no, J. Thromb. Haemost. 2 (2004) 394–399. PMID: 15009453.

[14] M.W. Mosesson, The fibrin cross-linking debate: cross-linked gamma chains in fibrin fibrils bridge 'transversely' between strands: yes, J. Thromb. Haemost. 2 (2004) 388–393. PMID: 15009452.

[15] E. Selmayr, M. Deffner, L. Bachmann, G. Müller-Berghaus, Chromatography and electron microscopy of cross-linked fibrin polymers—a new model describing the cross-linking at the DD-*trans* contact of the fibrin molecules, Biopolymers 27 (1988) 1733–1748, http://dx.doi.org/10.1002/bip.360271104. PMID: 3233327.

[16] K.R. Siebenlist, D.A. Meh, M.W. Mosesson, Position of gamma-chain carboxyterminal regions in fibrinogen/fibrin cross-linking mixtures, Biochemistry 39 (2000) 14171–14175, http://dx.doi.org/10.1021/bi000800m. PMID: 11087365.

[17] M.W. Mosesson, K.R. Siebenlist, D.A. Meh, The structure and biological features of fibrinogen and fibrin, Ann. N Y Acad. Sci. 936 (2001) 11–30, http://dx.doi.org/10.1111/j.1749-6632.2001.tb03491. PMID: 11460466.

[18] M.A. Rosenfeld, V.B. Leonova, M.I. Biryukova, M.V. Vasileva, Self-assembly of soluble unlinked and cross-linked fibrin oligomers, Biochemistry (Moscow) 76 (2011) 1155–1163, http://dx.doi.org/10.1134/S0006297911100099. PMID: 22098241.

[19] M.A. Rosenfeld, A.V. Bychkova, A.N. Shchegolikhin, V.B. Leonova, et al., Ozone-induced oxidative modification of plasma fibrin-stabilizing factor, Biochim. Biophys. Acta 1834 (2013) 2470–2479, http://dx.doi.org/10.1016/j.bbapap.2013.08.001. PMID: 23948453.

[20] J. Mcdonagh, W.G. Waggoner, E.G. Hamilton, B. Hindenbach, R.P. Mcdonagh, Affinity chromatography of human plasma and platelet factor XIII on organomercurial agarose, Biochim. Biophys. Acta 446 (1976) 345–357, http://dx.doi.org/10.1016/0005-2795(76)90002-7. PMID: 990283.

[21] M.A. Rosenfeld, K.B. Gershkovich, D.V. Kuznetsov, B.B. Meshkov, I.D. Gontar, The mechanism of self-assembly of soluble fibrin oligomers and the role of fibrinopeptides A and B in this process, Mol. Biol. (Moscow) 20 (1986) 1098–1110. PMID: 3762532.

[22] L. Svedsen, B. Blomback, M. Blomback, P.J. Olsson, Synthetic chromogenic substrates for determination of trypsin, thrombin and thrombin-like enzymes, Thromb. Res. 1 (1972) 67–278, http://dx.doi.org/10.1016/0049-3848(72)90023-0.

[23] P. Schuck, Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling, Biophys. J. 78 (2000) 1606–1619.

[24] E.F. Casassa, Light scattering from very long rod-like particles and an application to polymerized fibrinogen, J. Chem. Phys. 23 (1955) 596–597.

[25] S. Bernocco, F. Ferri, A. Profumo, C. Cuniberti, M. Rocco, Polymerization of rod-like macromolecular monomers studied by stopped-flow, multiangle light scattering: set-up, data processing, and application to fibrin formation, Biophys. J. 79 (2000) 561–583, http://dx.doi.org/10.1016/S0006-3495(00)76317-X. PMID: 10866981.

[26] G. Spraggon, S.J. Everse, R.F. Doolittle, Crystal structures of D fragment from human fibrinogen and its cross-linked counterpart from fibrin, Nature 389 (1997) 455–462, http://dx.doi.org/10.1038/38947. PMID: 9333233.

[27] V.C. Yee, K.P. Pratt, H.C. Cote, I.L. Trong, D.W. Chung, et al., Crystal structure of a 30 kDa C-terminal fragment from the gamma chain of human fibrinogen, Structure 5 (1997) 125–138, http://dx.doi.org/10.1016/S0969-2126(97)00171-8. PMID: 9016719.

[28] M.A. Rosenfeld, A.N. Shchegolikhin, A.V. Leonova, V.B. Bychkova, et al., Ozone-induced oxidative modification of fibrinogen: role of the D regions, Free Radic. Biol. Med. 77 (2014) 106–120, http://dx.doi.org/10.1016/j.freeradbiomed. PMID: 25224034.

[29] G.P. Samokhin, L. Lorand, Contact with the N-termini in the central E domain enhances the reactivities of the distal D domains of fibrin to factor XIIIa, J. Biol. Chem. 270 (1995) 21827–21832, http://dx.doi.org/10.1074/jbc.270.37.21827. PMID: 7665605.

[30] K.R. Siebenlist, D.A. Meh, J.S. Wall, J.F. Hainfeld, M.W. Mosesson, Orientation of the carboxy-terminal regions of fibrin gamma chain dimers determined from the crosslinked products formed in mixtures of fibrin, fragment D, and factor XIIIa, Thromb. Haemost. 74 (1995) 1113–1119. PMID: 8560422.

[31] M.W. Mosesson, K.R. Siebenlist, D.A. Meh, J.S. Wall, J.F. Hainfeld, The location of the carboxy-terminal region of γ chains in fibrinogen and fibrin D domains, Proc. Natl. Acad. Sci. U S A 95 (1998) 10511–10516. PMID: 9724734.

[32] M. Guthold, C. Carlisle, Single fibrin fiber experiments suggest longitudinal rather than transverse cross-linking: reply to a rebuttal, J. Thromb. Haemost. 8 (2010) 2090–2091, http://dx.doi.org/10.1111/j.1538-7836.2010.03985.x.

[33] J.W. Weisel, Biomechanics in hemostasis and thrombosis, J. Thromb. Haemost. 8 (2010) 1027–1029, http://dx.doi.org/10.1111/j.1538-7836.2010.03808.x.