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Longitudinal Orientation of Cross-Linked Polypeptide γ Chains in Fibrin Fibrils

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Abstract—The crosslinking of fibrin γ-polypeptide chains under the influence of the plasma fibrin-stabilizing factor (FXIIIa), which causes their conversion to γ-γ dimers, is the major enzyme reaction of covalent fibrin stabilization. We studied the self-assembly of soluble cross-linked fibrin oligomers. The results of analytical ultracentrifugation as well as elastic and dynamic light scattering showed that the double-stranded fibrin oligomers formed under the influence of moderate concentrations of urea are cross-linked only due to formation of γ-γ dimers, which can dissociate into single-stranded structure when the concentration of urea increases. This fact proves that γ-γ dimers are formed in the end-to-end manner.

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The treatment of fibrinogen with thrombin or other thrombin-like enzymes yields monomeric fibrin molecules. They can undergo spontaneous polymerization and arrange in rod-like double-stranded protofibrils, which laterally aggregate and branch to form a fibrin gel. Fibrin cross-linking catalyzed by the plasma fibrin-stabilizing factor (FXIIIa) is the key blood coagulation reaction, which ensures the mechanical strength of the fibrin gel, required for the hemostatic function, and its resistance to plasmin hydrolysis. Under the action of FXIIIa, covalent isopeptide bonds both between γ- and between α-polypeptide chains are formed, yielding γ-γ dimers and α-polymers [1]. One of the key problems in the mechanism of enzymatic fibrin stabilization is the determination of the morphology of the γ-γ dimers. Although different methodological approaches were used, the data on the spatial orientation of γ-γ dimers obtained to date are extremely controversial. Several studies suggest the longitudinal orientation of bonds, i.e., their formation between γ-polypeptide chains of two adjacent molecules of monomeric fibrin interacting in the "end-to-end" manner within the same fibrin filaments [2, 3], whereas others [4, 5] support the transverse orientation of bonds between the molecules belonging to two different fibrin strands (Fig. 1). To confirm the longitudinal orientation of γ-γ dimers, we used the approach based on the possibility of obtaining soluble equilibrium cross-linked double-stranded fibrin protofibrils in the presence of FXIIIa and moderate urea concentrations in medium [6]. The dissociation of double-stranded fibrin protofibrils to the single-stranded structures, which is observed with further increase in the urea concentration, would unambiguously testify to the longitudinal orientation of γ-γ-bonds. In this study, we attempted to confirm this orientation of cross-linked γ-chains.

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The FXIIIa factor was isolated from human blood plasma and activated with thrombin [7]. Fibrin was obtained from fibrinogen by activation with reptilase followed by dissolving in 1.6 M urea to form monomeric fibrin [8]. The self-assembly of monomeric molecules was initiated by reducing the concentration of urea to 1.45, 1.35, and 1.25 M. Covalent crosslinking of soluble fibrin protofibrils (oligomers) was induced by adding 10 μL (6.4 units) of FXIIIa solution. The formation of covalent bonds was assayed by electrophoresis under reducing conditions.

The molecular weights (M_w) and the spatial structure of soluble non-cross-linked (in the absence of FXIIIa) and cross-linked (in the presence of FXIIIa) fibrin oligomers were assessed by elastic light scattering (Malvern Instruments, United Kingdom) [9]. The translational diffusion coefficient D_w of fibrin samples was measured by dynamic light scattering with a Zetasizer Nano-S instrument (Malvern Instruments) [10]. The sedimentation velocity of fibrin samples was determined by analytical ultracentrifugation in a Beckman model E centrifuge (Beckman Coulter, Inc., Austria) [6].

Enzymatic crosslinking of soluble fibrin oligomers. In the presence of FXIIIa, γ-chains of soluble fibrin oligomers undergo enzymatic cross-linking to form γ-γ dimers, the amount of which increases as the con-

centration of urea decreases. However, any conversion of α-chains into α-polymers was not shown by electrophoresis throughout the concentration range of urea.

Self-assembly of soluble non-cross-linked and cross-linked fibrin oligomers. A decrease in the concentration of urea induced fibrin self-assembly. This was confirmed by analytical ultracentrifugation, demonstrating the appearance of a bimodal distribution of molecules depending on the sedimentation rate (Fig. 2). Along with retaining the monomeric fibrin (the slow-sedimenting fraction), the amount of which decreased with a decrease in the concentration of urea, a rapidly sedimenting fraction of high-molecular-weight products, formed as a result of the conversion of monomeric molecules to fibrin oligomers, was found. As the concentration of urea decreased, the sedimentation coefficient and the content of the oligomeric fraction increased.

The same pattern was observed for the distribution of molecules with respect to t he translational diffusion coefficient.

Taken together, the data of analytical ultracentrifugation and elastic light scattering allowed us to calculate the molecular weights of oligomers and characterize their spatial organization. Since $M_w = M_m W_m + M_{w,o} W_o$, where M_m and $M_{w,o}$ are the molecular weights of monomeric fibrin and its oligomers and W_m and W_o correspond to the weight contribution of the slowly and rapidly sedimenting fractions, respectively, the $M_{w,o}$ value can be calculated. For the control sample, $M_{w,o}$ at an urea concentration of 1.25 M was $(23.73 \pm 2.13) \times 10^5$ Da, which assumed the formation of oligomers consisting of seven or eight monomer units. The covalent cross-linking of fibrin oligomers increases their molecular weights, which, at an urea concentration of 1.25 M reached $(32.85 \pm 2.96) \times 10^5$ Da (i.e., the soluble cross-linked oligomers may comprise up to ten monomeric units).

Spatial organization of fibrin oligomers. The results of light scattering in Casassa coordinates [11] were used to determine the molecular weight per unit length of fibrin oligomers M_W/L_W. Experimental plots show a considerable degree of linearity at high angles, characteristic of an ensemble of long rod-shaped macromolecules (Fig. 3). However, linearity was not retained at small scattering angles because of the significant amount of monomeric fibrin in the samples. The M_W/L_W value increased with decreasing urea concentrations. This was obviously due to an increase in the weight contribution of oligomers with a large weight per unit length compared to that of the monomeric fibrin. 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 the weight-to-length ratio of monomeric and oligomeric molecules), the weight per unit length of oligomers can be calculated. The experimental data showed that, irrespective of the urea concentration, $M_{w,o}/L_{w,o}$ values obtained for non-cross-linked and cross-linked fibrin oligomers were $(1.27 \pm 0.24) \times 10^{11}$ g mol^{-1} cm^{-1}. These values correspond to the structures of rod-like double-stranded fibrin protofibrils [12].

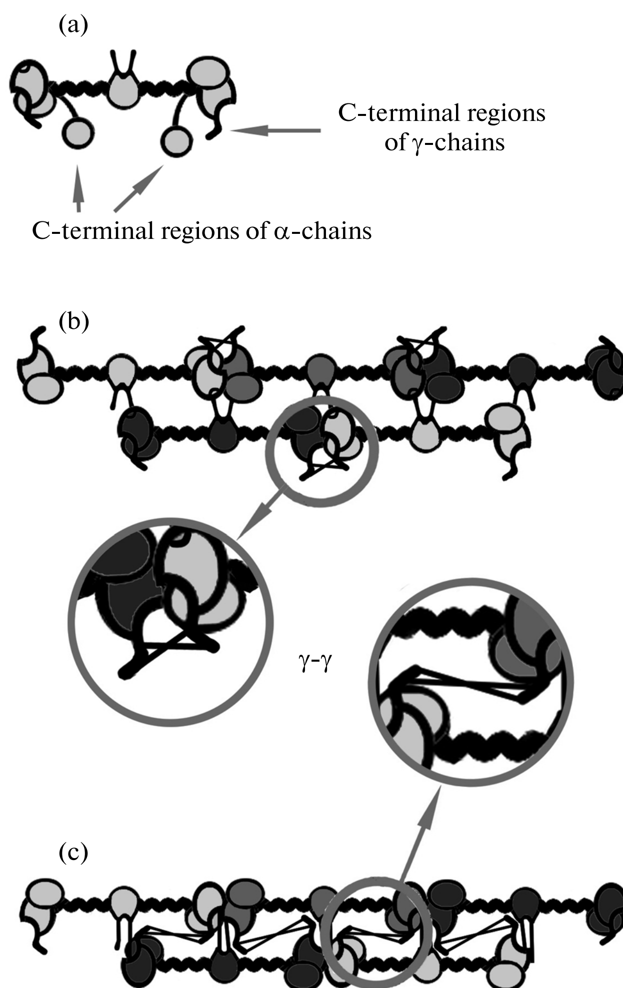

Fig. 1. (a) Schematic representation of the monomeric fibrin molecule and different models of the spatial orientation of γ-γ dimers stabilizing the structure of double-stranded fibrin protofibrils: the model of longitudinal orientation of γ-γ dimers, which covalently link adjacent monomeric fibrin molecules located in the same fibrin strand; (c) the model of transverse orientation of γ-γ dimers, which link the monomeric fibrin molecules in different protofibril strands. The C-terminal regions of α-polypeptide chains are not shown in Figs. 1b and 1c because they are not involved in the cross-linking reaction induced by FXIIIa.

Single-stranded soluble oligomers. Analytical ultracentrifugation and dynamic light scattering data showed that, when the urea concentration increased to 4.20 M, the bimodal distribution by the sedimentation rate and translational diffusion coefficient for the non-cross-linked oligomers became unimodal. This is a convincing confirmation of the complete degradation of the non-cross-linked fibrin oligomers to the original monomeric fibrin molecules. However, when the concentration of urea increased to 4.20 M, the bimodal

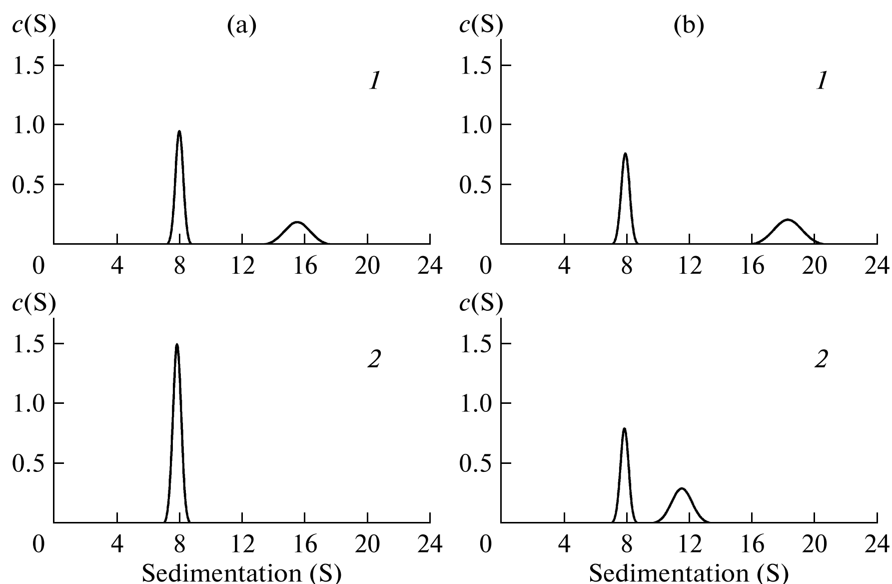

Fig. 2. Distribution of sedimentation coefficients c(S) for the samples of (a) non-cross-linked and (b) cross-linked fibrin oligomers at different concentrations of urea: (*1*) 1.25 M and (*2*) 4.20 M.

distribution for the cross-linked fibrin oligomers was retained. The slowly sedimenting fraction indicated the presence of monomeric fibrin, whereas the rapidly sedimenting fraction differed by its hydrodynamic properties both from the monomeric molecules and the rod-like double-stranded oligomers. Elastic light scattering data showed that the molecular weight of these oligomers was $(15.70 \pm 1.40) \times 10^5$ Da. A twofold decrease in the M_w of oligomers observed as the urea concentration increased to 4.20 M, as compared to the M_w value obtained for the cross-linked double-stranded oligomers in the presence of 1.25 M urea, clearly shows that the single-stranded oligomers were formed as a result of dissociation of the double-stranded cross-linked oligomers. Furthermore, the $M_{w,o}/L_{w,o}$ value, found from the angular dependence of the light scattering intensity in the Casassa coordinates, was $(0.68 \pm 0.08) \times 10^{11}$ g mol^{-1} cm^{-1}, which is evidence of the formation of rod-like single-stranded fibrin structures.

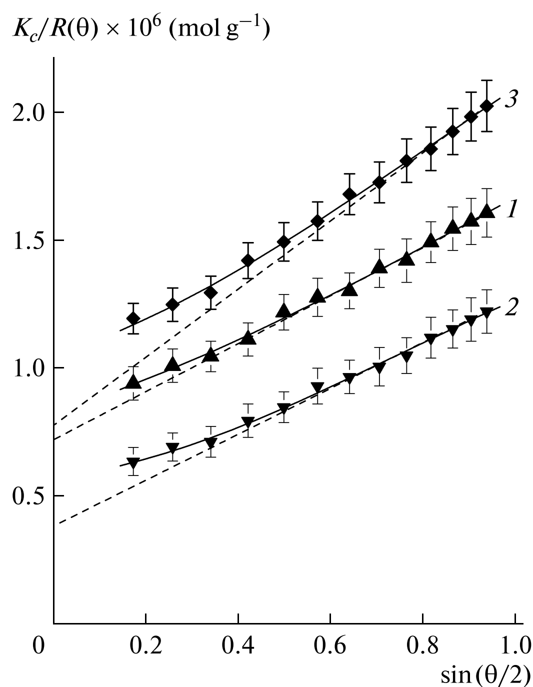

Fig. 3. Elastic light scattering plots obtained for (*1*) non-cross-linked and (*2*, *3*) cross-linked fibrin oligomers at urea concentrations of (*1*, *2*) 1.25 M and (*3*) 4.20 M. *1*—(0.88 ± 0.07), *2*—(0.93 ± 0.06), and *3*—(0.63 ± 0.07), $R(\theta)$—Rayleigh ratio, K—optical constant, c—protein concentration, θ—scattering angle. Data are represented as the mean value and the standard deviation. The values in parentheses are the M_w/L_w values expressed in 10^{11} g mol^{-1} cm^{-1}.

The absence of α-polymers according to the electrophoretic data is a sign that the cross-linked fibrin oligomers were covalently stabilized only by the γ-γ dimers formed in the presence of FXIIIa. In this case, the dissociation of the double-stranded fibrin oligomers to form the rod-like single-stranded fibrin structures is possible only in the case of the longitudinal orientation of bonds between the adjacent monomeric fibrin molecules belonging to the same protofibril strand (Fig. 1). Thus, the results of our study completely refute the model of transverse γ-γ bonds [4, 5, 13] and provide convincing evidence for the model of the longitudinal arrangement of γ-γ bonds formed by the end-to-end manner [2, 3, 14].

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