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Self-Assembly of Soluble Unlinked and Cross-Linked Fibrin Oligomers

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Abstract—Self-assembly of soluble unlinked and cross-linked fibrin oligomers formed from des*A*-fibrin monomer under the influence of factor XIIIa was studied in the presence of non-denaturing urea concentrations. By methods of elastic and dynamic light scattering combined with analytical ultracentrifugation, des*A*-fibrin oligomers formed in both the presence and absence of the factor XIIIa were shown to be ensembles consisting of soluble rod-like double-stranded protofibrils with diverse weight and size. Unlinked and cross-linked soluble double-stranded protofibrils can reach the length of 350-450 nm. The structure of soluble covalently-linked protofibrils is stabilized by isopeptide γ-dimers. Electrophoretic data indicate a complete absence of isopeptide bonds between α-chains of des*A*-fibrin molecules. The molecular mechanism of formation of soluble rod-like fibrin structures and specific features of its covalent stabilization under the influence of factor XIIIa are discussed.

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Key words: soluble fibrin, double-stranded protofibrils, factor XIIIa, γ-dimers, α-polymers

Fibrinogen is a soluble protein of blood plasma that is mainly responsible for generation of an insoluble fibrin gel in the presence of thrombin. A fibrinogen molecule is generally thought to be a rod-like 45-nm particle with asymmetry degree of about 5 [1-3]. Fibrinogen is strongly hydrated and contains 5-6 g water per gram protein [4]. The fibrinogen molecule is a dimer consisting of three pairs of polypeptide chains $(A\alpha, B\beta, \gamma)_2$. Molecular weights of the polypeptide chains *A*α, *B*β, and γ connected by disulfide bonds are ~67.5, 55, and 46.5 kDa, respectively [2]. A fibrinogen molecule is divided into several structurally and functionally important regions: the central *E* region formed by NH_2-terminal sites of all three pairs of the polypeptide chains; two peripheral *D* regions consisting of COOH-terminal sites of the *B*β and γ polypeptide chains; two α*C* regions including a significant part of the COOH-terminal sites of *A*α chains; and two *B*βN regions consisting of NH_2-terminal sites of *B*β chains [5]. In turn, *E* and *D* regions of fibrinogen have multidomain structure [6-9]. Treatment with thrombin initially results in detachment from fibrinogen *A*α-polypeptide chains of fibrinopeptides *A* (*A*α1-16) with production of fibrin monomer molecules (des*A*-fibrin) containing exposed polymerization center knobs "*A*". This polymerization center is formed by amino acid residues belonging to two different polypeptide chains of a fibrin molecule. One part includes amino acid residues of the α-chain *N*-terminal site, α17-20GPRV, whereas the other part is located in the β-chain of fibrin between residues 15Gly and 42Arg [10]. The polymerization center knob "*A*" is complementary to sites of holes "*a*" that are located in the COOH-terminal site of the γ-polypeptide chain in the *D* region between amino acid residues γ337Asn-379Lys [11, 12]. Monomer fibrin molecules are connected end-to-middle with a displacement of 22.5 nm, and the *D* region of one fibrin monomer molecule interacts with the *E* region of another molecule and also contacts end-to-end with the *D* region of the third molecule, thus forming a multidomain node. This results in production of structurally rigid rod-like double-stranded fibrin protofibrils. Each strand of a protofibril consists, on average, of 7-8 fibrin monomers [13, 14]. On reaching this critical length, protofibrils display a tendency for lateral association and form fibrils, which later divide in branches and form the network for the three-dimensional structure of the fibrin gel. At the stage of protofibril formation the rate of fibrinopeptide *B* (*B*β1-14) cleavage by thrombin significantly increases,

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with subsequent conversion of des*A*-fibrin into des*AB*-fibrin [1, 2]. This results in opening of new self-assembly regions knobs "*B*" that interact with polymerization sites holes "*b*" located in the COOH-terminal site of β-polypeptide chain of the *D* region. The knob "*B*"-into-hole-"*b*" interaction is thought to be insignificant for generation of protofibrils but can be manifested mainly by increasing the lateral association of protofibrils [15].

In the presence of the fibrin-stabilizing plasma transglutaminase (factor XIIIa), fibrin polymers are subjected to covalent linking via formation of ε/γ-glutamyl–lysine isopeptide bonds. This is associated with the covalent binding of COOH-terminal sites of γ-chains, contacting *D* regions, to one another with production of intermolecular γ-dimers. These dimers are produced as a result of binding the lysine γ406 residue of the first molecule with the glutamine residue γ398 or γ399 of the other molecule and of binding γLys406 of the second molecule with γGln398 or γGln399 of the first molecule. Factor XIIIa also catalyzes formation of isopeptide bonds between α-chains of neighboring fibrin molecules providing the interaction of the α-chain of one molecule with the α-chains of two other molecules with resulting production of α-polymers consisting of more than five α-polypeptide fibrin chains [1-3].

Generation of equilibrium, soluble, rod-like oligomers was earlier observed in the presence of moderate urea concentrations in cases of both des*AB*- and des*A*-fibrin prepared from fibrinogen, respectively, under the influence of thrombin or the thrombin-like enzyme reptilase, which detaches from fibrinogen only fibrinopeptides *A* [16, 17]. Because such oligomers were prepared in the absence of factor XIIIa, the monomer molecules interacted to one another only via weak noncovalent bonds that made the self-assembly process completely reversible. Thus, a reasonable question arises, whether soluble cross-linked fibrin oligomers can be generated under the influence of factor XIIIa in the presence of urea. Up to now such structures have not been described in the literature. This paper presents a response to this question, which concerns molecular mechanisms of polymerization and covalent stabilization of fibrin.

MATERIALS AND METHODS

Fibrinogen prepared from citrated blood plasma by alcohol precipitation was used [18]. It was additionally purified from admixtures of plasminogen and fibrin-stabilizing factor by chromatography on DEAE cellulose with subsequent precipitation with ammonium sulfate [19]. The resulting fibrinogen was transferred by gel filtration with Sephadex G-25 into 0.05 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl.

The fibrin-stabilizing factor (factor XIII) was prepared from platelet plasma by fractional precipitation with ammonium sulfate and subsequent ion-exchange chromatography on DEAE-Toy Pearl M650 (Japan) [20]. Factor XIII was converted into the active form XIIIa under the influence of thrombin (Roche, France) as described in [21]. The residual thrombin was inactivated with antithrombin III–heparin mixture. The completeness of thrombin inactivation was monitored with the chromogenic substrate S-2238 as described in [22]. The factor XIIIa activity determined by the Lorand method [20] was 640 standard units (S.U.) per ml (1 S.U. corresponds to the factor XIIIa activity in 1 ml of donor blood serum).

Monomeric des*AB*-fibrin and des*A*-fibrin were prepared by activating fibrinogen with thrombin or a reptilase analog – ancistron (Tekhnologiya-Standart, Russia) using a method described in detail earlier [16]. Monomeric des*AB*-fibrin was dissolved in 3.5 M urea, and des*A*-fibrin was dissolved in 1.6 M urea. The urea solutions were prepared in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl. Polymerization of des*AB*-fibrin and des*A*-fibrin in the absence of factor XIIIa was initiated by a decrease in the urea concentration from the initial value of 3.5 to 2.2 M in the first case and from 1.6 to 1.2 M in the second case. The urea concentration was decreased by the addition to the initial solutions of fibrin monomers of a calculated amount of Tris-HCl buffer. The urea concentration in the samples of des*AB*-fibrin under study was 3.5 and 2.2 M, and in the samples of des*A*-fibrin it was 1.6, 1.4, 1.3, and 1.2 M; the monomeric fibrin concentration in all samples was balanced with the corresponding solutions of urea and was 0.75 mg/ml. The samples were incubated at 25°C for 24 h to reach equilibration in the system. Polymers were covalently linked by adding factor XIIIa to the samples of fibrin monomers in the presence of calcium ions, i.e. 1 ml of the polymer solution in urea was simultaneously supplemented with 10 μl (6.4 S.U.) of factor XIIIa and 5 μl of 10% $CaCl_2$ (5 mM). The control samples (without XIIIa) were supplemented with 10 μl of Tris-HCl buffer and 5 μl of 10% $CaCl_2$. The cross-linking reaction was performed during 24 h. The ability for depolymerization of the control and experimental (XIIIa-containing) polymers of des*A*-fibrin prepared in 1.2 M urea was studied after increasing the urea concentration in the samples to 1.8 M.

The cross-linking of polypeptide chains was determined by electrophoresis of reduced samples in 7.5% polyacrylamide gel. The samples were treated with a mixture of 7 M urea, 2% SDS, and 1% 2-mercaptoethanol.

Average molecular weights M_ω of polymers and their spatial structures were assessed using Rayleigh light scattering with a 4400 spectrometer (Malvern, England) supplied with a multi-bit 64-channel K7025 correlator in cylindrical cells in the scattering angle θ range of 20-130° [17]. A helium-neon laser with wavelength of 632.8 nm was used as the light source. All protein solutions were

carefully purified from dust by ultrafiltration. Incremental values of the protein refraction coefficients were determined with a Chromatix KMX-16 differential refractometer (USA).

Z-Mean coefficients of the translational diffusion D_z of samples before and after their incubation with the fibrin-stabilizing factor were determined by dynamic light scattering using a Zetasizer Nano-S spectrometer (Malvern) with the detection angle of 173° [23]. The data were processed using the Origin 7.0 program.

Sedimentation studies were performed using a Beckman model *E* analytical centrifuge (Austria) equipped with a photoelectric scanning absorption optical system at the rotor rotation rate of 48,000 rpm at 20°C.

Experimental results were processed with the SEDFIT computer program created by P. Schuk at the National Institutes of Health (USA), which is available free at (http://www.analyticalultracentrifugation.com/default.htm).

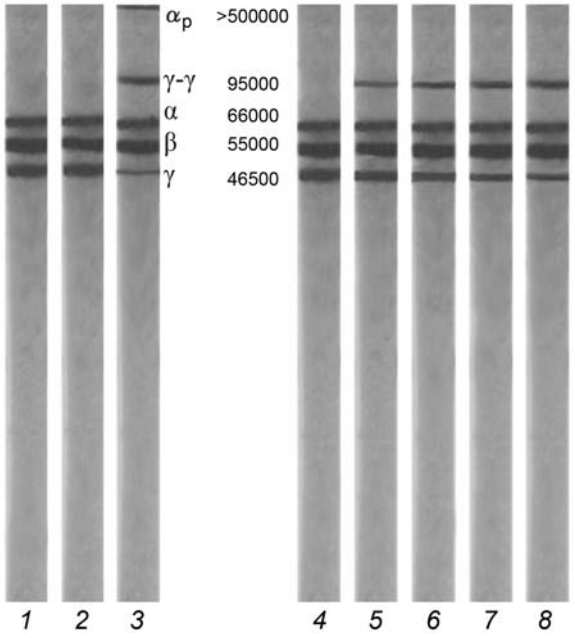

Fig. 1. Electrophoresis of reduced fibrin specimens. *1*) Monomeric fibrin-des*AB* in 3.5 M urea; *2*) fibrin-des*AB* upon incubation for 24 h with factor XIIIa at urea concentration 2.2 M; *3*) the same sample in 1.6 M urea; *4*) monomeric fibrin-des*A* in 1.6 M urea; *5-8*) covalently-linked fibrin-des*A* prepared at urea concentrations of 1.6, 1.4, 1.3, and 1.2 M, respectively.

RESULTS

Soluble fibrin polymers from des*AB*-fibrin were prepared at urea concentrations in the range 3.5-2.2 M. At the lower urea concentrations gelation occurred. Electrophoresis revealed a complete absence of the enzyme activity on the incubation of soluble fibrin polymers with factor XIIIa even at the lowest possible urea concentration of 2.2 M. No accumulation of γ-dimers or α-polymers was detected even after incubation for 24 h (Fig. 1). However, electrophoregrams indicated that enzymatic activity of factor XIIIa appeared on decreasing urea concentration to 1.6 M. Consequently, covalently linked soluble fibrin polymers cannot be prepared under the action of thrombin on fibrinogen. However, we showed earlier [16] and confirmed in the present work that soluble fibrin polymers formed from des*A*-fibrin can exist at significantly lower urea concentrations, even at 1.2 M. Such polymers can be involved in the cross-linking process catalyzed by factor XIIIa. Therefore, fibrin polymers formed only from des*A*-fibrin are characterized in this work.

Hydrodynamic parameters of soluble fibrin in the absence of factor XIIIa (the control sample) in the presence of 1.6 M urea had translational diffusion and sedimentation coefficients, respectively, $D_z = (2.40 \pm 0.22)\cdot10^{-7}$ cm^2/sec and $s = (7.90 \pm 0.37)$ S, which corresponds to the physicochemical parameters of fibrin monomers [16, 24].

The decrease in the urea molarity initiates polymerization. This is confirmed by data of analytic ultracentrifugation (Fig. 2) demonstrating a bimodal distribution of molecules by sedimentation rate in the urea concentration range 1.4-1.2 M. Along with the retention of monomeric des*A*-fibrin, the concentration of which decreases with a decrease in the urea molarity, a rapidly sedimenting fraction appears that represents the accumulation of fibrin oligomers. The increase in the sedimentation coefficient and enlargement of the sedimentation peak at its base with a decrease in urea concentration suggest an increase in the molecular weight and polydispersity of the self-assembly products. In factor XIIIa was present the general type of the sedimentation curves is retained, but the contents of high molecular weight products increases relative to the control at correspondingly equal urea concentrations (Fig. 2, the curves *6-9*).

This regularity is revealed on investigation of the oligomer structure using dynamic light scattering. In the urea concentration range 1.4-1.2 M for the control samples the distribution of macromolecules by the translational diffusion coefficient D_z, as in the case of ultracentrifugation, is characterized by two peaks corresponding to the translational diffusion coefficients of fibrin monomer and oligomers (Fig. 3). A decrease in the urea molarity is associated with a decrease in the translational diffusion coefficients, which suggests the polymerization of fibrin. In the presence of factor XIIIa the histogram is also characterized by a bimodal distribution of translational diffusion coefficients with a relatively greater contribution (as compared to the control) of oligomers on a decrease in the urea concentration (Fig. 3, curves *6-9*).

Overall, the data of analytic ultracentrifugation and elastic light scattering allow us to characterize the molec-

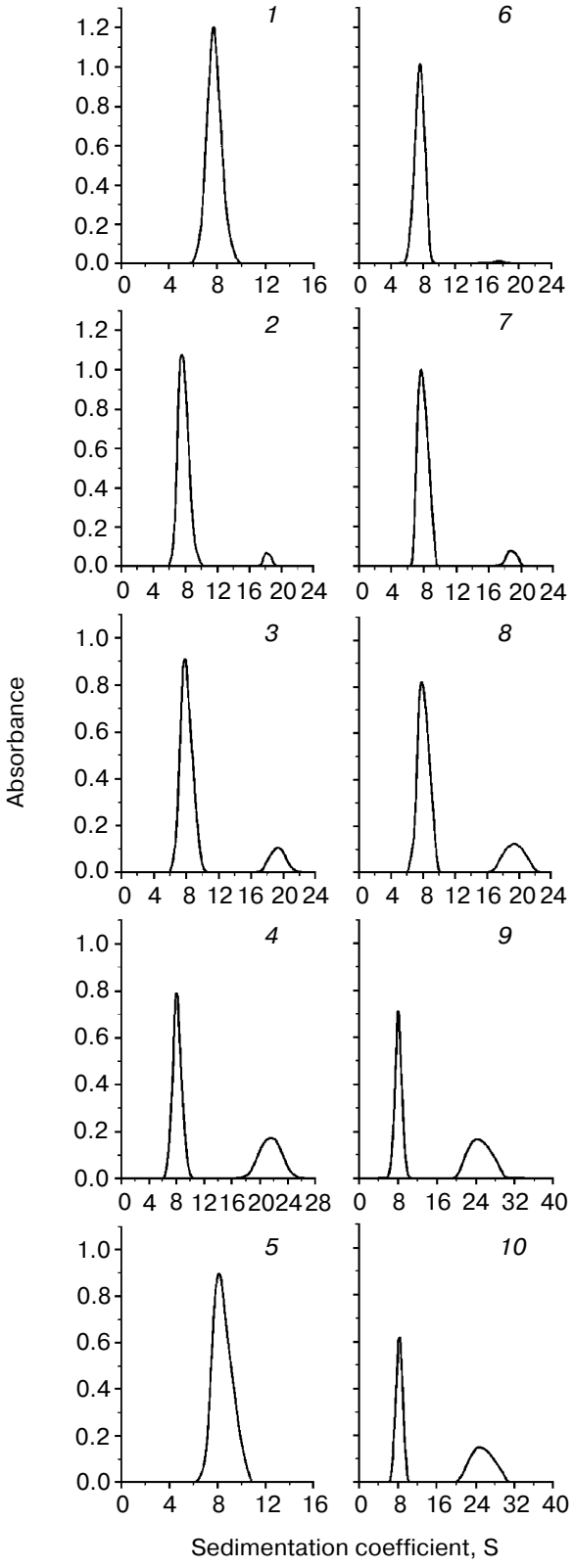

Fig. 2. Results of analytical centrifugation of control (*1-5*) and experimental (*6-10*) samples of fibrin-des*A* oligomers prepared at different urea concentrations: *1*, *6*) 1.6 M; *2*, *7*) 1.4 M; *3*, *8*) 1.3 M; *4*, *9*) 1.2 M; *5*, *10*) 1.8 M.

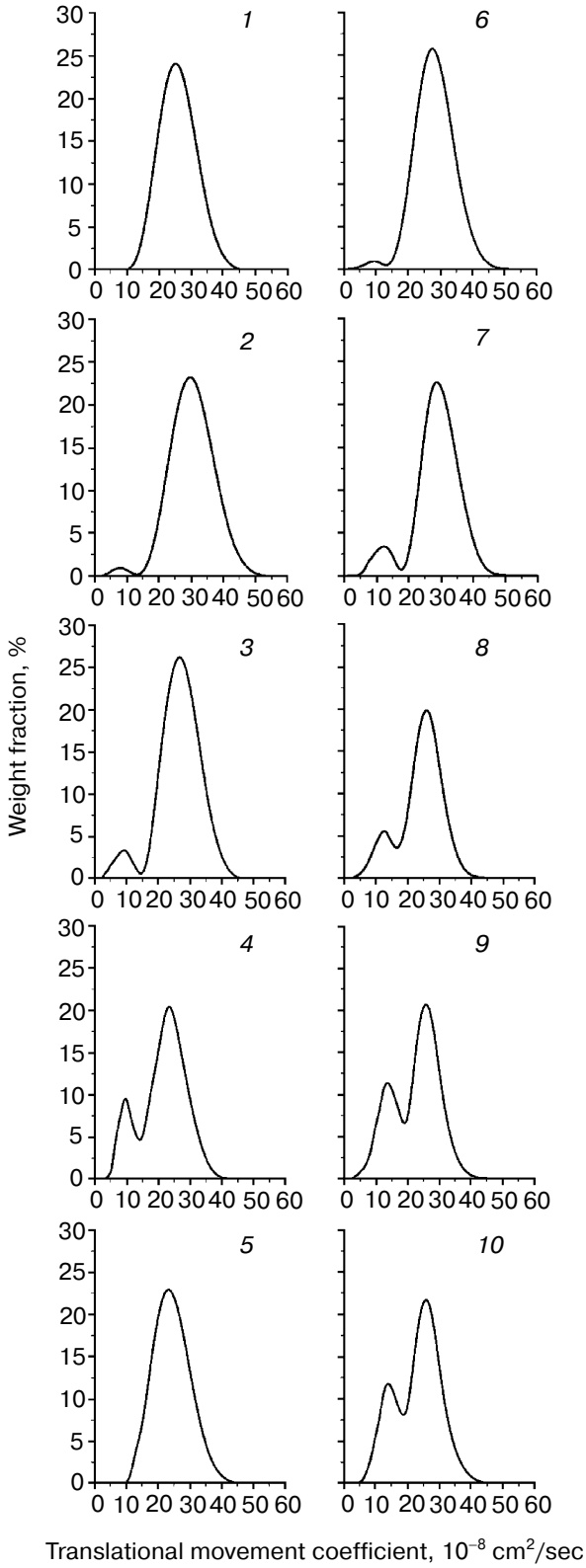

Fig. 3. Distribution curves for translational diffusion coefficient D_z of control (*1-5*) and experimental (*6-10*) soluble fibrin-des*A* oligomers prepared at different urea concentrations: *1*, *6*) 1.6 M; *2*, *7*) 1.4 M; *3*, *8*) 1.3 M; *4*, *9*) 1.2 M; *5*, *10*) 1.8 M.

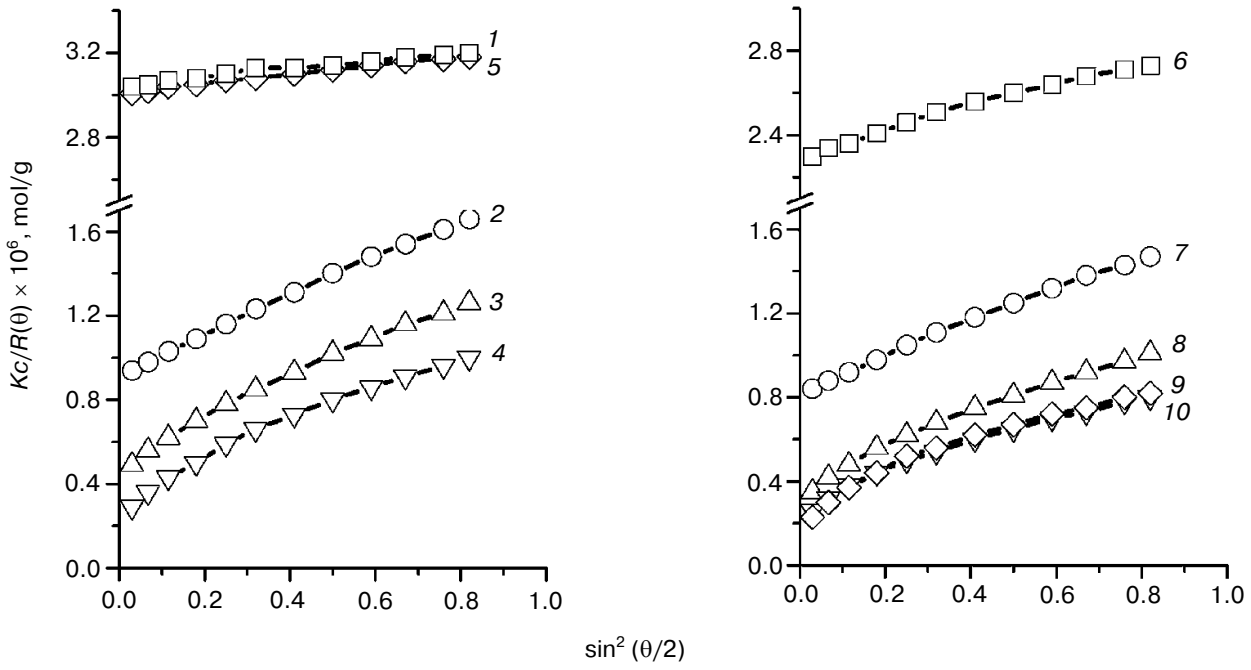

Fig. 4. Angular dependences in Zimm coordinates of elastic light scattering intensity for control (*1-5*) and experimental (*6-10*) fibrin-des*A* oligomers prepared at different urea concentrations: *1*, *6*) 1.6 M (3.3 ± 0.14 and 4.4 ± 0.18); *2*, *7*) 1.4 M (10.9 ± 0.45 and 12.3 ± 0.6); *3*, *8*) 1.3 M (21.3 ± 1.3 and 31.2 ± 1.6); *4*, *9*) 1.2 M (35.7 ± 1.8 and 43.5 ± 3.5); *5*, *10*) 1.8 (3.4 ± 0.17 and 46.7 ± 2.9). In parentheses M_ω values in 10^5 Da are presented.

ular weight and spatial organization of polymers constituting the rapidly sedimenting fraction. Angular dependences of the light scattering plotted in Zimm coordinates allow us to determine mean values of molecular weights M_ω of the polydisperse ensemble of macromolecules (Fig. 4). Data of elastic light scattering indicate that a decrease in the urea concentration is accompanied by an increase in values of mean molecular weight M_ω. Because the weight contribution of oligomers is proportional to the area under the sedimentation diagram curve, and $M_\omega = M_i\omega_i + M_k\omega_k$, where M_i and M_k are mean molecular weights of fibrin-des*A* monomer and its oligomers, and ω_i and ω_k correspond to weight contributions of slowly and rapidly sedimenting fractions, it is possible to calculate M_k values. For the control sample the mean molecular weight of oligomers at the least possible urea molarity of 1.2 M preceding gelation is $(55.5 \pm 3.5)\cdot10^5$ Da. Based on the resulting value of the molecular weight of soluble oligomers, they occur to consist of about 16 monomeric links. The covalent cross-linking of des*A*-fibrin oligomers under the influence of factor XIIIa increases their critical weight to $(63.3 \pm 4.5)\cdot10^5$ Da; thus, covalently-linked soluble oligomers can include up to 20 monomeric links.

Angular dependences of elastic light scattering intensity (Fig. 5) plotted in Casassa coordinates give the ratio of the mean weight to the mean length M_ω/L_ω for rather long rod-like fibrillar structures of biopolymer [25]. Similarly to the case of calculation of the mean molecular weights of fibrin oligomers, the data of elastic light scattering and analytic centrifugation together allows us to design curves $Kc_k/R_k(\theta) = f[\sin(\theta/2)]$ assigned only to the rapidly sedimenting fraction of oligomers, where c_k and $R_k(\theta)$ correspond to the concentration and the Rayleigh ratio of oligomers. The resulting experimental data show that for the whole range of urea concentration for both control and experimental samples the M_ω/L_ω values were, on average, $(1.20 \pm 0.14)\cdot10^{11}$ g/mol·cm. Because molecular weight of fibrinogen is 340 kDa and the rod-like molecule length is 45 nm [2], the weight/length ratio for the protein is $0.63\cdot10^{11}$ g/mol·cm. Consequently, soluble unlinked and covalently cross-linked des*A*-fibrin oligomers are double-stranded protofibrils of 350-450-nm length. Note that even at the lowest possible urea concentration no soluble fibrin fibrils were detected close to the gelation point. This means that the lateral association of double-stranded protofibrils preceding the gelation can occur only in a very narrow range of urea concentrations. Possible explanations for this will be discussed further.

In the presence of factor XIIIa the spatial structure of fibrin oligomers completely corresponds to unlinked structures. In other words, in the whole range of urea concentrations factor XIIIa is unable to covalently cross-link soluble double-stranded protofibrils to one another with production of fibrin fibrils. But it follows from data presented in Fig. 1 that the enzymatic activity of the fib-

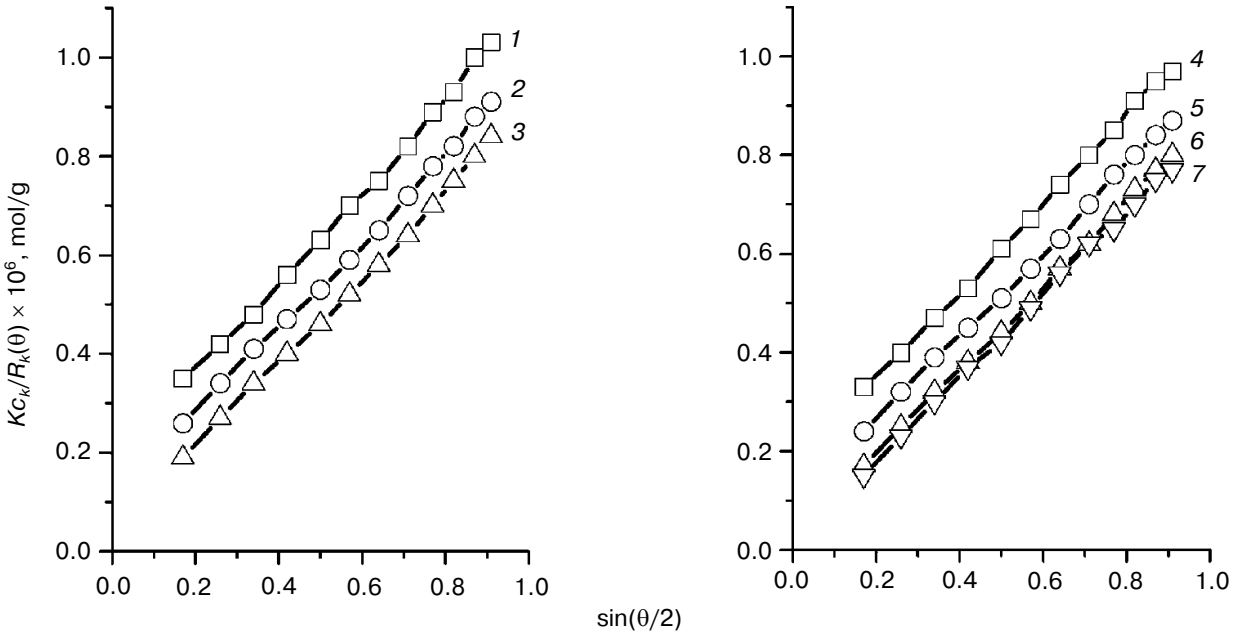

Fig. 5. Angular dependences in Casassa coordinates of elastic light scattering intensity for soluble control (*1*-*3*) and experimental (*4*-*7*) fibrin-des*A* oligomers prepared at different urea concentrations: *1*, *4*) 1.4 M; *2*, *5*) 1.3 M; *3*, *6*) 1.2 M; *7*) 1.8 M.

rin-stabilizing factor manifests itself at the maximal urea concentration of 1.6 M. This is supported by accumulation of γ-dimers over time. Consequently, monomeric fibrin molecules not incorporated into protofibrils are covalently cross-linked, because the above-presented data show that at the urea concentration of 1.6 M des*A*-fibrin remains a monomer. With a decrease in the urea concentration the rate of involvement of γ-chains of fibrin monomers into covalent cross-linking increases. However, electrophoretic data indicate a complete absence of accumulation of α-polymers after 24 h of the enzymatic reaction.

The ability for depolymerization of unlinked and covalently-linked des*A*-fibrin oligomers initially formed at 1.2 M urea was studied. For this purpose the oligomer structure in the control and experimental samples was analyzed on increasing the urea molarity from 1.2 to 1.8 M. The physical reason for these experiments is based on fundamentally different resistance of unlinked and covalently-linked structures to the action of urea. As was noted earlier that fibrin formation in the absence of factor XIIIa is fully reversible, whereas enzymatic cross-linking can deprive the structures of their ability for dissociation. Moreover, at the urea concentration of 1.8 M the enzymatic activity of factor XIIIa is completely inhibited, and thus those covalently cross-linked structures are analyzed that were generated before the increase in the urea concentration. Data of analytic ultracentrifugation and dynamic light scattering indicate that upon increase in the urea concentration in the control sample the bimodal distribution of macromolecules is changed to a unimodal one (Figs. 2 and 3, curves *5*). Values of the sedimentation and translational diffusion coefficients equal to 8.15 S and $2.40 \cdot 10^{-7}$ cm^2/sec for the molecules suggest the complete depolymerization of fibrin oligomers. However, the peak corresponding to the fraction of double-stranded protofibrils formed in the presence of factor XIIIa is not noticeably changed upon the increase in the urea concentration (Figs. 2 and 3, curves *10*). Initial values of the molecular weight M_ω and the weight/length ratio M_ω/L_ω for the linked protofibrils are also retained (Figs. 4 and 5).

DISCUSSION

The studies have shown that double-stranded des*A*-fibrin oligomers are formed in the control and experimental samples in urea-containing medium. Non-stabilized fibrin is dissolved and soluble polymers are depolymerized under the action of urea before formation of initial molecules of fibrin monomer retaining all physicochemical properties inherent in the native protein. In other words, the fibrin formation is completely reversible. Data that were recently obtained on involvement of different polymerization centers in fibrin assembly allow us to analyze in detail the mechanism of formation of soluble double-stranded protofibrils. The formation of double-stranded protofibrils is mainly contributed by knobs "*A*"-into-holes-"*a*" interactions. The knob "*A*" is the so-called combined polymerization center formed by amino acid residues belonging to two different α- and β-polypeptide chains of fibrin molecules closely located to one another [10]. Two holes "*a*" located in the COOH-

terminal part of the γ-polypeptide chain of the *D* region, which are responsible for the protofibril formation on interactions with the complementary knobs "*A*", are located on one side of the fibrin molecule, whereas fibrin molecules belonging to two chains of the protofibril are located "face-to-face" [9]. In the forming double-stranded protofibrils the neighboring monomeric links of each chain are located "end-to-end", whereas molecules belonging to different chains are displaced by half of its length. X-Ray crystallography reveals an intermolecular interaction of *D* regions of fibrin molecules contacting "end-to-end" in the each chain of double-stranded protofibril. This interaction is asymmetric relative to the longitudinal axis of the two neighboring monomeric links [6]. It has been shown recently that self-assembly centers different from the knobs "*A*"-into-holes-"*a*" sites are also involved in formation of double-stranded protofibrils [26]. The so-called "*C*" site is located in the fibrin fragment *B*β12-46 and functions until the detachment of fibrinopeptide *B*. The site "*c*" complementary to site "*C*" is located in the *N*-terminal part of the γ-chain of the fibrin *D* region, as differentiated from holes "*a*" located in the *C*-terminal part of the γ-chain of the *D* region [26]. Upon the detachment from a fibrinogen molecule of fibrinopeptide *A*, treatment with thrombin results in detachment of fibrinopeptide *B*. However, it has been convincingly shown that the bulk of fibrinopeptide *B* is detached only after the protofibril formation and initiation of their lateral association [27]. Interactions of the polymerization center knobs "*B*" exposed as a result of the detachment of fibrinopeptides *B* with the complementary holes "*b*" located in the *C*-terminal part of the β-polypeptide chain of the *D* region are mainly responsible for strengthening the lateral association of protofibrils [2, 3]. In the present work we have studied the self-assembly of fibrin oligomers on the treatment of fibrinogen with ancistron, which is incapable of detaching fibrinopeptide *B*. Consequently, the interaction between the knobs "*B*" and holes "*b*" cannot be realized. The lateral association of such protofibrils mainly occurs through binding sites located in fragments of the COOH-terminal part of the des*A*-fibrin γ-polypeptide chain, γ350-360 and γ370-380 [28]. In the fibrin molecules these sites are directed differently from the longitudinal axis of the molecule, and this promotes the thickening. Thus, it seems that the assembly of double-stranded protofibrils and their subsequent lateral association are realized through interaction of the ensemble of strictly selective polymerization centers. In the present work it is shown that soluble protofibrils formed from des*AB*-fibrin monomers can exist at urea concentrations in the range 2.6-2.2 M, whereas the self-assembly of soluble protofibrils formed from des*A*-fibrin monomers occurs only at significantly lower concentrations of urea. A possible explanation is as follows. The thickness of fibrin fibrils formed under the influence of thrombin on fibrinogen is always greater than the thickness of fibrils formed as a result of polymerization of des*AB*-fibrin monomers [29]. On the self-assembly of fibrin monomers under the action of thrombin on fibrinogen, the knobs "*B*" become exposed after the protofibril formation, and this provides for the interprotofibrillar interaction of the knobs "*B*" and holes "*b*". On the polymerization of des*AB*-fibrin monomers the knobs "*B*" and holes "*b*" interact mainly inside the protofibrils [26]. Thus, the presence only of knobs "*A*" in des*A*-fibrin makes it more sensitive than des*AB*-fibrin to the action of urea.

The depolymerization activity of urea is known to be due to inhibition of formation of noncovalent hydrogen, hydrophobic, and electrostatic bonds. Just hydrogen and electrostatic bonds mainly contribute to interaction of the main assembly centers of double-stranded protofibrils – polymerization center knobs "*A*" and holes "*a*" [28]. However, urea destabilizes the protein structure mainly due to solubilization of nonpolar lateral amino acid residues. It is suggested that these influences result in the affecting by urea of complementarity of the polymerization center knobs "*A*" and holes "*a*" (possibly, also of the sites "*C*"–"*c*"). Thus, depending on the urea concentration the interaction of *D* and *E* regions of des*A*-fibrin to one another on the assembly of double-stranded protofibrils is partially or completely inhibited. It seems that this can be also assigned to intermolecular interactions of the *D* regions of fibrin molecules contacting "end-to-end" in each chain of the double-stranded protofibril with involvement of two amino acid residues. The action of urea on fibrin assembly is reversible; therefore, an increase in the inhibitor concentration is accompanied by recovery of the ability of knobs "*A*" and holes "*a*" to interact, which involves fibrin monomers into a self-assembly process of double-stranded protofibrils. However, certain thermodynamic conditions prevent a lateral association of double-stranded protofibrils, which is associated with overcoming an entropy barrier. The dimerization of structurally rigid rod-like short or long protofibrils must be accompanied by the same entropy losses [17]; therefore, protofibrils must reach a critical length. In this case the total energy of weak noncovalent bonds will compensate the unfavorable entropy effect, and the dimerization process will be spontaneous. The energy dependence on chain length causes cooperativity of the lateral association of double-stranded protofibrils. The above-said explains the causes that prevent formation of separate small aggregations of short fibrils and promote appearance of long polymeric fibrils. The present work shows (Fig. 5a) that double-stranded soluble protofibrils reach the length of about 350 nm that precedes lateral association, and this is in agreement with the literature data [13, 14].

According to the electrophoresis data (Fig. 1), in the presence of factor XIIIa covalently cross-linked soluble fibrin oligomers are produced. All physicochemical

approaches used in the present work show the greater contribution of double-stranded protofibrils as compared to the control samples at the same urea concentrations (Figs. 2-5). Double-stranded protofibrils are an ideal substrate for factor XIIIa because all molecules of fibrin monomers are arranged similarly along the polymer chains of the protofibril. And the COOH-terminal sites of γ-polypeptide chains of the *D* regions of the neighboring monomeric links are located immediately close to one another, and this promotes a rapid inclusion of the chain in the enzymatic dimerization. Therefore, at the urea concentration of 1.6 M γ-chains in des*AB*-fibrin are completely included in the dimerization (Fig. 1). In this connection, it is interesting that at the urea concentration providing for retention in the control of des*A*-fibrin as monomers, γ-dimers can also be generated in the presence of the fibrin-stabilizing factor (Fig. 1). The molecular mechanism of cross-linking of monomeric molecules unable to interact with one another seems to be similar to the mechanism of enzymatic cross-linking of fibrinogen molecules. The *C*-terminal part of γ-polypeptide chain of fibrinogen (as if of fibrin monomer) contains the binding site of factor XIIIa a_2-subunits [1]. On spatial approaching of molecules and in the case of contacting COOH-terminal parts of γ-polypeptide chains of the *D* regions, factor XIIIa is responsible for their enzymatic cross-linking. Because not all molecules on approaching occupy the position required for the enzymatic reaction, fibrinogen molecules are cross-linked significantly less rapidly that fibrin protofibrils, and some γ-polypeptide chains of the protein remain intact [2, 21]. This is the reason for the presence of a certain amount of des*A*-fibrin monomers uninvolved in the enzymatic cross-linking.

To understand the architecture of the resulting covalent bonds, we have analyzed structures of fibrin oligomers formed on the increase in urea concentration from 1.2 to 1.8 M in the experimental sample on its incubation with factor XIIIa and in the control sample. As differentiated from the unlinked structures, the double-stranded protofibrils covalently cross-linked under the influence of factor XIIIa lose the ability to dissociate due to formation of covalent bonds. Obviously, the dissociation of protofibrils into double-stranded fragments is impossible at any configuration of isopeptide covalent bonds resulting between the COOH-terminal parts of γ-chains of the contacting *D* regions of the neighboring monomeric links. The disjoining of a double-stranded protofibril at the longitudinal position of covalent bonds into two one-stranded protofibrils could be most demonstratively recorded using analytical centrifugation. In fact, at the resulting value of the mean molecular weight of a covalently cross-linked protofibril equal to $(63.3 \pm 4.5)\cdot 10^5$ Da two rod-like one-stranded structures would be produced, each with the weight of about $3.0\cdot 10^6$ Da. According to theoretical hydrodynamic calculations [30], the sedimentation coefficient of one-stranded oligomers $s \sim s_i(M_k/M_i)^{0.2}$, where M_k is molecular weight of oligomers, s_i and M_i are, respectively, the sedimentation coefficient and molecular weight of the monomeric link. Inserting the experimental data into this expression we would obtain the *s* value approximately equal to 12 S. However, there is no such peak in sedimentation diagrams (Fig. 2). Dissociation of a double-stranded protofibril into hydrodynamically more compact flexible-chained oligomers also does not occur because data of elastic light scattering show (Figs. 4 and 5) that M_ω and M_ω/L_ω values are not changed on the increase in the urea molarity to 1.8 M. Consequently, our findings have shown the inability of double-stranded protofibrils to dissociate and to produce oligomeric structures. Do these findings allow us to consider the geometry of isopeptide γ-dimers?

The available data on the architecture of isopeptide γ-dimers are very contradictory. According to one standpoint, isopeptide bonds are formed between γ-polypeptide chains of two fibrin molecules contacting end-to-end, or longitudinally, within the same chain of a protofibril [31-34]; according to the other standpoint these bonds are formed between γ-polypeptide chains of the neighbor monomeric links from the different chains of a protofibril, i.e. located side-to-side, or transversally [35]. The lateral cross-linking of two chains of a protofibril seemed to explain the ability of a cross-linked protofibril to completely recover its initial form after the nearly twofold elongating, as differentiated from an unlinked protofibril [1, 35]. However, together the X-ray crystallography and electron microscopy data about production of one-stranded fragments as a result of plasmin hydrolysis of fibrin stabilized by the factor XIIIa convincingly confirm the production of the end-to-end formed γ-dimers [31-34].

Unfortunately, our findings that soluble covalently cross-linked double-stranded protofibrils are unable to dissociate with an increase in the urea concentration do not present information that would sufficient to suppose the geometry of isopeptide γ-dimers. The resistance of protofibril structures to the action of urea seemed to suggest the lateral orientation of covalent bonds because in this case isopeptide bonds resulting between γ-polypeptide chains of the neighboring monomeric links belonging to the different chains of the protofibril would prevent their dissociation. However, even at the end-to-end geometry of γ-dimers no dissociation of protofibrils can be observed. Protofibrils possess a helical conformation [36, 37]. Isopeptide bonds between γ-polypeptide chains within the same chain of a protofibril stabilize coils of the helix and thus prevent their untwisting and subsequent separation. Thus, at any possible geometry of γ-dimers protofibrils can be resistant to the action of urea.

According to data of electrophoresis, α-polypeptide chains of soluble structures of fibrin oligomers are not involved in formation of α-polymers. Now it is well known that formation of isopeptide bonds between α-

chains of neighbor fibrin molecules occurs an order of magnitude slower than dimerization of γ-chains [2]. In our experiments this can be the reason for the absence of accumulation in the urea-containing medium of a noticeable amount of α-polymer. However, this can also be due to the ability of factor XIIIa to covalently cross-link α-chains of molecules only at the stage of protofibril joining into fibrils. There are no data in the literature about the possibility of the covalent stabilization of protofibrils due to cross-linking of α-chains; therefore, at present this idea is purely hypothetical. We think that glutamine and lysine residues of fibrin α-polypeptide chains involved in α-polymerization are sterically available to one another only in the structure of a fibril. This hypothesis is supported by our findings on the presence of α-polymers in des*AB*-fibrin produced in the presence of 1.6 M urea. Moreover, there are also direct confirmations that the production of α-polymers correlates in time with the rate of gelation [38]. Thus, it is supposed that γ-dimers with any orientation should stabilize double-stranded protofibrils due to presence of isopeptide bonds, whereas α-polymers appear between protofibrils and stabilize fibrils. In general, this is responsible for formation in fibrin of a strong carcass of covalent bonds.

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