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Self-Assembly of Fibrin Monomers and Fibrinogen Aggregation during Ozone Oxidation

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Abstract—The mechanism of self-assembly of fibrin monomers and fibrinogen aggregation during ozone oxidation has been studied by the methods of elastic and dynamic light-scattering and viscosimetry. Fibrin obtained from oxidized fibrinogen exhibits higher average fiber mass/length ratio compared with native fibrin. Fibrinogen ozonation sharply reduced the latent period preceding aggregation of protein molecules; however, the mechanism of self-assembly of ozonated and non-ozonated fibrinogen cluster was identical. In both cases flexible polymers are formed and reaching a certain critical length they form densely packed structures and aggregate. Using infrared spectroscopy, it has been shown that free radical oxidation of amino acid residues of fibrinogen polypeptide chains catalyzed by ozone results in formation of carbonyl, hydroxyl, and ether groups. It is concluded that fibrinogen peripheral D-domains are the most sensitive to ozonation, which causes local conformational changes in them. On one hand, these changes inhibit the reaction of longitudinal polymerization of monomeric fibrin molecules; on the other hand, they expose reaction centers responsible for self-assembly of fibrinogen clusters.

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Key words: fibrinogen, fibrin, self-assembly, oxidation, structure

Fibrinogen is a soluble plasma membrane protein playing a central role in the process of fibrin formation. The fibrinogen molecule is a dimer, which consists of three pairs of polypeptide chains ($A\alpha$, $B\beta$, and $\gamma)_2$, forming major domains: central E-domain, two peripheral D-domains, and two αC-domains representing C-terminal regions of $A\alpha$-chains. E- and D-domains are linked together by rigid supercoiled regions, which include segments from all three polypeptide chains. The fibrinogen molecule has a unique structure, because being a globular protein it has structural elements of fibrillar proteins, particularly, high asymmetry (about five) and at high longitudinal length (~45 nm) this allows the formation of highly organized rod-like two-stranded fibrin protofibrils. The central E-domain contains two polymerization sites, A and B "knobs", shielded by fibrinopeptides A and B. Peripheral domain D has initially opened polymerization sites *a* and *b* "holes", which are complementary to polymerization sites in the E-domain. The action of thrombin on fibrinogen results in cleavage of fibrinopeptides and exposure A and B "knobs". Self-assembly of protofibrils occurs due to E–D interdomain interaction of adjacent monomeric molecules, which associate in a half-staggered manner. Upon reaching a critical length, protofibrils undergo lateral association and form fibrils, which subsequently branch and represent a basis for three-dimensional structure of the fibrin gel [1, 2].

Based on previous experimental data on structure and function of fibrinogen, it is suggested that fibrinogen molecules can undergo local conformational transitions in solution during their incubation under conditions close to physiological ones [3, 4]. This results in accumulation of so-called "defective" molecules with impaired native spatial organization of peripheral D-domains; this causes opening of new reaction sites, which principally differ from the polymerization sites *a* and *b* "holes". This initiates a self-assembly process in which fibrinogen molecules interacts in a tail-to-tail manner with formation of flexible chain polymers [5]. It has also been demonstrated that fibrin formed from "defective" fibrinogen is characterized by a rougher structure and higher average fiber mass/length ratio compared with native fibrin [6]. However, the physicochemical mechanism of molecular aging of fibrinogen (i.e. accumulation of its "defective" molecules) remains to be clarified.

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Now it is generally accepted that many amino acid residues of proteins are sensitive to oxidation by various reactive oxygen species (ROS). Free radical oxidation of proteins can be accompanied by cleavage of polypeptide chains, modifications of amino acid residues, and protein conversion into derivatives highly sensitive to proteolytic degradation [7, 8]. Proteins subjected to oxidative modification exhibit age-related accumulation in the body, as well as accumulation in oxidative stress and various diseases [9]. It was shown that fibrinogen is 20-times more sensitive to oxidative modification than other major plasma proteins (albumin, immunoglobulins, transferrin, and ceruloplasmin) [10]. Fibrinogen is easily involved in reactions of free radical oxidation; this results in formation of oxidized forms of this protein, which differ from the native form by both chemical composition and structural organization. This causes changes in the functional properties of fibrinogen. During free radical attack, it can form a macromolecular cluster due to formation of noncovalent bonds [11]. Oxidized fibrinogen inhibits formation of a fibrin clot catalyzed by thrombin [12, 13], adhesion and aggregation of platelets [14]; it stimulates activity of tissue plasminogen activator [15], exhibits negative influence on hemorheological parameters [16]; oxidized fibrinogen is actively involved in the process of vascular wall inflammation; it is more effective than native fibrin in induction of interleukin-8 production by primary culture of human vascular endothelium [17, 18].

One may suggest that all the above-mentioned properties of oxidized fibrinogen are determined by conformational conversions in its molecules accompanied by appearance of new reaction centers. We do believe that some of these reaction centers are the self-assembly sites, which are opened during incubation of native fibrinogen in solution.

Thus, the goal of this study was to demonstrate that the physicochemical mechanism of molecular aging of fibrinogen is determined by processes that are identical to either its spontaneous oxidation (during incubation of this protein in solution) or induced oxidation. In this study, we have investigated kinetics of aggregation of oxidized fibrinogen and formation of fibrin gel (in order to compare spatial organization of resultant structures with those obtained from "defective" fibrinogen).

MATERIALS AND METHODS

Bovine fibrinogen was obtained from citrated plasma and additionally purified from plasminogen contaminations and a fibrin stabilizing factor as described earlier [19]. The resulting fibrinogen preparation was transferred into 0.06 M phosphate buffer, pH 7.4, containing 0.15 M NaCl by gel filtration using Sephadex G-25.

Fibrinogen solutions (concentration of 7 mg/ml) were ozonated in a reactor ($3.3 \cdot 10^{-3}$ liter) equipped with quartz windows by insufflation of ozone/oxygen mixture through the free volume of the reactor. The ozone content in the reactor monitored spectrophotometrically at 254 nm varied from $2 \cdot 10^{-7}$ to $6 \cdot 10^{-7}$ M. The kinetic curve of ozone absorption by protein suggests exponential mode of ozone absorption (Fig. 1).

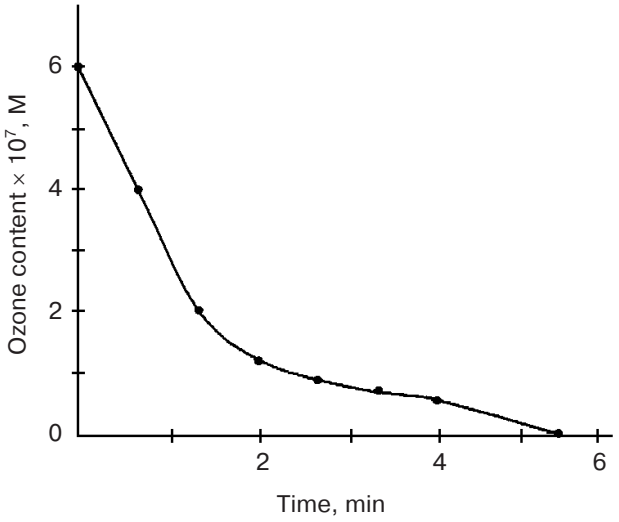

Fig. 1. Kinetic curve of ozone absorption by fibrinogen.

Functional group composition was determined using a Brucker VERTEX 70 FTIR spectrophotometer (Brucker, USA) with resolution of 4 cm^{-1}. IR spectra of aqueous solutions (suspensions) of initial and ozonated fibrinogen were recorded in transmission mode. Protein aliquots (10 μl; concentration 7 mg/ml) were layered onto a silica surface and carefully dried under identical conditions (air, room temperature). The silica support with applied sample was fixed at a magnetic holder for film samples and the IR spectrum was recorded. Molecular masses of $A\alpha$, $B\beta$, and γ polypeptide chains of these proteins were evaluated by electrophoretic analysis of reduced samples.

Fibrinogen was converted into fibrin by a method described earlier [6]. Kinetics of fibrinogen aggregation in solution was evaluated by elastic and dynamic light-scattering using a Malvern spectrometer (England) with the range of angle scattering θ of 20-140° and a helium–neon laser light source ($\lambda = 632.8$ nm). Dust was removed from solutions by ultrafiltration. Distilled benzene was used as the standard; the Rayleigh ratio of R_{90} for benzene measured at this wavelength was assumed to be $8.5 \cdot 10^{-6}$ cm^{-1} [20]. The increment of the refraction parameter was determined using a KMX-16 differential refractometer (Chromatix, USA).

The coefficient of translational diffusion $\overline{D}$ was determined by dynamic light scattering based on values of autocorrelation functions of fluctuations of intensity of scattered light as described earlier [19]. The Z-average

diffusion coefficient $\overline{D}_Z$ was determined by graphic extrapolation of $\overline{D}$ to zero scattering angle. Average molecular masses M_w and angle dependence of the average scattering factor $P(\theta)$ were determined from elastic light scattering data [20].

Viscosimetric studies were carried out using Ostwald thermostatted viscosimeters.

Plots were prepared using the Origin 6.0 program.

RESULTS AND DISCUSSION

Ozonated fibrinogen was subjected to biochemical analysis. Electrophoregrams of reduced samples demonstrate that all three pairs of polypeptide chains $A\alpha$, $B\beta$, and γ of the oxidized protein are identical to native fibrinogen, i.e. oxidation conditions used in our experiments did not cause either intermolecular covalent cross-links or intra-chain fragmentation (Fig. 2, a and b).

Figure 3 shows the differential IR spectrum of ozonated versus native fibrinogen. There were changes in protein absorbance bands in the region 1650-1550 cm^{-1}, typical for carbonyl amides. Appearance of a new band at 1740 cm^{-1} suggests that fibrinogen oxidation by ozone is accompanied by accumulation of carbonyl groups, and also by OH- and C–O-groups indicating formation of ethers or alcohols. Analysis of IR-spectroscopy data has shown that the absence of characteristic bands in the spectrum at 1655-1625 cm^{-1} suggests maintenance of fibrinogen α- and β-structures during oxidation [21]; on the other hand, changes in protein absorbance bands in the region 1650-1550 cm^{-1} indicate that oxidation results in local conformational changes in this protein possibly due to appearance of new polar chemical groups that can form additional intramolecular hydrogen bonds. Our results on fibrinogen oxidation are consistent with results of studies on ozonation of other proteins [22]. Their oxidation was also accompanied by accumulation of carbonyl and other polar groups and sensitivity of amino acid residues of these proteins to oxidation decreased in the following order: Met, Trp, Tyr, His, Phe.

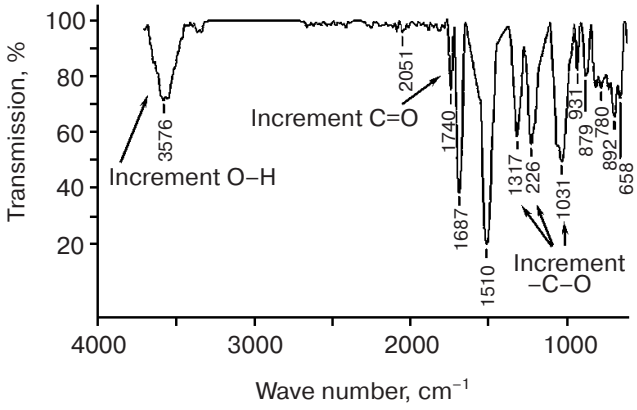

Fig. 3. Differential IR spectrum of ozonated versus native fibrinogen.

Oxidized fibrinogen could be also converted into a fibrin gel after thrombin addition, but the time required for its formation (45 sec) significantly increased compared with control (15 sec). Angle dependencies of the Rayleigh ratio $R(\theta)$ for normal and oxidized fibrin gels were obtained by elastic light scattering. These dependencies represent straight lines passing through the origin and differing by the tangent of the slope angle (Fig. 4). It was already discussed [6] that according to the theory of light scattering [20, 23], in the case of long and thin rod-like particles the Rayleigh ratio is described by the following expression:

$$R(\theta) = cK\lambda_0\mu/4n\sin(\theta/2), \quad (1)$$

where c is protein concentration, λ_0 is light wavelength in vacuum, $\mu = M_w/l_w$ is a ratio of average mass to average length of a polymer chain expressed in g/(mol·cm), and n is refraction coefficient in solution. K, the constant for a vertically polarized beam is written as follows:

$$K = 4\pi^2 n_0^2 (dn/dc)^2 (N_A\lambda_0^4), \quad (2)$$

where N_A is Avogadro's number, dn/dc is the increment of the refraction parameter. According to formula (1), the μ

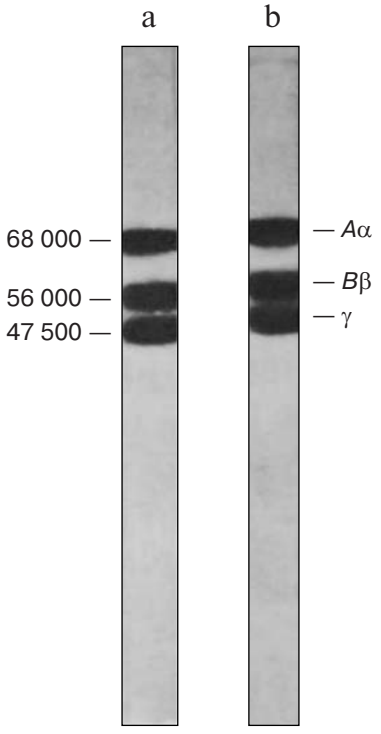

Fig. 2. Electrophoregrams of reduced samples of nonoxidized (a) and oxidized (b) fibrinogen. On the left, molecular mass in Daltons.

value can be calculated from the slope (tanα). In the case of fibrin polymers formed from native fibrinogen (Fig. 4, line *1*) this value is $14.5 \cdot 10^8$ g/(mol·cm), for fibrin polymers formed from ozonated fibrinogen (line *2*) it is $25.0 \cdot 10^8$ g/(mol·cm). Since for two-stranded fibrin protofibrils $\mu = 1.3 \cdot 10^{11}$ g/(mol·cm) [24], it is clear that fibrils represent rod-like formations with cross-sections significantly (tens folds) higher than μ of protofibrils. Fibrinogen ozonation determines formation of relatively "rougher" gels with thicker fibers than in native gel. Since first of all gel structure is determined by kinetic factors (i.e. the ratio of rate constants for reactions of longitudinal polymerization of monomeric fibrin and lateral aggregation of protofibrils), it appears that ozonation impairs the interaction between E- and D-domains of monomeric fibrin molecules.

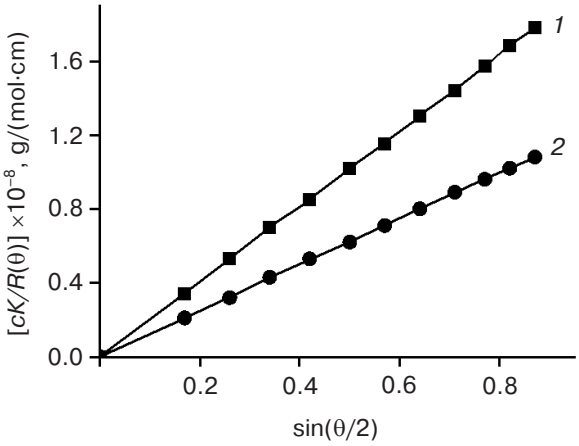

Fig. 4. Angular dependences of light scattering intensity for various fibrin gels: *1*) obtained from native fibrinogen; *2*) fibrin formed from ozonated fibrinogen.

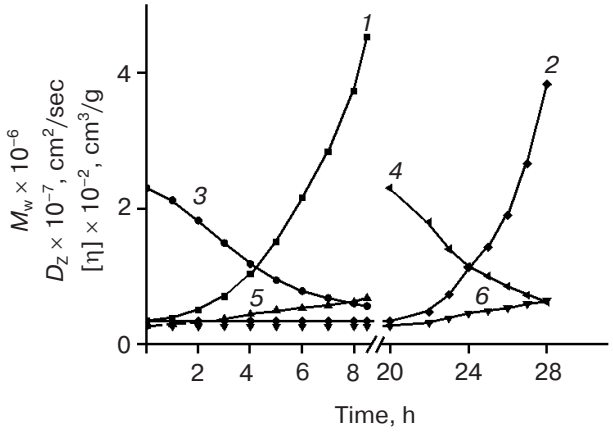

Fig. 5. Time dependences of light scattering intensity (curves *1* and *2*), translational diffusion coefficient (curves *3* and *4*), and characteristic viscosity (*5* and *6*). *1*, *3*, *5*) Induced oxidized fibrinogen; *2*, *4*, *6*) native fibrinogen.

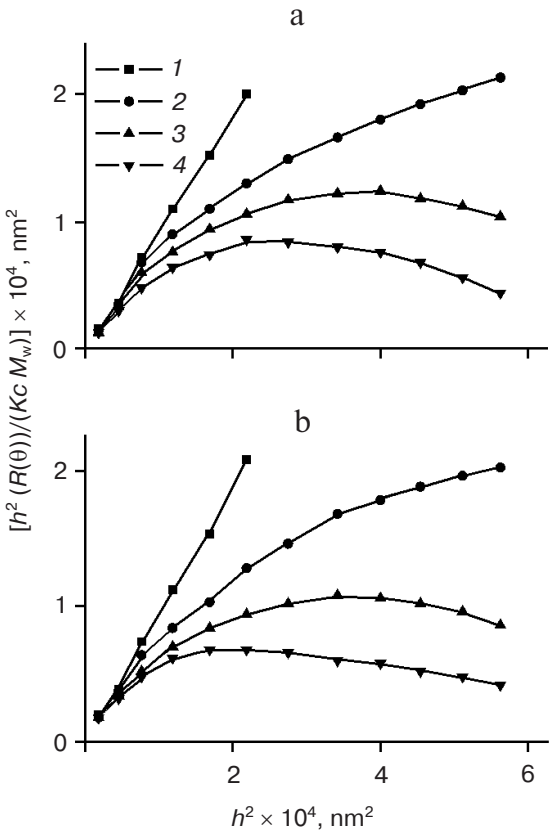

Fig. 6. Kratky–Porod plots for ozonated (a) and non-ozonated (b) fibrinogen polymers at various incubation times. a: *1-4*) 0, 2, 4, and 8 h, respectively. b: *1-4*) 0, 22, 26, and 28 h, respectively.

Oxidized fibrin acquires ability for self-association (Fig. 5). However, right after fibrinogen oxidation its physicochemical characteristics (molecular mass of 340 ± 10 kD, translational diffusion coefficient of $(2.30 \pm 0.10) \cdot 10^{-7}$ cm²/sec, characteristic viscosity of 27 ± 2 cm³/g) perfectly correspond to native fibrinogen. Studies of hydrodynamic and optical properties of ozonated fibrinogen (curves *1*, *3*, and *5*) show significant shortening of the latent period preceding aggregation of protein molecules compared with control (curves *2*, *4*, and *6*). Gradual increase in average molecular masses calculated by angle dependences of light scattering intensity of polydisperse (by size and mass) macromolecules in Zimm plots, the increase in characteristic viscosity, and the decrease in translational diffusion coefficient indicate formation of high molecular weight products due to aggregation reactions of oxidation-induced and native fibrinogen.

The Kratky–Porod plots of angle dependence of light scattering intensity can be used to evaluate spatial organization of fibrinogen polymers formed during the self-assembly process (Fig. 6). Near zero time both ozonated and control protein (curves *1*) are close to a straight line. According to calculations made for various molecular models [5, 25], this indicates rod-like shape of the fibrino-

gen molecule. During assembly the angle dependences deviate from straight line (curves *2*); this corresponds to polymers lacking structural rigidity. According to the extreme mode of light scattering curves at M_w values exceeding ~$3 \cdot 10^6$ Daltons (curves *3* and *4*), polymers are folded into rather compact branched structure.

Independent information about spatial organization of biopolymers can be obtained by hydrodynamic methods. It is known that in first approximation changes in translational diffusion coefficient and characteristic viscosity in a polymer homolog series are determined by the Mark–Kuhn–Houwink expressions:

$$D_Z = K_D M_w^{-b}, \quad (3)$$

$$[\eta] = K_\eta M_w^{\alpha}, \quad (4)$$

where K_D, K_η, b, and α are constants for a selected polymer–solvent system. Use of average molecular masses in Eqs. (3) and (4) for analysis of a series of homologous fibrinogen polymers brings minor inaccuracy in b and α [5]. The dependences of characteristic viscosity [η] (Fig. 7, curves *1*) and $\overline{D}_Z$ (Fig. 6, curves *2*) on average molecular mass of polymer homologs show the slope of the curves plotted using Eqs. (3) and (4) monotonously decreases with increase in M_w. This suggests that polymeric chains lacking backbone rigidity are formed on the early stages of self-assembly of fibrinogen molecules. The decrease in b and α values from initial 0.60 and 0.63 to final 0.35 and 0.20, respectively, is an indicator for progressing compactness of molecular structures (formed during assembly) that is determined by strong chain branching. However, we have not found any principal differences in spatial organization of fibrinogen polymers formed from ozonated or non-ozonated protein. It should be noted that under our experimental conditions concentration effects on optical and hydrodynamic properties of polymer fibrinogen solutions can be ignored [19].

Thus, results of physicochemical studies suggest that both spatial organization of oxidized fibrinogen polymers and structure of resultant fibrin are identical to those structures of fibrinogen and fibrin aggregates which were formed from "defective" fibrinogen preincubated in solution.

Earlier using elastic light scattering, it was shown that fibrin formed from "defective" fibrinogen is characterized by a rougher structure with higher ratio of average fiber mass/length ratio compared with native fibrin [6]. The authors suggested that a putative reason for appearance of such abnormal fibrin structures consists in conformational lability of fibrinogen molecules especially in the region of D-domains. We suggest that oxidation of fibrinogen molecules causes structural changes in terminal regions of the fibrinogen molecules.

It is known that fibrinogen conversion into fibrin involves two reactions. An enzymatic reaction, catalyzed

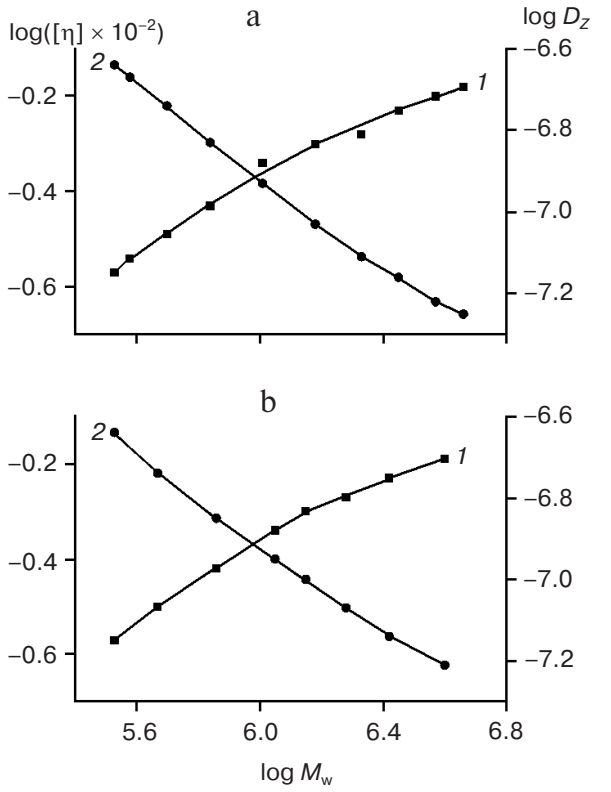

Fig. 7. Dependence of characteristic viscosity (curves *1*) and Z-average coefficient of translational diffusion (curves *2*) on average molecular mass for ozonated (a) and non-ozonated (b) fibrinogen.

by thrombin, involves cleavage of two pairs of fibrinopeptides A and B; this opens two polymerization sites, *A* and *B* "knobs". Self-assembly of monomeric fibrin molecules is determined by interaction of *A* and *B* "knobs" (domain E) with *a* and *b* "holes", localized in the region of D-domains. This results in formation of two-stranded rod-like protofibrils, which aggregate, branch, and finally form gel network. The E-domain is more resistant to external treatments compared with D-domain [6]. Damage of native structure of D-domains well explains our results on formation of "rough" fibrin gels from oxidized fibrinogen. It appears that oxidation causes impairments in structure of D-domains (particularly γC-subdomains related to the polymerization site *a* "holes"). In this case, the interactions *A* "knobs and *a* "holes" become weaker, the rate of longitudinal polymerization, which occurs as "tail-to-center" reduces, whereas the ability of protofibrils for lateral aggregation remained unaltered.

This viewpoint is consistent with data on inhibition of fibrin formation during metal-catalyzed fibrinogen oxidation [13]. Those authors have shown that fibrinogen oxidation does not cause fibrinopeptide cleavage and the decrease in clotting is determined only by impairments of

polymerization capacity of monomeric fibrin molecules. It was also found that fibrinogen oxidized by chloramine sharply increases activity of tissue plasminogen activator due to structural conversions in D-subunits of fibrinogen [15].

Conformational changes that occur in the region of D-domains of the fibrinogen molecule during its oxidation explain well the phenomenon of formation of flexible branched fibrinogen polymers. It was already mentioned that during oxidative stress fibrinogen molecules acquire the ability to aggregate due to noncovalent bonds [11]. We suggest an ozone effect on amino acid residues (first of all of aromatic and sulfur-containing residues, as the most sensitive to ozone oxidation [22]) results in exposure of reaction centers responsible for axial contacts in D-domain. In our viewpoint, E-domain is the most resistant to oxidation. Comparative study of proteins with similar molecular mass and amino acid composition of their subunits has shown that sensitivity to ozone oxidation depends not only on primary but also secondary and tertiary structures [22]. This may be the case. It is possible that aromatic and sulfur-containing amino acid residues localized in E-domain (which significantly differs in its three-dimensional structure from spatial organization of D-domain) are less susceptible to the action of ozone. Interaction of two oxidized fibrinogen molecules results in formation of dimers due to contacts of D-domains of adjacent molecules in a "tail-to-tail" manner. Subsequent growth of the chain is determined by sequential attachment of protein molecules and structural flexibility of polymers determines their subsequent folding. Such structures totally coincide with those observed during aggregation of "defective" fibrinogen [19].

Thus, based on results of our study we conclude that induced oxidation of fibrinogen results in impairments of its functional properties due to conformational changes involving D-domains. Since spatial organization of polymers of oxidized fibrinogen and structure of fibrin obtained from this fibrinogen are identical to those aggregates of fibrinogen and fibrin, which have been formed from "defective" fibrinogen, one can conclude that the physicochemical mechanism of fibrinogen aging is determined by the process of its spontaneous oxidation during protein incubation in solution. Such interpretation can also explain earlier obtained data [19] on inhibition of fibrinogen aggregation in the presence of albumin. The thing is that at high albumin/fibrinogen molar ratios, albumin acts as an antioxidant scavenging ROS. "Defective" fibrinogen may be involved in reaction of cross-linking catalyzed by fibrin stabilizing factor [3]. Consequently, ozonated fibrinogen should also exhibit such property. Validation of this suggestion will be investigated in our subsequent studies.

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