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Oxidized Modification of Fragments *D* and *E* from Fibrinogen Induced by Ozone

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Abstract—Ozone-induced free-radical oxidation of fragments *D* and *E* from fibrinogen has been studied. The methods of elastic and dynamic light scattering in combination with electrophoresis of unreduced samples have shown the acceleration of enzymatic covalent crosslinking of molecules of oxidation-modified fragment *D* under the action of factor XIIIa. UV and IR spectroscopy shows that free-radical oxidation of amino acid residues of polypeptide chains catalyzed by ozone affects the cyclic and amino groups, giving rise to generation of mainly oxygen-containing products. Comparison of the IR spectra obtained for the oxidation-modified *D* and *E* fragments revealed more significant transformation of functional groups for the *D* fragment. EPR spectroscopy showed that the rotational correlation time of spin labels bound to the ozonized proteins decreased in comparison with the non-ozonized proteins. The rotation correlation time of the radicals covalently bound to the ozonized *D* and *E* fragments suggests that *D* fragment of fibrinogen is more sensitive to free-radical oxidation followed by local structural changes. Possible causes of different degrees of oxidation for fragments *D* and *E* are discussed.

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Key words: fibrinogen, fragments *D* and *E*, factor XIIIa, oxidation, structure

The fibrinogen molecule is a high molecular weight and structurally complex protein comprising two sets of three non-identical polypeptide chains: *A*α, *B*β, and γ. The molecular core including the NH_2-terminal regions of all six polypeptide chains forms central domain *E* containing two polymerization centers (*A* and *B* "knobs") screened by fibrinopeptides *A* and *B*. Domain *E* is connected via superhelical structures with two peripheral domains *D* with originally open polymerization centers (*a* and *b* "holes") complementary to the centers in domain *E*. Also, the COOH-terminal regions of *A*α-chains are packed in α*C*-domains. Under the influence of thrombin, monomeric fibrin is formed from fibrinogen due to the cleavage of fibrinopeptides *A* and *B* from the *A*α- and *B*β-polypeptide chains of the *E*-domain with exposure of polymerization centers: *A* and *B* "knobs". The latter are complementary to the *a* and *b* "holes" localized in the γ*C*- and β*C*-subdomains in the COOH-terminal regions of the *D*-domain, respectively. Monomeric fibrin molecules are bound to each other in such a way that the central domain *E* of one molecule interacts with two *D*-domains of other molecules, which in the end causes formation of structurally rigid rod-like fibrin fibrils. Fibrin polymers are an ideal substrate for fibrin-stabilizing factor (XIIIa). Factor XIIIa, a plasmic transglutaminase, catalyzes the formation of ε/γ-glutamyl-lysine isopeptide covalent bonds. At the same time, the COOH-terminal regions of γ-chains, γ*C*-subdomains contacting each other, are responsible for the emergence of intermolecular γ-dimers, while α-chains of one molecule interact with α-chains of two other molecules leading to formation of multivariate structures, the so-called α-polymers [1-3].

Previously it has been shown that when fibrinogen is incubated for a long time in solution under near-physiological conditions, it acquires the ability to self-associate end to end, i.e. by contacting peripheral *D*-domains of the neighboring protein molecules. As a result, single-stranded flexible chain homopolymers of fibrinogen are formed [4]. Besides, the fibrin formed under the action of thrombin on fibrinogen incubated in solution for a long time before addition of the enzyme [5] has a coarser gel structure with greater mass per fibril length unit. It seems to be due to a decrease in the rate of longitudinal polymerization of monomeric fibrin molecules resulting from

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disturbance of interaction between domains *D* and *E* [1, 5]. Incubation of fibrinogen also determines acceleration of the reaction of its enzymatic cross-linking in the presence of factor XIIIa with formation of a large quantity of γ-dimers [6]. Since all of the above reactions involve domain *D*, the findings suggest that fibrinogen during incubation may undergo local structural transformations apparently touching primarily upon the region of *D*-domains [4-6].

The results of investigation into ozone-induced free radical oxidation of fibrinogen demonstrate that oxidized fibrinogen is a more preferred substrate than non-oxidized protein in the reaction of fibrinogen cross-linking under the influence of factor XIIIa [7]. The spatial organization of the polymers of oxidized fibrinogen and structure of the fibrin obtained from the latter are absolutely identical to the structures of fibrinogen and fibrin aggregates formed from fibrinogen during its incubation [8]. Consequently, the probable physicochemical mechanism of local structural rearrangements of fibrinogens may be determined by processes identical either to spontaneous free-radical oxidation during protein incubation in solution or to induced oxidation.

We believe that the effect of ozone on amino acid residues of fibrinogen, first of all aromatic residues as most susceptible to ozone oxidation [9], may result in local conformational rearrangements in the *D*-domain responsible for the above effects. In our view, the *E*-domain seems to be more resistant to oxidation. This is due mainly to the much lower content of aromatic and other highly reactive amino acid residues compared to the *D*-domain [10]. Besides, as shown by comparative study of proteins with subunits similar in molecular weight and amino acid composition, sensitivity to ozone oxidation depends not only on the primary but also on the secondary and tertiary structures [9]. It is probable that oxidation-susceptible amino acid residues in the *E*-domain, which is significantly different from the *D*-domain in 3D configuration, may be less accessible for ozone. Strict evidence of different oxidizing abilities of the domains can be obtained only when using a fibrinogen molecule without its fragmentation. However, in a first approximation, one can study ozone-induced oxidation of the final products of plasmin-mediated hydrolysis of fibrinogen: fragments *D* and *E*, which are closest in structural organization and chemical composition to domains *D* and *E*.

Under the influence of plasmin, fibrinogen undergoes enzymatic hydrolysis with possible formation of the final reaction products, fragments *D* and *E*, as a result of breaking the peptide bonds between domains *D* and *E*. Being globular proteins, they preserve, to a large extent, the structural organization of domains *D* and *E* of the parent fibrinogen molecule [11]. Fragment *D*, similar to the peripheral domain *D*, consists of three polypeptide chains $A\alpha_D$, $B\beta_D$, and γ_D. During the moderate enzymatic hydrolysis of fibrinogen by plasmin, fragment *D* with the highest molecular weight of 100 kDa is formed at a certain stage due to reaction block [10]. It is known that with such molecular weight its structure corresponds to the fragments of *A*α105-197, *B*β134-461, and γ63-411 chains of fibrinogen [12, 13]. Possessing the originally spatially-open polymerization centers, i.e. *a* and *b* "holes" localized in the γ*C*- and β*C*-subdomains, fragment *D* formed under moderate fibrinogen hydrolysis can participate as an inhibitor in self-assembly of monomeric fibrin, competing with its *D*-domains for the complementary regions located in domain *E*. Besides, fragment *D* preserves intact γ-polypeptide chains in the COOH-terminal region and thus is able to form *D*-dimers in the presence of factor XIIIa [14]. Fragment *D* molecules are dimerized through formation of peptide bridges between lysine in position 406 of one γ-chain and glutamine in position 398 of the other chain [1]. More extensive plasmin hydrolysis of fibrinogen leads to formation of a lighter fragment *D* due to γ-chain degradation in its COOH-terminal region. As a result, such fragment *D* loses its anti-polymerization activity and the ability to be involved in the enzymatic reaction of dimerization [15]. Fragment *E* originates from the central part of the fibrinogen molecule. It is a chemical dimer and includes the *N*-terminal regions of all six polypeptide chains connected by a system of disulfide bonds. The structure of fragment *E* of fibrinogen with the highest molecular weight of 50 kDa, which is formed under the moderate effect of plasmin, can be written as: $[A\alpha 1\text{-}78,\ B\beta 53\text{-}121,\ \text{and}\ \gamma 1\text{-}62]_2$ [16]. It contains fibrinopeptides *A*, which screen the self-assembly centers *A* "knobs" complementary to the centers *a* "holes". Besides, similar to domain *E*, it preserves a thrombin-binding region and thereby can inhibit enzymatic transformation of fibrinogen into fibrin [1].

This paper presents a study of ozone-induced free-radical oxidation of the final products of fibrinogen hydrolysis: fragments *D* and *E* formed under the moderate effect of plasmin. The goal of this work was to obtain evidence of much higher sensitivity of fragment *D* to free-radical oxidation compared to fragment *E*. In turn, it must promote local conformational changes in fragment *D* as a result of oxidative modification and its more complete involvement in the process of *D*-dimer formation compared to non-oxidized fragment.

MATERIALS AND METHODS

Fibrinogen used for obtaining the final products of plasmin hydrolysis, fragments *D* and *E*, was isolated from citrated bovine blood plasma followed by additional purification from the admixtures of plasminogen and fibrin-stabilizing factor [4]. The quantity of coagulable protein in the preparation was 98%. Fibrinogen preparation was transferred into 0.05 M Tris/0.15 M NaCl buffer, pH 7.2, by gel filtration on Sephadex G-25.

Lys-plasminogen was obtained from donor blood by affinity chromatography on lysine-CNBr-Sepharose 4B (Amersham, England) by the method described previously [17]. For removal of aminocaproic acid and concentration of the protein, Lys-plasminogen was precipitated with $(NH_4)_2SO_4$ (0.31 g per ml of solution); the precipitate was dissolved in 0.05 M Tris/0.15 M NaCl buffer, pH 7.2, and dialyzed against the same buffer. The resulting Lys-plasminogen was converted into plasmin by streptokinase activation (Bechringwerke, Germany). Streptokinase was added to Lys-plasminogen at 125 units of activity per ml of proenzyme solution. Activation was performed for 10 min at 37°C. The activity of the resulting plasmin was measured by the method of Robbins and Summaria [18]; it was 11.3 caseinolytic units (C.U.) per mg of the protein.

The final products of fibrinogen cleavage, fragments *D* and *E*, were formed as a result of moderate plasmin hydrolysis of fibrinogen and isolated as described [19]. Plasmin hydrolysis of fibrinogen was performed by adding plasmin at 3 C.U. per ml of the mixture at 37°C for 6 h in 0.05 M Tris/0.15 M NaCl buffer, pH 7.2, in the presence of Ca^{2+} at the final concentration of $5 \cdot 10^{-3}$ M. The reaction was stopped by adding aprotinin (Sigma, USA) to the reaction mixture: 20 kallikrein units per ml of lysate. Then the hydrolysate was released from plasmin by affinity chromatography on lysine-CNBr-Sepharose 4B under the same conditions as during the isolation of Lys-plasminogen [17]. Fibrinogen degradation products were separated by ion-exchange chromatography on CM-Sephadex C-50 [20]. The fractions corresponding to each fragment were combined, concentrated by ammonium sulfate at 50% saturation, and dialyzed against 0.15 M NaCl solution containing $5 \cdot 10^{-3}$ M $CaCl_2$, pH 7.4. Concentrations of the fragments were determined by spectrophotometry with coefficient $A_{280}^{0.1\%} = 1.02$ for fragment *E* and 2.08 for fragment *D*. The purity of fragments *E* and *D* was tested in the electrophoresis of unreduced samples in 5% polyacrylamide gel in the presence of 0.1% SDS and 8 M urea. The molecular weights of fragments *E* and *D* were 50 and 98 kDa, respectively. The anti-polymerization and antithrombin activities of fragments *D* and *E* were estimated by increase in the time of fibrinogen conversion into fibrin [19]. At equimolar fibrinogen/fragment *D* ratio, the time of fibrin formation increased by more than 40% compared to the control. The time of fibrin formation increased by almost 10% at the fibrinogen/fragment *E* molar ratio of 1 : 0.5 and by 15% at their equimolar ratio.

Fibrin-stabilizing factor (factor XIII) was isolated from bovine blood by the method of Lorand et al. [21]. Conversion of factor XIII into active form (XIIIa) under the influence of thrombin (Roche, France) and subsequent inactivation of thrombin by antithrombin III–heparin mixture were performed as described previously [6]. Complete inactivation of thrombin was verified on chromogenic substrate *S*-2238 according to the method of Svendsen et al. [22]. The activity of factor XIIIa was 320 standard units per ml (1 standard unit (SU) corresponds to the activity of factor XIIIa in 1 ml of donor blood serum).

The intact and oxidized *D* fragments were cross-linked in 0.15 M NaCl, $5 \cdot 10^{-3}$ M $CaCl_2$, pH 7.4. Factor XIIIa (0.02 ml or 6.4 SU) was added to 1 ml of fragment *D* (ozonized and non-ozonized samples) at the concentration of 2 mg/ml. The reaction of cross-linking was stopped after 30 and 60 min by adding a mixture of urea (8 M) and SDS (2%). Cross-linking of the fragments was detected during electrophoresis of unreduced samples in 5% polyacrylamide gel in the presence of urea and SDS. A commercial protein kit (Amersham) was used as molecular weight markers.

We have chosen ozone as an oxidizer. This natural reagent, an active oxygen forms, is extremely convenient for model system studies as has been mentioned before [7]. Its half-life time in water solutions in the presence of oxidation substrate is less than 1-2 min. Oxidation degree is strictly regulated because the amount of ozone reacting with the reducer is accurately estimated by spectrophotometry at 254 nm. The solutions of degradation fragments (7 mg/ml) were ozonized in a reactor ($3.3 \cdot 10^{-3}$ liter) by blowing the ozone–oxygen mixture through the free volume of the reactor [7]. The amount of ozone in the reactor was $3 \cdot 10^{-7}$ M.

The UV protein spectra before and after ozonation were recorded using an SF-2000 spectrophotometer (Russia) in 1-cm quartz cells.

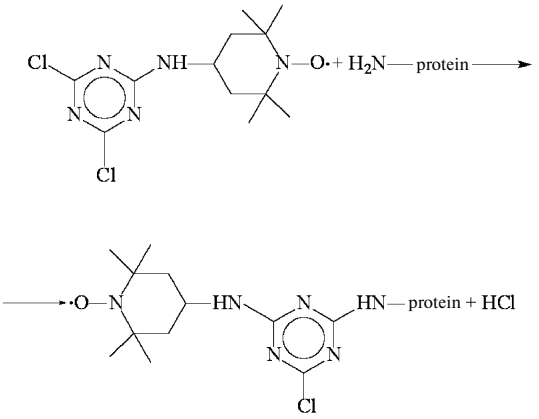
The IR spectra of the samples of initial and ozonized fibrinogen fragments *D* and *E* were recorded using a VERTEX-70 Fourier-IR spectrometer (Bruker, Germany) with a DTGS detector, with averaging of 256-512 accumulated scans with a resolution of 4 cm^{-1} [8]. The samples for measurement were prepared by applying 5-10 μl of aqueous sample solution onto the surface of a silicon plate (2 × 2 × 0.1 cm) followed by evaporation of the water at room temperature to constant weight. The IR spectra of dry residue were recorded in pass-through mode using a Microfocus add-on device (Perkin-Elmer, England) equipped with a CaF_2 lens.

Values of *Z*, the mean coefficients of translational diffusion D_z of the degradation fragments *D* and *E* before and after ozonation and the products of association of fragment *D* molecules during their incubation with fibrin-stabilizing factor, were determined by the dynamic light scattering in a Zetasizer Nano-S spectrometer (Malvern, England) with a detection angle of 173°. The weight average molecular weights M_w of the proteins were found by the method of Rayleigh light scattering based on the values of light scattering intensity in a 4400 spectrometer (Malvern) with a K7025 multi-bit 64-channel correlator in cylinder cells in the scattering angle range of θ: 30-130° [4]. The light source in both devices was a helium-neon laser with a wavelength of 632.8 nm. All protein solutions with concentrations of 2 mg/ml in the experiment were

thoroughly freed of dust by ultrafiltration. The refractive index increments of the proteins were determined using a Chromatix KMX-16 differential refractometer (USA).

The structural and dynamic changes in fragments *D* and *E* during ozonation were estimated by EPR spectroscopy using a spin label [23-25]. The stable chlorine-containing nitroxyl radical was used as the label.

Label solution (25 μl) in 96% ethyl alcohol (~$7\cdot10^{18}$ spin/ml) was mixed with 1 ml of protein fragment solution (2 mg/ml) in phosphate buffer, pH 8.5, at room temperature. The label-containing solution was allowed to stand for 2 h and then dialyzed for 12 h against the initial phosphate buffer. The covalent binding of radicals with protein macromolecules proceeded by the following Scheme [26]:



The number of labels on the macromolecules was estimated by comparing the intensities of signals from the EPR spectra of radicals before and after dialysis: ~12 and ~5 label molecules on fragments *D* and *E*, respectively.

The EPR spectra were recorded using a Bruker EMX 2.7/8 spectrometer (Germany) in the X-range at 20 ± 0.5°C. SHF power was 10 mW; modulation amplitude was 1 G. Calcined magnesium oxide powder with Mn^{2+} was used as an external standard. The following parameters were determined on the basis of EPR spectra: the intensity of extreme components of the nitrogen triplet ($I_{+1,-1}$) and the width (ΔH_{+1}) of low-polar component of the nitrogen triplet; on their basis, the correlation times of label rotation (τ) in the interval of 0.1-1.0 nsec were calculated by the formula [22]:

$$\tau = 6.65\cdot10^{-10}\,\Delta H_{+1}\,[\{I_{+1}/I_{-1}\}^{1/2} - 1].$$

The correlation times and the share of slowly rotating labels (τ > 1 nsec) were analyzed by the tabulated spectra [24].

RESULTS

The values of Z-average coefficients of translational diffusion and weight-average molecular weights for the isolated fibrinogen fragments *D* and *E* formed under the moderate effect of plasmin were obtained by dynamic and elastic light scattering: $(4.95 \pm 0.25)\cdot10^{-7}$ and $(6.25 \pm 0.34)\cdot10^{-7}$ cm^2/sec, 99 ± 4.1 and 50 ± 3.5 kDa, respectively. The values of these physicochemical parameters are in good agreement with the literature data [27]. Induced protein oxidation does not alter these values immediately after ozonation, because neither formation of intermolecular covalent cross-links nor intra-chain fragmentation can be observed under the selected oxidation conditions [8]. Besides, ozonation has no effect on the values of hydrodynamic radii of the molecules, indicating the absence of large-scale structural rearrangements in the proteins and maintenance of the degree of their hydration. Incubation of ozonized fragment *E* is not attended by changes in its physicochemical parameters. However, during incubation of oxidized fragment *D* in solution, the weight-average molecular weight increases and the coefficient of translational diffusion decreases (Fig. 1), indicating the tendency of macromolecules to self-association. On addition of fibrin-stabilizing factor to the solution of both non-oxidized and oxidized *D* fragments, D_z and M_w change due to the reaction of enzymatic cross-linking of molecules. At the same time, as one can see from Fig. 1, ozonation accelerates this reaction. Based on the values of weight-average molecular weights connected by a simple ratio with the molecular weights of fragments *D* and *D*-dimer: $M_w = w_1M_D + w_2M_{2D}$, where w_1 and w_2 are mass fractions of fragments *D* and *D*-dimer, it is not difficult to calculate the content of fragment *D*-dimer in the control (non-oxidized) and experimental samples. In 1 h after the enzymatic reaction it was 0.18 and 0.27 mg/ml, respectively, i.e. only 9% (in the control) and about 14% (in the experiment) of fragment *D* molecules were subject to covalent cross-linking.

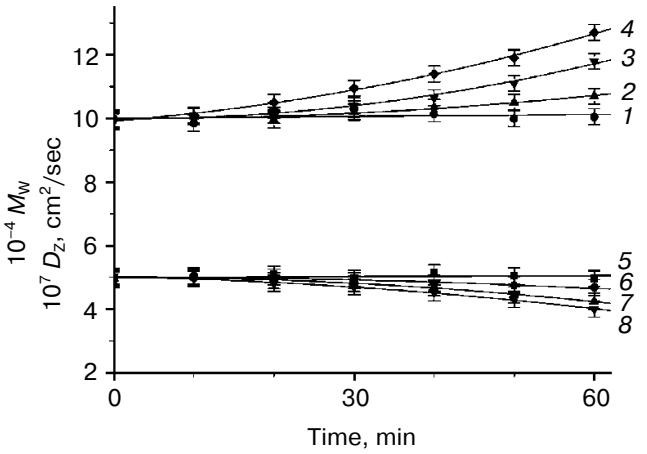


Fig. 1. Kinetic curves of variations in weight-average molecular weight (*1-4*) and mean coefficient of translational diffusion *Z* (*5-8*) for fragment *D* before (*1*, *3*, *5*, *7*) and after ozonation (*2*, *4*, *6*, *8*); in the presence of factor XIIIa (*3*, *4*, *7*, *8*).

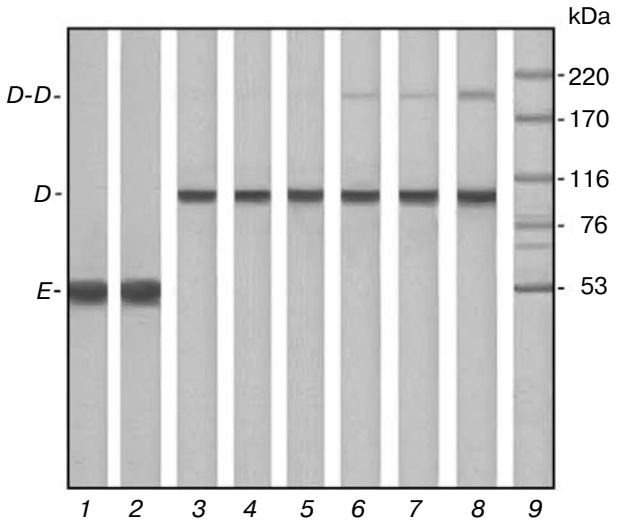


Fig. 2. Electrophoregrams of unreduced samples of non-ozonized and ozone-induced oxidized fragments *E* and *D*: *1*, *2*) control and oxidized fragment *E*, respectively; *3*, *4*) control and ozonized fragment *D*, respectively; *5*, *6*) control and ozonized fragment *D* in the presence of factor XIIIa after 30 min of enzymatic reaction; *7*, *8*) the same samples after 60 min; *9*) molecular weights of the markers.

Formation of covalently bound fragments *D* is confirmed by the data of electrophoresis of unreduced samples (Fig. 2). As one can see from the electrophoregram, the preparation of fragment *D* is actually homogenous and contains the major component with a molecular weight of 98 kDa. The available minor quantity of the lighter fraction is due to the polyvariance of degradation of the γ-polypeptide chain residue in the COOH-terminal region [28]. Ozonation of both fragment *D* and fragment *E* has no effect on their electrophoretic mobilities.

D-Dimer molecules of 200 kDa are formed in the presence of factor XIIIa, and their quantity in experimental samples is higher than in the control. After 30 min, even traces of *D*-dimer cannot yet be found in the control samples, while the oxidized samples contain a high molecular weight product. Acceleration of the enzymatic reaction of covalent crosslinking of oxidized fragment *D* is in good agreement with the above light scattering results.

The pattern of changes in the UV spectra of fragments in the region of 280 nm (Fig. 3) demonstrates that ozone actively interacts with aromatic amino acid residues of tryptophan, tyrosine, and phenylalanine of the *E* and *D* protein molecules. The shift of absorption peaks to shorter wavelength and decrease in their intensities are due to formation of quinoid structures from the phenoxyl and imidazole nuclei of amino acid residues under the influence of ozone. Besides, as can be seen from the spectra, fragment *D* is oxidized to a much greater extent than fragment *E*. The findings correlate rather well with the IR spectroscopy data presented in Fig. 4. Differential IR spectra show noticeable transformation of functional groups due to oxidation reactions under the influence of ozone on macromolecules of fragments *E* and *D*, while the general pattern of changes in the spectra of ozonized proteins *E* and *D* actually coincides with those observed previously for oxidized fibrinogen [8]. In particular, in the high-frequency region (3600-2800 cm^{-1}) both fragments *E* and *D* show a considerable decrease in the content of N–H- (3307-294 cm^{-1}) and phenoxy-OH groups (3400-3500 cm^{-1}). A certain decrease in absorption of C–H groups in the region of 3100-3050 cm^{-1} in the spectrum of fragment *E* it probably associated with transformation of a share of phenoxyl rings into quinoid structure. The death of some part of N–H groups as a result of ozonation of both fragment *E* and fragment *D* is accompanied by decrease in the intensity of amide-I and amide-II bands (the maxima of the bands are close to 1660 and 1550 cm^{-1}, respectively), characterizing the micromobility of the environment of N–H groups and the packing of α-helical configuration of polypeptide chains. In addition, the differential IR spectrum of fragment *D* shows an increment of intensity of absorption bands at frequencies close to 1685 and 1630 cm^{-1}, which are known to be typical of β-structures. Thus, conspicuous changes in the region of amide bands I and II (1690-1500 cm^{-1}) may be evidence both of possible rearrangement of secondary structures of fragments *E* and *D* after induced free-radical oxidation and of local conformational changes in the proteins. However, the equality of coefficients of translational diffusion before and after ozonation of fragments *E* and *D* leads to a conclusion in favor of local conformational changes in the proteins due to emergence of new polar chemical groupings capable of forming additional hydrogen bonds. The minor peaks in differential spectra close

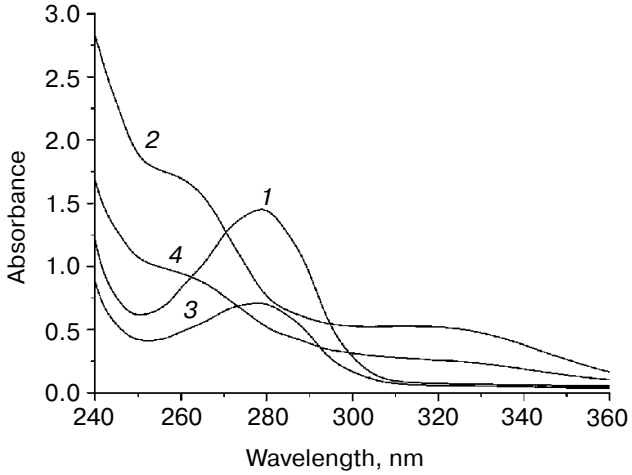


Fig. 3. UV spectra of fragments *E* and *D* before and after ozonation: *1*, *3*) control samples of fragments *D* and *E*, respectively; *2*, *4*) ozone-induced oxidized proteins.

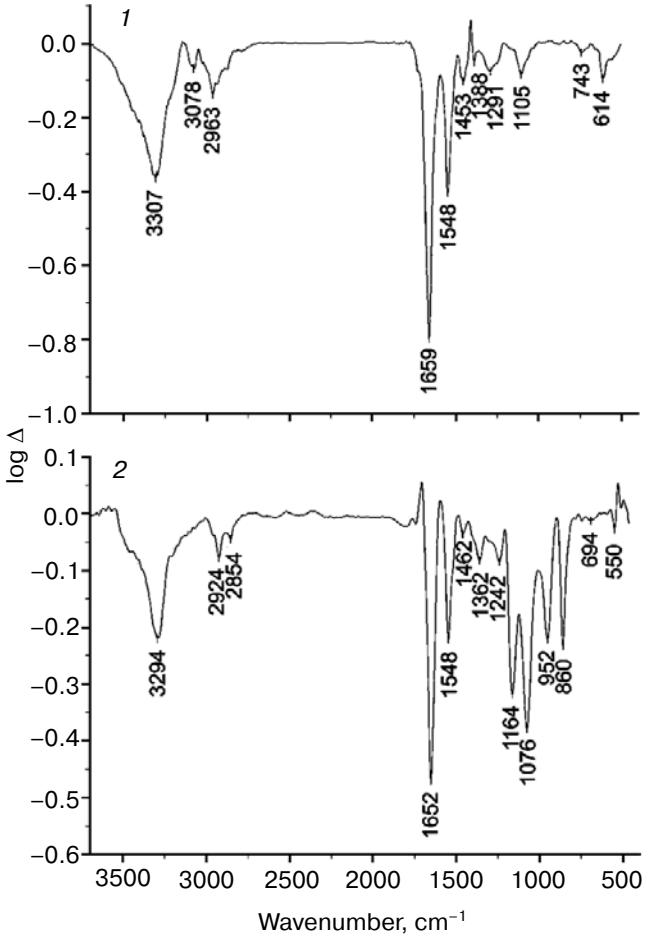


Fig. 4. Differential IR spectra of ozone-induced oxidized relative to intact proteins: *1*) fragment *E*; *2*) fragment *D* (Δ is the difference in transmission, %).

to 1460 and 1370 cm^{-1} indicate the possibility of interaction between ozone and CH_2 groups of amino acid residues of the proteins. The presence of rather intense peaks in the region of 1200-800 cm^{-1} in the differential spectrum of fragment *D* seem to be evidence of interaction between ozone and the amino acid residues of methionine, tryptophan, histidine, cysteine, and phenylalanine, which, as is known, have absorption bands in this frequency range and are liable to ozone-induced oxidation to a greater extent than other amino acids [29]. On the whole, comparison of differential IR spectra of the *E* and *D* fragments of fibrinogen demonstrates much greater susceptibility of fragment *D* to ozone-induced oxidation. The pattern of chemical modification of the proteins is in good agreement with the data obtained from the study of ozone effect on the amino acid residues of glutamine synthetase and bovine serum albumin [9].

As shown by EPR spectroscopy of spin labels, the correlation times of rotation of the radicals covalently bound to macromolecules change as a result of free-radical protein oxidation. The spectrum of EPR radicals covalently bound to amino acid residues of fragment *D*, in contrast to the EPR spectrum of spin-labeled fragment *E* (Fig. 5), shows the existence of two major regions of localization of the labels characterized by two correlation time values: ~10^{-9} and 10^{-7} sec. The share of slowly rotating labels is approximately 40% [23, 24]. It seems that the slowly rotating radicals are localized in the internal regions of fragment *D* characterized by high microviscosity. During ozonation, the share of slowly rotating labels is reduced to ~10%, probably due to partial loosening of the protein globule, at least in the region of localization of spin labels. The correlation times of quickly rotating labels were calculated on the basis of EPR spectra and their variation as a result of free-radical oxidation of the proteins was analyzed. So, for fragments *D* and *E* (Fig. 5) in the control, the correlation times calculated in accordance with the above equation are 0.83 and 0.50 nsec, respectively, while after ozonation they are 0.30 and 0.26 nsec (the error in τ determination is less than 10%). Since the correlation time in fragment *D* during ozonation changes more (2.8-fold) than in fragment *E* (2-fold), one may draw a conclusion about more significant local changes in the *D* fragment conformation. The number of radicals covalently bound to fragments *D* and *E* after their

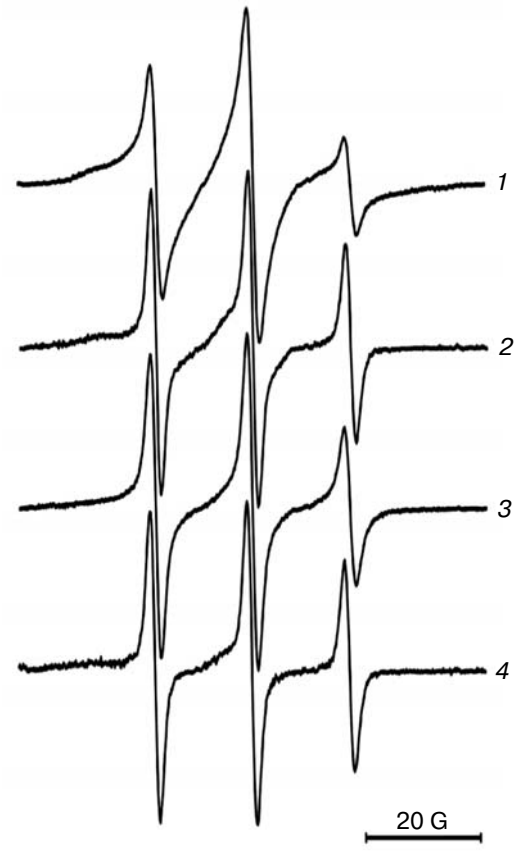


Fig. 5. EPR spectra of spin-labeled non-ozonized (*1*, *3*) and ozonized (*2*, *4*) fragments *D* and *E*, respectively.

oxidation is 2.5-3.0 times less than before oxidation. This seems to be a consequence of decrease in the number of amino groups able to react, which directly conforms to the data of IR spectroscopy.

DISCUSSION

Thus, the cumulative result demonstrates that fragment *D* of fibrinogen is more sensitive to ozone-induced free-radical oxidation than fragment *E*. Most probably, this is generally determined by features of their primary structure. Fragment *D* carries many more aromatic and cyclic amino acid residues, which, as has been shown by the UV and IR spectroscopy (Figs. 3 and 4), are most oxidizable as confirmed by the findings of other authors [9]. The content of tryptophan residue (the most sensitive to oxidation) in fragment *D* is more than fivefold higher than in fragment *E*, and the number of tyrosine, phenylalanine, and histidine residues is almost threefold higher [10]. Besides, it likely that these amino acid residues are differently accessible for ozone depending on the secondary and tertiary protein structures. It is likely that oxidation-susceptible amino acid residues in fragment *D*, which substantially differs in its 3D configuration from fragment *E*, are more accessible for to ozone. The data of EPR spectroscopy of spin labels obtained in this work also confirm the dependence of free-radical oxidation of protein fragments on their spatial configuration (Fig. 5). To all appearances, the absence of slowly rotating labels with the time of correlation 10^{-7} sec in fragment *E* and the lesser decrease in the correlation time values of quickly rotating radicals after oxidation result from the denser packing of polypeptide chains compared to fragment *D*. At the same time, the γ*C*- and β*C*-subdomains of fragment *D* contain structurally and functionally significant three-loop structures forming cavities [30]. Probable localization of spin labels in these cavities may determine the appearance of a slow rotation component in the spectrum. Besides, the structure of fragment *E* with its molecular weight of about 50 kDa is stabilized by eleven disulfide bonds, whereas fragment *D* with its nearly twice higher molecular weight is stabilized by only eight disulfide bonds [12, 13]. Thus, high oxidizability of fragment *D*, causing the emergence of new polar chemical groupings that can form additional intramolecular hydrogen bonds, is responsible for its liability to local conformational rearrangements. What region of fragment *D* can be most susceptible to free-radical oxidation? The EPR spectroscopy gives no direct answer to this question, because spin label interacts with all accessible amino groups of the protein. Nevertheless, the data on acceleration of the formation of γ-dimers (Figs. 1 and 2) obtained for the oxidized fragment are indirect evidence of possible local conformational disturbance in the region of γC-subdomains. It is known that γC-subdomain carries the binding region for factor XIIIa; its enzymatic action results in formation of γ-dimers due to covalent isopeptide binding of γ406 lysine residue of one molecule of the fragment with the γ398 or γ399 glutamine residue of another molecule [14]. It is probable that the conformation of the COOH-terminal region of γ-chain becomes more spatially open for the enzyme because of accumulation of oxygen-containing oxidation products.

To what extent can the conclusions about free-radical oxidation of fragments *D* and *E* be referred to domains *D* and *E* of fibrinogen? Strictly speaking, the final products of plasmin hydrolysis of fibrinogen (fragments *D* and *E*) differ from their original domains both in chemical composition and in secondary and tertiary structures as a result of exarticulation from the parent molecule. During moderate enzymatic hydrolysis, the peptide bonds between domains *D* and *E* break with formation of fragments *D* and *E*, which are the closest chemical and structural homologs of the domains, to a large extent preserving their functional properties [11, 31]. Fragment *D* possessing the initially spatially open polymerization centers, *a* and *b* "holes", localized in the γ*C*- and β*C*-subdomains, can participate as an inhibitor in self-assembly of monomeric fibrin and compete with its *D*-domains for the complementary binding sites. Besides, the presence of intact γ-polypeptide chains in the COOH-terminal region, as has been mentioned previously, allows fragment *D*, similar to the *D*-domain, to form *D*-dimers in the presence of factor XIIIa. Fragment *E*, like the *E*-domain, preserves the thrombin binding site and, as a result, is a competitive inhibitor of thrombin [32].

Fragments *D* and *E* obtained in this work during the moderate hydrolysis of fibrinogen had molecular weights of 99 and 50 kDa, respectively. Since fibrinogen hydrolysis also results in exarticulation from the COOH-terminal region of α-chain of a large (40 kDa) fragment corresponding to the α*C* domain [33], the total weight of these fragments formed from fibrinogen makes up about 330 kDa. This means that altogether no more than 3-4% of the initial weight of fibrinogen is lost due to removal of short peptides. However, not very great discrepancy between the primary structures of the fragments and their domains cannot play the key role in possible identity of oxidative abilities of the proteins. The differences in oxidative abilities of the fragments and their respective domains can be explained by at least two factors. The first is that, as has been shown by the example of oxidation of glutamine synthetase and bovine serum albumin [9], susceptibility to ozone oxidation of proteins with subunits similar in molecular weight and amino acid composition depends not only on the primary structure, but also on the secondary and tertiary structures. All the above may be referred in full to the oxidation of fibrinogen fragments and domains different not only in chemical composition but also in spatial organization. Another possible reason is that a fibrinogen molecule contains two α*C* domains, the

effect of which on oxidation of domains *D* and *E* is absolutely unknown. All these facts lead to a conclusion that the general patterns of oxidation of domains *D* and *E* and the respective fragments may be significantly different. Therefore, the notion of high oxidative ability of domains *D*, based on the results of oxidation of fragment *D*, is as yet merely hypothetical. This notion is attractive as a good explanation for oxidation features of fibrinogen and disturbance of its functional properties. High oxidizability of domains *D*, similarly to fragment *D*, explains the fact that fibrinogen is at least 20 times more susceptible to oxidative modification than other basic plasma proteins: albumin, immunoglobulins, transferrin, and ceruloplasmin [34]. Therefore, fibrinogen is considered as an important antioxidant that protects β-lipoproteins in blood plasma [35]. Fibrinogen easily enters the reaction of free-radical oxidation, causing the formation of oxidized protein forms different in chemical composition and structural organization from the native form. As a result, the functional properties of fibrinogen change. During free-radical attack, it becomes able to form macromolecular clusters due to "end-to-end" interaction of peripheral domains *D* and to be more completely involved in the crosslinking reaction under the influence of factor XIIIa [7, 8].

The high sensitivity of fragment *D* to oxidation suggests that the two peripheral *D*-domains of fibrinogen during its free-radical attack can serve as interceptors of free radicals, maximally protecting domain *E* from oxidation. We believe that the biological sense of this phenomenon is probably assurance of reliability of the main function of fibrinogen: its participation as a substrate in the reaction of fibrin formation. Transformation of fibrinogen into monomeric fibrin is known to be performed due to enzymatic cleavage of fibrinopeptides *A* and *B* from the *A*α- and *B*β-polypeptide chains of domain *E* with exposure of polymerization centers *A* and *B* "knobs". The regions responsible for thrombin binding are localized in the so-called "funnel-shaped" subdomain of domain *E* and include the residues of polypeptide chains *A*α38-46 and *B*β75 [36, 37]. The Phe38 residue of *A*α-chain plays a special role in formation of the binding center because it is spatially located at the very edge of the subdomain, maximally close to the amino acid residue Ala75 of *B*β-chain, and is accessible for solvent [38]. It is quite realistic to suggest that domains *D* protect, in particular, this easily oxidizable amino acid residue, imperative for the enzymatic reaction of cleavage of fibrinopeptides. Indeed, during free-radical oxidation of fibrinogen, thrombin-catalyzed cleavage of fibrinopeptides remains normal [39], which seems to be evidence of maintenance of local conformation in the region of the "funnel-shaped" subdomain of domain *E*. Oxidative modification of fibrinogen determines the experimentally observed inhibition of fibrinogen formation [8, 40, 41]. It results in formation of a cruder gel with higher ratio of weight-average weight to weight-average length of the fibrils [8]. As is known, a two-stranded protofibril of fibrin is formed in such a way that domain *D* of one molecule of monomeric fibrin is bound to the central domain *E* of another molecule and contacts domain *D* of a third molecule by "end-to-end" type, forming a three-domain *D*-*E*-*D* node. On reaching the critical length, protofibrils show the tendency to lateral association with formation of fibrils. According to recent data [42, 43], γ*C*- and β*C*-subdomains of domain *D* carry not only polymerization centers *a* and *b* "holes" but also the centers responsible for the lateral association of protofibrils. Since gel structure is determined primarily by kinetic factors, i.e. the rate constant ratio for the reactions of longitudinal polymerization of monomeric fibrin and lateral aggregation of protofibrils, it is probable that ozonation results in disturbance of the interaction between domains *E* and *D* of monomer fibrin molecules. This seems to be caused by the change in local conformation in the region of domains *D*, supposedly due to their high oxidative ability.

Finally, we would like to note that the conclusions regarding the oxidative ability of domains *E* and *D* based on the results of oxidation of fragments *E* and *D* of fibrinogen, as has been mentioned before, are still tentative. Direct evidence of different oxidative abilities of the domains may be obtained from studies only at a level of intact fibrinogen molecule without its preliminary fragmentation.

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