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Chemiluminescence Study on Peroxide Damage of Albumin, Initiated by Fenton's Reagent

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Abstract—Peroxide damage of albumin by the action of iron(II) sulfate–hydrogen peroxide was studied by the chemiluminescence method. Orders of the reaction with respect to particular reactants were determined, and side processes complicating the peroxidation were identified.

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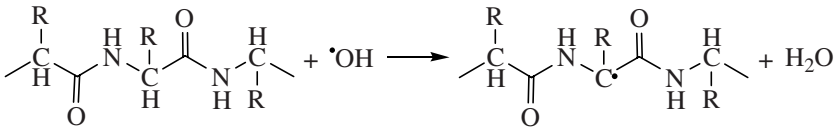
Numerous published data indicate participation of radical species, mainly oxygen radicals, in peroxide damage of proteins [1–5]; therefore, this process may be regarded as radical chain reaction like degradation of lipids constituting another important class of biopolymers [6–8]. We previously proposed a scheme for the radical decomposition of a polypeptide chain, involving no degenerate chain branching [9–11].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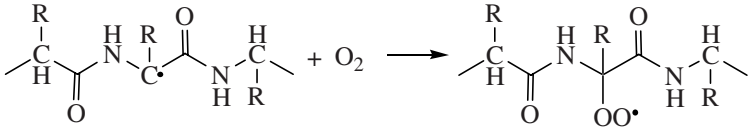
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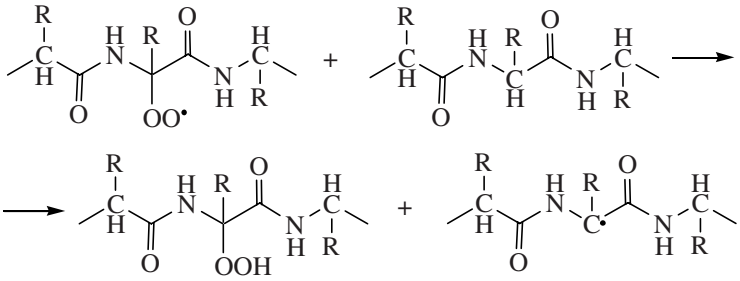
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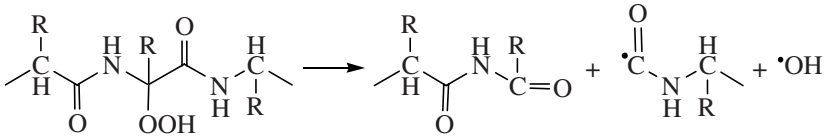
(4)

† Deceased.

The key steps in the peroxidation of polypeptide chain are the following: attack by hydroxyl radical on a protein molecule at the α-carbon atom [reaction (1)] with generation of carbon-centered radical species, formation of polypeptide peroxyl radical [reaction (2)], hydrogen abstraction by the latter from another protein molecule with formation of hydroperoxide [reaction (3)], and its subsequent transformations [reaction (4)]. The radical chain process may be initiated in several ways; however, the only conditions ensuring further development of the process is generation of hydroxyl radical which attacks polypeptide chain.

Peroxide-induced degradation of proteins is commonly studied by spectrophotometry; this method is applicable to any protein and is widely used in medical and biological practice to determine the amount of final protein degradation products, i.e., carbonyl-containing fragments [3, 12–16]. The latter are identified as colored adducts with 2,4-dinitrophenylhydrazine, the corresponding 2,4-dinitrophenylhydrazones (2,4-DNPH). The generality of this procedure was proved [3] by studying electronic absorption spectra of peroxidation products obtained from various protein enzymes after treatment with a solution of 2,4-dinitrophenylhydrazine [12–16]. In all cases, the spectra were almost identical, regardless of the initial protein structure. Using glutamine synthetase as example, it was shown [3] that the results of spectral studies did not depend on the mode of initiation of polypeptide chain degradation. The spectra of the peroxide oxidation products of polypeptides formed by the action of $HO^{\cdot}$ radicals generated both in a chemical system (Fenton's reagent) and from native neutrophils were identical. Therefore, the character of products formed by fragmentation of polypeptide chain does not depend on the system generating active oxygen species, so that different systems can be used to generate radicals while studying peroxidation of proteins by the above spectrophotometric technique.

In the recent years, studies on the mechanisms of radical chain oxidation of bioorganic structures by highly sensitive chemiluminescence methods have been extensively developed. These procedures ensure determination of highly reactive radicals responsible for chain propagation in a very low concentration [2, 16–18].

The chemiluminescence procedure used in the present work is based on recording the overall chemiluminescence intensity within a definite period of time in the reaction of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) with hydroxyl radical. Hydroxyl radicals are generated in the system iron(II) sulfate–hydrogen peroxide according to the following scheme:

$$H_2O_2 + Fe^{+2} = Fe^{+3} + {}^{\bullet}OH + {}^{-}OH. \quad (5)$$

In any case, hydroxyl radicals generated by reaction (5) but not reacting with protein according to reaction (1), are trapped by luminol giving rise to photons that are detected using a chemiluminescentce setup. In fact, we observe chemiluminescence quenching by a protein, which is well known from the literature [19]. With account taken of reactions (5) and (1), the kinetic equation for hydroxyl radicals is given by Eq. (6) [20]:

$$d[{}^{\bullet}OH]/d\tau = k_5[Fe^{+2}][H_2O_2] - k_1[{}^{\bullet}OH][Alb]. \quad (6)$$

Here, k_5 and k_1 are, respectively, rate constants for the formation and consumption of hydroxyl radicals. By applying the stationary concentration principle [20] we obtain Eq. (7); then, equating of the left and right parts of Eq. (6) to zero, we arrive at Eq. (8), and simple transformation of the latter leads to Eq. (9) for the stationary concentration of $^{\cdot}OH$ in the system under study:

$$d[{}^{\bullet}OH]/d\tau = 0, \quad (7)$$

$$k_5[Fe^{+2}][H_2O_2] = k_1[{}^{\bullet}OH][Alb], \quad (8)$$

$$[{}^{\bullet}OH] = k_5[Fe^{+2}][H_2O_2]/k_1[Alb]. \quad (9)$$

Equation (9) suggests the first order of the reaction with respect to albumin. In fact, the plots of the overall chemiluminescence intensities (I_{chl}) versus time at different albumin concentrations (Fig. 1) follow pseudofirst-order kinetic equation with $k_{ap} = 0.034\ s^{-1}$. Thus, the chemiluminescence method allows us to study peroxidation of proteins at the initial stage involving formation and consumption of hydroxyl radicals) [17–19]. Insofar as the light yield is determined by reaction of luminol with those $^{\cdot}OH$ radicals which did not react with the protein [reaction (1)], the chemiluminescence intensity appropriately reflects the concentration of hydroxyl radicals in the system Fenton's reagent–albumin and provides very important information for analyzing the mechanism of processes initiating protein peroxidation.

As follows from Fig. 1, the major part of the overall chemiluminescence intensity is detected within 30 s after addition of hydrogen peroxide. Therefore, in the further study, variation in the overall chemiluminescence intensity depending on the reactant

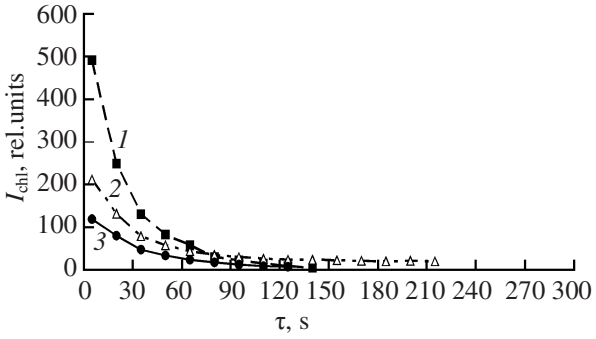

Fig. 1. Variation of the overall chemiluminescence intensity (I_{chl}, relative units) with time (τ, s) at different albumin concentrations: c_{Alb}, M: (*1*) 0.30×10^{-6}, (*2*) 7.69×0^{-6}, (*3*) 15.39×10^{-6}; the overall chemiluminescence intensity was measured over a period of 5 s every 10 s.

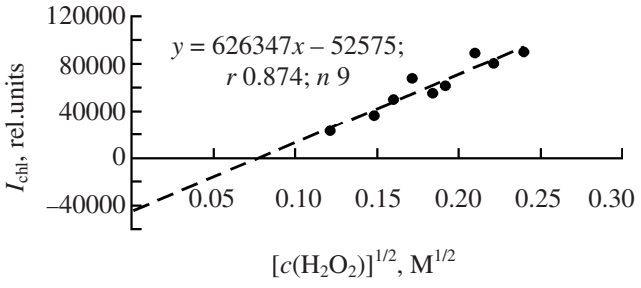

Fig. 3. Plot of the overall chemiluminescence intensity (I_{chl}, relative units) vs. square root of the hydrogen peroxide concentration (M), τ = 30 s; I_{chl} ranges from 23 180 to 90 030 relative units upon variation of hydrogen peroxide concentration in the range $(14.71–57.60)\times10^{-3}$ M; c(Alb) = 15.38×10^{-6} M, $c(Fe^{2+}) = 0.28\times10^{-3}$ M.

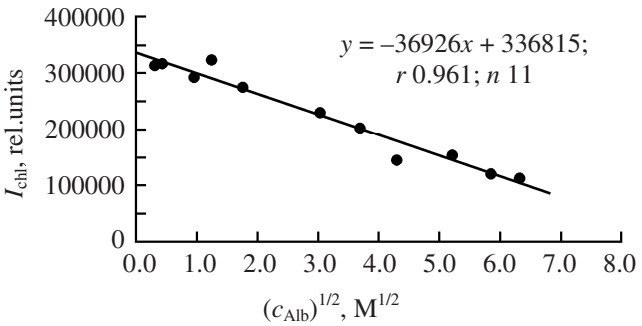

Fig. 2. Plot of the overall chemiluminescence intensity (I_{chl}, relative units) vs. square root of the albumin concentration (10^{-6} M), τ = 30 s; I_{chl} ranges from 313 580 to 120 050 relative units upon variation of albumin concentration in the range $(0.09–34.22)\times10^{-6}$ M; $c(H_2O_2) = 80.9\times10^{-3}$ M, $c(Fe^{2+}) = 0.28\times10^{-3}$ M.

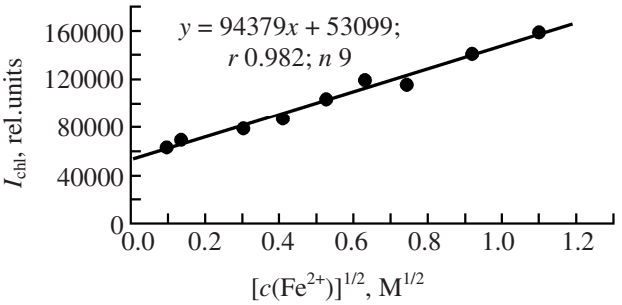

Fig. 4. Plot of the overall chemiluminescence intensity (I_{chl}, relative units) vs. square root of the iron(II) concentration (10^{-3} M), τ = 30 s; I_{chl} ranges from 63 020 to 158 740 relative units upon variation of iron(II) concentration in the range $(0.009–1.21)\times10^{-3}$ M; c(Alb) = 15.38×10^{-6} M, $c(H_2O_2) = 80.9\times10^{-3}$ M.

concentration was recorded just within the first 30 s after addition of hydrogen peroxide.

The chemiluminescence intensity (I_{chl}) was found to be proportional to the square root of the albumin concentration (Fig. 2). While speaking about the first step of albumin peroxidation, which involves generation and consumption of hydroxyl radicals, it should be noted that the experimental plot of I_{chl} versus square root of the hydrogen peroxide concentration is described by a straight line with a negative intercept on the y axis (–52600 rel. units; Fig. 3). In the same system (Alb–H_2O_2–Fe^{2+}), the plot of the chemiluminescence intensity versus square root of the iron(II) ion concentration is characterized by a positive intercept on the y axis (53000 relative units; Fig. 4). We presumed that these intercepts, being approximately equal in absolute value, are related to reactions (10)–(12) that are concurrent to the main process [21, 22].

$$^{\bullet}OH + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O, \quad (10)$$

$$HO_2^{\bullet} + Fe^{2+} \rightarrow HO_2^- + Fe^{3+}, \quad (11)$$

$$HO_2^- + H^+ \rightarrow H_2O_2. \quad (12)$$

As the concentration of hydrogen peroxide increases, a larger amount of $^{\bullet}OH$ radicals is consumed, and the overall chemiluminescence yield continuously decreases. Raising the concentration of iron(II) ions leads to generation of an additional amount of hydrogen peroxide, which gives rise to new $^{\bullet}OH$ radicals and gain in the overall chemiluminescence intensity. As a result, the intercepts on the y axis for the experimental dependences (Figs. 3, 4) are characterized by equal lengths but opposite signs.

Thus, our chemiluminescence study on albumin peroxidation in the system H_2O_2–Fe^{2+} has revealed an important side reaction pathway which complicates the overall process.

EXPERIMENTAL

Lyophilized human serum albumin was dissolved in doubly distilled water to a concentration of 1%, phosphate buffer (pH 7.4) was added to the freshly prepared solution to an overall volume of 2.7 ml, and 0.3 ml of a 1×10^{-4} M solution of luminol (chemiluminescence activator) in dimethyl sulfoxide (stored in a refrigerator at 4°C) was added. The protein peroxidation process was initiated by adding 2.0–5.0 ml of a freshly prepared 1:1 (by volume) mixture of a 0.06% aqueous solution of iron(II) sulfate and a 0.06% aqueous solution of ethylenediaminetetraacetic acid. The mixture was transferred into a cell placed in the probe of a chemiluminometer, and a 0.5% solution of hydrogen peroxide was injected in a volume of 5.0 to 2.0 ml to adjust the final volume of the reaction mixture to 10 ml. The chemiluminescence intensity was estimated by the overall chemiluminescence intensity within a definite period of time.

Variations in the overall chemiluminescence intensities at different reactant concentrations were measured within 30 s after addition of hydrogen peroxide using a setup design and supplied by Associate Professor I.V. Yudin. The kinetics of the process were studied on a Flyuorat-02-3 instrument (Lyumeks). The overall luminescence intensities (rel. units) were measured in 10 s after addition of hydrogen peroxide and then every 5 s until chemiluminescence decay. Insofar as the cell volume of the instrument was 4 ml, the amounts of all components were reduced thrice.

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