Russian Journal of General Chemistry, Vol. 71, No. 6, 2001, pp. 968–963. Translated from Zhurnal Obshchei Khimii, Vol. 71, No. 6, 2001, pp. 1030–1036.

Some Problems of Spontaneous Protein Peroxidation

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Received May 16, 2000

Abstract — General regularities of spontaneous peroxide fragmentation of polypeptide chains are considered with human serum albumin as a model. A radical chain mechanism of this reaction is proposed. The chain process is initiated by reaction of ionogenic peptide groups with dissolved oxygen.

It is well known that proteins in aqueous solutions undergo substantial changes [1]. Even slight variations in temperature and pH, as well as action of trace amounts of various chemicals strongly affect chemical behavoir of proteins, including such an important plasma proteins as serum albumin [2].

Spontaneous peroxidation of proteins is considered one of the routes of protein modification in aqueous solutions, which is consistent with the commonly accepted notion of autooxidation processes in C–H acids [3–8], specifically primary and secondary nitroalkanes [3, 8–11].

Peroxidation processes in biological molecules are radical reaction, which has been unequivocally proved for lipids [12–20] and proteins [21–29], including serum albumin [30]. Thus, albumin under ionizing radiation gives long-lived radicals [30] and ehibits antioxidant properties, playing the role of a "sacrifical" antioxidant attacked by active oxygen species [31–32]. Nucleic acids [27, 33–40] and varios low-molecular biologically important compounds [31–48], such as tromboxanes [20], amino acids and their derivatives [43, 48], uric acid [44], and biologically important aromatic compounds [42, 46–47], too, are subject to radical peroxidation.

At present lipid peroxidation is treated as a radical chain process [12, 49–51] having much in common with oxidation of low-molecular organic compounds [52–58]. The main difference between these two processes lies in the stage of chain initiation. In oxydation of low-molecular organic compounds generation of radical centers involves mostly molecular oxygen [52–58]. In lipid peroxidation chain initiation most commonly occurs via formation of active oxygen species. The latter can be generated by the Fenton reaction, involving hydrogen peroxide [51, 59], via the generation of superoxide radical anion, involving of the reduced form of nicotine adeninedinucleotide phosphate (NADFH) [60], or via the generation of complexes of chelated iron with superoxide radical anion, involving adenine dinucleotide phosphate (ADF) [61, 62]. The intermediacy of radical species in the peroxide damage of nucleic acids and proteins is indisputable, though there are no generally accepted schemes of processes, expect for hemoglobin [63–65]. However, chain mechanisms of reactions involving this heme-containing protein have been proposed only for oxidation of the heme group and have nothing to do with the polypeptide moiety of the molecule.

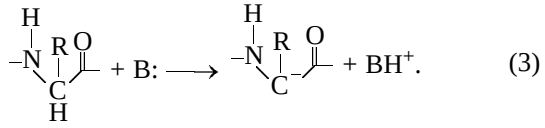
In the present paper we made an attempt to treat fragmentation of the polypeptide moiety as a radical chain process.

The mode of production of radical species, i.e. of the mechanism of chain initiation, is the key problrm to be solved in examining any chain process [52–57]. Dissolved protein molecules are a single source of free radicals in aqueous solution. Therewith, initially the protein molecoule should form a nucleophilic reagent to act as an electron donor on dissolved oxygen. The source of these nucleophilic reagents are the potentially ionogenic protein fragments capable of dissociation by schemes (1)–(3):

$$\text{–SH} + \text{B:} \rightleftharpoons \text{–S}^- + \text{BH}^+, \quad (1)$$

$$\text{–OH} + \text{B:} \rightleftharpoons \text{–O}^- + \text{BH}^+. \quad (2)$$

The –SH groups are supplied by the cysteine fragments of the polypeptide chain (pK_a 8.3) [66]. The –OH groups belong to the serine, treonine and thyrosine fragments of the polypeptide chain. The pK_a of the thyrosine OH group is 10.9 [66]. Since serine and threonine are aliphatic amino acids, their OH groups should have much higher K_a values.



(3)

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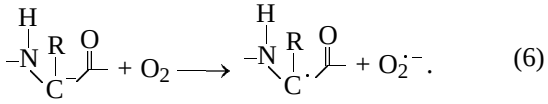
Based on published data [67], the pK_a value of the methine fragment (–CHR) of the polypeptide chain can be estimated at about 20, because the pK_a value for acetone which, like the polypeptide molecule in question, has the (–C=O) group α to the deprotonated carbon is 20 [67]. The pK_a of the CH_3 group of acetamide (the amide group α to the deprotonated carbon atom) is 25 [68]. Since the polypeptide chain in question have both the C=O and C(NH_2)=O groups α to the –(CHR)– fragment, the pK_a value for the latter fragment can be expected to lie in the range 20–25. The acidity may be higher due to the presence in the β position of one more carbonyl fragment bound with the deprotonated carbon atom via an –NH– bridge. However, the effect of the β-carbonyl group on the acidity of the (–CHR–) fragment should not be significant, since the Nα, Cα, Cβ atoms are not coplanar. Had they been coplanar, the pK_a value for the methyne fragment –(CHR)– would have been about 13, which is unlikely. However, in any case this group shoul be the most acidic among acidic groups of the polypeptide chain, and its pK_a shoul be higher than that of hydrogen peroxide (11.82) [69].

Based on the above facts, the –SH fragments of cysteine are the most probable candidates to participate in reactions (1)–(3).

The resulting nucleophilic fragments act as electron donors towards dissolved oxygen [reactions (4)–(6)]:

$$–S^- + O_2 \rightleftarrows –S^- + O_2^{\cdot-}, \quad (4)$$

$$–O^- + O_2 \rightleftarrows –O^- + O_2^{\cdot-}, \quad (5)$$

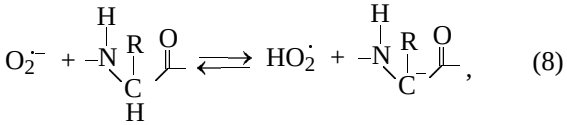


(6)

The probability of participation in chain initiation decreases in the reaction order (4) > (5) > (6), in parallel with decreasing ability of the acidic fragments to dissociation.

Once superoxide radical anion has been produced, the chain process can further develop via the generation of hydroperoxide radical by reactions (7)–(10):

$$O_2^{\cdot-} + BH^+ \rightleftarrows B: + HO_2^{\cdot}, \quad (7)$$

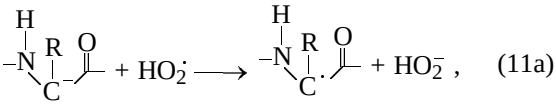


(8)

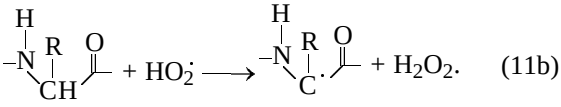
$$O_2^{\cdot-} + –SH \rightleftarrows HO_2^- + –S^-, \quad (9)$$

$$O_2^{\cdot-} + –OH \rightleftarrows HO_2^- + –O^-. \quad (10)$$

Taking account of the high acidity of $HO_2^{\cdot}$ radical (pK_a = 4.88) [70], the equilibria of reactions (7)–(10) can be axpected to be strongly shifted to the right. But even in the presence of traces of hydroperoxide radical which is a fairly strong electron acceptor [59, 60], its reaction with the protein carbanion [reaction (11)] or with the initial protein molecule [reaction (11b)] are not excluded.

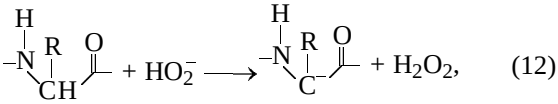


(11a)



(11b)

The resulting hydroperoxide anion is capable to split off a proton from the –(CHR)– protein fragment, or from OH and SH groups of the polypeptide chain, producing hydrogen peroxide [reactions (12)–(14)]:



(12)

$$–SH + HO_2^- \rightleftarrows –S^- + H_2O_2, \quad (13)$$

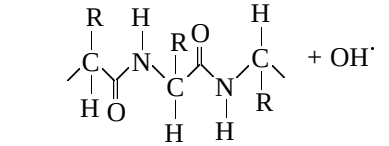
$$–OH + HO_2^- \rightleftarrows –O^- + H_2O_2. \quad (14)$$

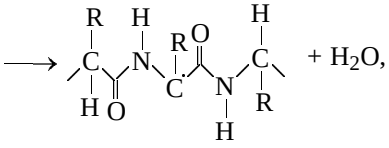
Reaction (12) is the least probable one because of the low CH acidity of the –(CHR)– fragment of the polypeptide chain, whose pK_a, as stated above, is considerably higher than that of hydrogen peroxide. This difference hampers proton transfer to the hydroperoxide ion. Reactions (13) and (14) are more probable because of the higher acidity of the corresponding sites of protein molecule and provide the main route of proton transfer to the hydroperoxide anion to give hydrogen peroxide involved in further chain peroxide damage of the polypeptide chain. One more way to H_2O_2 generation is the reaction $HO_2^{\cdot}$ with the native protein by scheme (11a).

Hydrogen peroxide via reactions with ionized OH, SH, or –(CHR)– fragments of the protein molecule (Nu^-) generates $^{\cdot}OH$ [scheme (15)]. The lattes ensures further development of the chain peroxide damage of the protein molecule [reactions (16)–(19)].

The ionized $–S^-$, $–O^-$ and –(CR)– nucleophilic fragments of the polypeptide molecule may be involved into reaction (19) according to the above mentioned tendency to take part in such processes.

$$H_2O_2 + Nu^- \longrightarrow {}^-OH + {}^-OH + Nu^{\cdot}, \quad (15)$$





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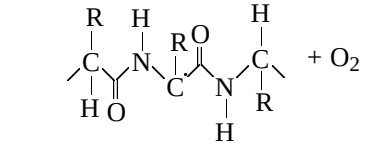

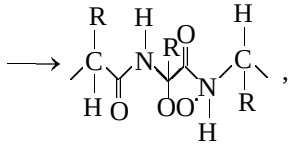

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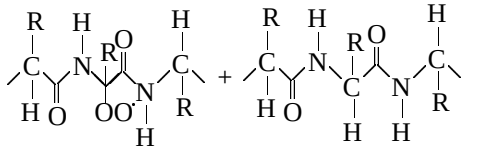

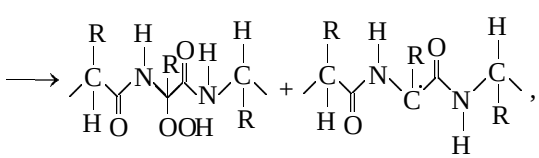

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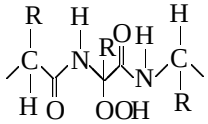
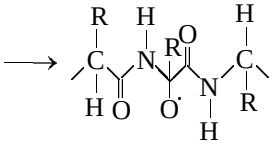
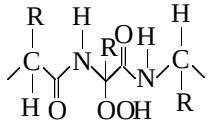


(19a)





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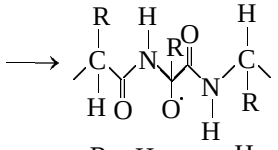
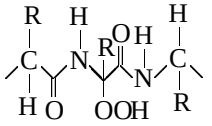
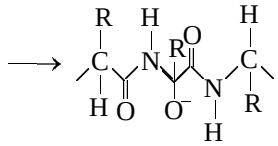
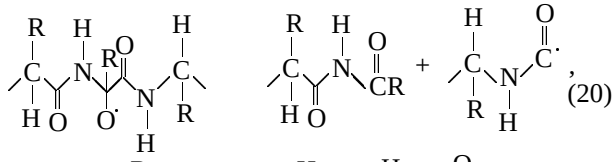




(19c)



The alkoxy radical formed by reactions (19a) and (19a) initiates the main route of the peroxide fragmentation, leading to the generation of carbonyl fragments [reactions (20)–(22)] via unimolecular cleavage [reaction (20)] followed by reactions of the resulting fragments with the native protein [reaction (21)]. The alkoxy radical also can directly react with the initial protein molecule [reaction (22)].



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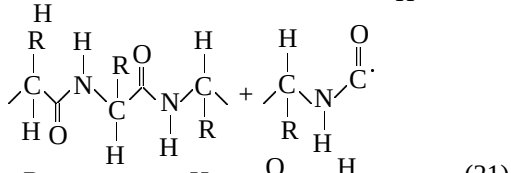
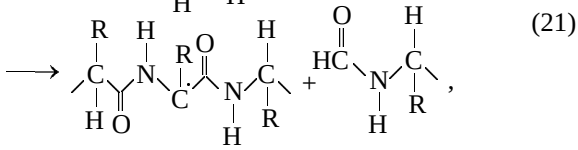
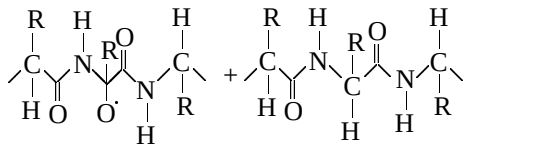
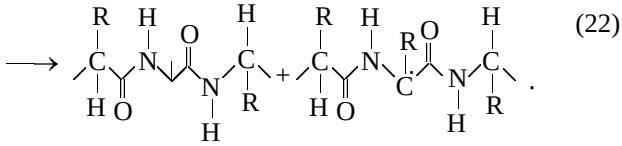




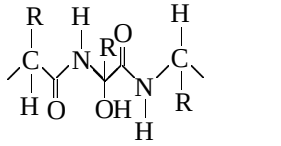
(21)

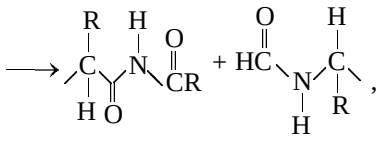




(22)

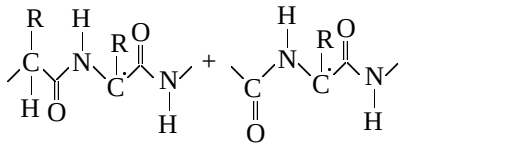
The hydroxyderivative of the protein obtained in these reactions, apparently splits producing carbonyl fragments.

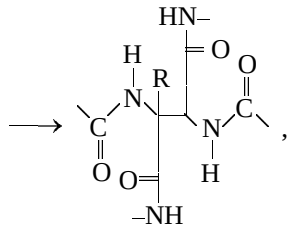




(23)

The mechanism of chain termination is quite an important problem. The most probable route of chain termination are reactions (24) and (25), i.e., recombination of carbon-centered radicals generated from the native protein to give dimeric C–C bonded aggregates [reaction (24)] and recombination of –(CR)– radical with the alkoxyl radical to give C–O–C bonded agregates [reaction (25)].





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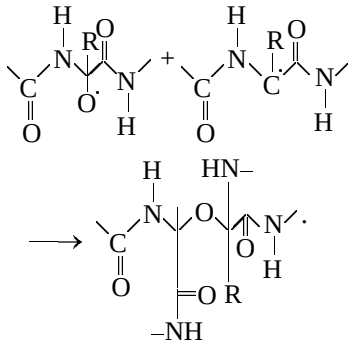

(25)

To decide between alternative routes of chain termination, one should establish dependence of the rate of accumulation of peroxidation products on reagent concentration [71, 72]. We establish such dependence dor spontaneous fragmentation of serum human albumin (**I**) in aqueous solution in the presence of dissolved oxygen (**II**).

Provided chain initiation involves reactions (4)–(6), the rate of accumulation of fragmentation products v (detected as dinitrophenyl hydrazones **III**) should be proportional to the square root of the chain initiation rate (v_0) [Eq. (26)] and can be described by Eq. (27):

$$v = av_0^{1}/2, \quad (26)$$

$$v = bc_{\mathrm{I}}^{1/2}. \quad (27)$$

In the case of chain termination by reaction (24) (quadratic chain termination) stage (17) is the limiting step of chain unit and the reaction rate v gas Eq. (28). Under these conditions, Eq. (29) must hold, which is actually the case (Fig. 1).

$$v = dv_0^{1/2}c_{\mathbf{II}}, \quad (28)$$

$$v = mc_{\mathbf{I}}^{1/2}. \quad (29)$$

When the albumin concentration considerably increases, a change in the kinetic law for the accumulation of the products of the protein peroxide fragmentation takes place. When the albumin concentration exceeds 30 μM, equation (30) holds:

$$v = nc_{\mathbf{I}}. \quad (30)$$

Such a change in the kinetic law can be explained by a change in the chain termination step. In the latter case, reaction (25) takes place, and the limiting steps are reactions (17) and (22). Consequently, cross chain termination occurs, and the reaction rate can be described by Eq. (31):

$$v = pv_0^{1/2}(c_{\mathbf{I}}c_{\mathbf{II}})^{1/2}. \quad (31)$$

Taking into account equation (32), finally we come to Eq. (33).

$$v_0 = kc_{\mathbf{I}}, \quad (32)$$

$$v = qc_{\mathbf{I}}. \quad (33)$$

In Eqs. (26)–(33), a, b, d, m, n, p, k, and q are proportionality coefficients.

The experimentally dependence of the rate of accumulation of peroxidation products on albumin concentration (Fig. 2) fits Eq. (33).

The change of the chain termination step with increasing albumin concentration increases is probably explained by a sharp increase the concentration of the alkoxyl radical with increasing initial concentration of the protein.

Equilibria (1)–(5) are shifted to the right with increasing pH, and reaction (6) is thereby accelerated. However, as the pH decreases, equilibria (7)–(10) are shifted to the left, resulting in reduced generation of the chain-propagating radical $HO_2^{\cdot}$. Thus, the opposite effects the pH of the reaction medium exerts on the main stages of the process under investigation led us to expect that the final result, i.e. the accumulation of peroxide fragmentation products, will only slightly pH-dependent. This proved the case. Indeed, over the range of pH 5.8–8.0, the accumulation od carbonyl fragments detected as their 2,4-dinitrophenylhydrazones varied only slightly.

Based on the aforesaid, the overall scheme of the spontaneous chain albumine peroxidation can be described by a scheme including reactions (1)–(6) as chain initiation steps, reactions (7)–(23) as chain proparation steps, and reactions (24) and (25) as chain termination steps.

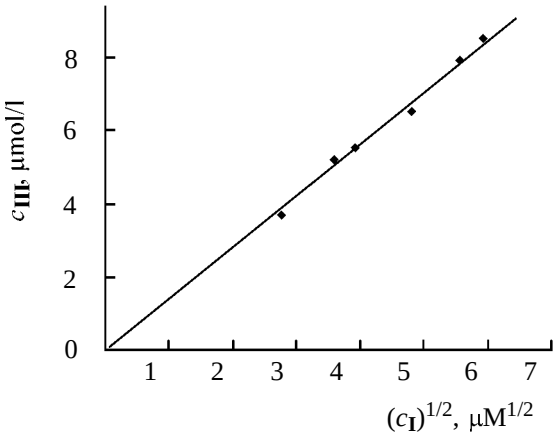

Fig. 1. Plot of the concentration of carbonyl-containing protein fragments determined as 2,4-dinitrophenylhydrazones (c_{III}) vs. the square root of albumin concentration c_I (aqueous phosphate buffer, pH 7.4, c_I 7.69–30.77 μM).

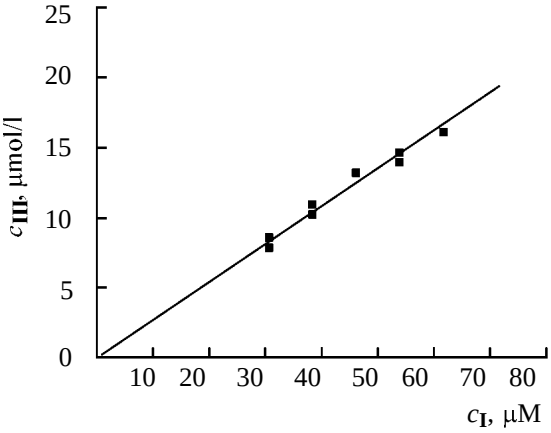

Fig. 2. Plot of the concentration of carbonyl-containing protein fragments determined as 2,4-dinitrophenylhydrazones (c_{III}) vs. albumin concentration c_I (aqueous phosphate buffer, pH 7.4, c_I 30.77–61.54 μM).

EXPERIMENTAL

The posphate buffer (pH = 7.4) was added to a freshly prepared 1% solution of liophylized human serum albumin in twice-distilled water. The total volume of the mixture was brought to 1.0 ml. The prepared solution was kept in a water bath (37°C) for 15 min.

The peroxidation reaction was terminated by addition of 1 ml of 20% trichloroacetic acid, and the mixture was created with 1 ml of a 2% solution of 2,4-dinitrophenylhydrazones in 2 N HCl to obtain colored 2,4-dinitrophenylhydrazine of the carbonyl-containing albumin fragments and left to stand form 1 h at room temperature.

The resulting precipitate was separayed by centrifugation (15 min, 3000 rpm), washed from excess 2,4-dinitrophenylhydrazine with 3 ml of a freshly prepared 1:1 (by volume) mixture of ethanol and ethyl acetate, and the centrifugation was repeated. The precepite was then dried and treated with 5 ml of 8 M urea with 1–2 drops of 2 N HCl (for better solution). The mixture was warmed on the water bath at 37°C until the precepitate had dissolved completely.

The polypeptide chain damage was followed spectrophotometrically on an SF-46 spectrometer by the accumulation of colored hydrazones (λ^{max} 363 nm).

The concentration of the produced hydrazones was calculated using the extinction coefficient ε 2.2×10^4 l mol^{-1} cm^{-1} [18].

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