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Superoxide Formation as a Result of Interaction of L-Lysine with Dicarbonyl Compounds and Its Possible Mechanism

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Abstract—The EPR signal recorded in reaction medium containing L-lysine and methylglyoxal is supposed to come from the anion radical (semidione) of methylglyoxal and cation radical of methylglyoxal dialkylimine. These free radical intermediates might be formed as a result of electron transfer from dialkylimine to methylglyoxal. The EPR signal was observed in a nitrogen atmosphere, whereas only trace amounts of free radicals were registered under aerobic conditions. It has been established that the decay of methylglyoxal anion radical on aeration of the medium is inhibited by superoxide dismutase. Using the methods of EPR spectroscopy and lucigenin-dependent chemiluminescence, it has been shown that nonenzymatic generation of free radicals including superoxide anion radical takes place during the interaction of L-lysine with methylglyoxal – an intermediate of carbonyl stress – at different (including physiological) pH values. In the course of analogous reaction of L-lysine with malondialdehyde (the secondary product of the free radical derived oxidation of lipids), the formation of organic free radicals or superoxide radical was not observed.

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Key words: free radicals, malondialdehyde, methylglyoxal, modification of amino acids

Reactive oxygen species and carbonyl compounds are able to modify amino acid residues in protein molecules [1-7]. Modified proteins can serve as markers of atherosclerosis, diabetes mellitus, neurodegenerative diseases, and other pathologies developing under oxidative stress [1-4, 7-11]. The increased level of reactive oxygen species under oxidative stress induces peroxidation of lipids and formation of chemically reactive carbonyl compounds similar to 4-hydroxynonenal and malondialdehyde (MDA) [2, 7, 11]. Besides, diabetic hyperglycemia can cause the development of the so-called "carbonyl stress" as a result of accumulation of carbonyl compounds of other structure, which are products of glucose autooxidation and metabolism of triose phosphates including glyoxal, methylglyoxal, and 3-deoxyglucosone [6, 7, 11, 12]. All of these carbonyl compounds react with lysine, arginine, and cysteine residues with the formation of Schiff bases, ketamines, and thiohemiacetals, respectively [2, 5-7]. Protein modification under carbonyl stress finally results in the formation of fluorescent compounds such as pentosidines, pyrrole, pyrazine, and imidazole derivatives [1, 6, 8, 12]. Recently we have shown that α-oxoaldehydes (glyoxal, methylglyoxal), like 4-hydroxynonenal and MDA, modify L-lysine and apoprotein B of low density lipoproteins (LDL) [11]. Data on the formation of free radical intermediates on the interaction of methylglyoxal with L-alanine are available in literature [12], but the mechanism of this reaction has not been fully ascertained. It has also been revealed that the formation of superoxide by mitochondrial respiratory chain in rats with streptozotocin-induced diabetes increases in parallel with glycation of the proteins of these organelles [10]. Consequently, the study of free radical processes with the involvement of various active carbonyl compounds might be very important for understanding of molecular mechanisms of modification of protein molecules under oxidative and carbonyl stresses. Based on this,

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Abbreviations: LDL, low density lipoproteins; MDA, malondialdehyde; MG, methylglyoxal; NBT, nitro blue tetrazolium; SOD, superoxide dismutase.

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in this work we have studied in detail the formation of free radical products of L-lysine interaction with carbonyl compounds of different origin: MDA and methylglyoxal.

MATERIALS AND METHODS

Superoxide dismutase from bovine erythrocytes, methylglyoxal, L-lysine, 1,1,3,3-tetraethoxypropane, nitro blue tetrazolium (NBT), lucigenin, and other reagents produced by Sigma (USA) were used in the work. Malondialdehyde was obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane as described in [2].

EPR spectra were recorded at room temperature in an E-109E spectrometer (Varian, USA). Recording settings were as follows: microware power 20 mW, microware frequency 9.15 GHz, high frequency modulation amplitude 0.2 mT. Spectrum recording was started 1 min after the mixing of reaction components. The reaction mixture (120 μl) was introduced into PTFE 22 gas-permeable capillaries (Zeus Industrial Products, USA). The capillaries were placed into a quartz tube for continuous nitrogen or air flow during the measurement. EPR spectra were simulated by SimFonia software (Bruker, Germany). The EPR signal of the stable synthetic free radical diphenylpicrylhydrazine was used as a standard [13].

Generation of superoxide anion radical ($O_2^{\dot{-}}$) was detected using two independent methods: reduction of nitro blue tetrazolium by the superoxide and $O_2^{\dot{-}}$ induced chemiluminescence of lucigenin. The kinetics of accumulation of NBT reduction product, formazan, was determined by absorption at 560 nm in a Hitachi-557 spectrophotometer (Japan) at 25°C. The reaction was initiated by adding 10 mM methylglyoxal or 10 mM MDA to the medium containing 100 μM NBT and 10 mM L-lysine in 100 mM carbonate buffer, pH 9.5. Chemiluminescence was measured by a Lum-5773 chemiluminometer (Russia) in medium containing 20 μM lucigenin, 15 mM L-lysine, and 15 mM methylglyoxal in 100 mM K,Na-phosphate buffer, pH 7.8. Measurements were performed at 37°C under continuous stirring of the reaction medium. Statistical treatment of the data was performed using Student's *t*-criterion.

RESULTS AND DISCUSSION

For the comparative study of the interaction of L-lysine with carbonyl compounds, we used the major secondary product of lipid peroxidation (MDA) and its isomer α-ketoaldehyde (α-oxoaldehyde) — methylglyoxal. In mammals, methylglyoxal is formed in the course of glycolysis and on autooxidation of glucose [6, 8, 14]. L-Lysine was used because this amino acid is one of the main targets of the action of active carbonyl compounds in protein molecules [1, 6, 8, 15]. Figure 1 shows the results of EPR spectroscopic study of the products of L-lysine reactions with methylglyoxal and MDA. The data presented in this figure demonstrate that free radical intermediates are formed under anaerobic conditions in the reaction of L-lysine with methylglyoxal but not with MDA (Fig. 1, spectra *1* and *3*). The EPR spectrum recorded during the reaction of L-lysine with methylglyoxal has a multicomponent hyperfine structure. Previously, in work [12] such EPR spectrum was recorded in reaction mixture containing L-alanine and methylglyoxal. In this work, using C^{13}- and N^{15}-substituted and deuterated L-alanine derivatives it has been shown that the EPR spectrum is a superposition of signals of methylglyoxal anion radical ($MG^{\dot{-}}$) and Schiff base cation radical (dialkylimine) appearing on the interaction of methylglyoxal with the amino acid. Based on this, we suggest that the EPR spectrum observed in our experiments is also a superposition of signals of $MG^{\dot{-}}$ and the cation radical of methylglyoxal dialkylimine with lysine.

It is important to note that only trace quantities of free radical intermediates were registered under aeration of the reaction mixture (Fig. 1, spectrum *2*). Substitution of air for nitrogen after incubation of methylglyoxal and L-lysine mixture under aerobic conditions results in a significant (nearly by an order of magnitude) increase in the level of free radicals, supposedly dialkylimine and methylglyoxal (Fig. 2). It is significant that under these conditions the content of free radical intermediates increases on addition of superoxide dismutases (SOD) to the reaction mixture (Fig. 2, curve *2*). The effect of SOD might be due to the fact that this enzyme removes the

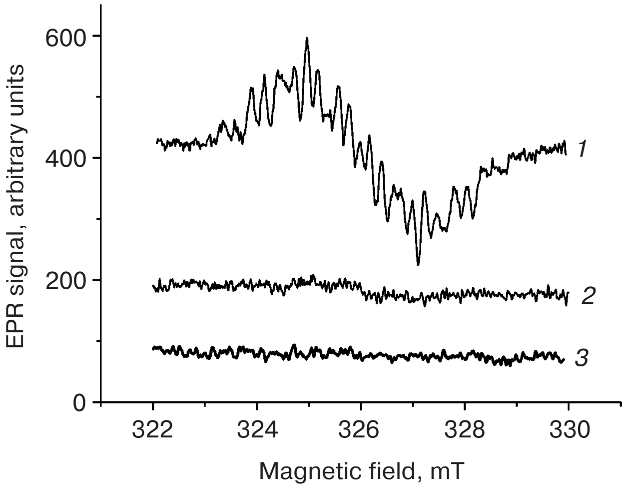

Fig. 1. EPR spectra of free radical intermediates of the reaction between L-lysine and dicarbonyl compounds. The reaction medium contained 160 mM L-lysine and 160 mM methylglyoxal (spectra *1* and *2*) or 160 mM MDA (spectrum *3*) in K,Na-phosphate buffer (0.2 M, pH 7.8). EPR signals were registered 4 min after mixing the components under aeration (spectrum *2*) or under nitrogen (spectra *1* and *3*).

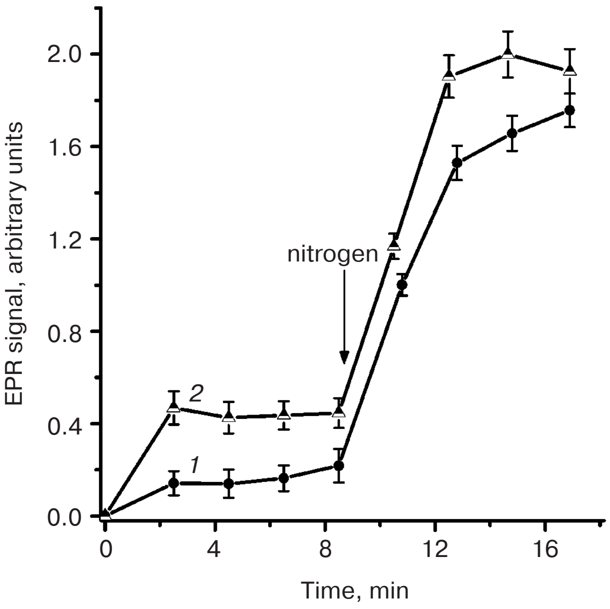

Fig. 2. Effect of aeration and SOD on the kinetics of accumulation of free radical intermediates recorded by EPR. The reaction medium contained: *1*) 160 mM L-lysine and 160 mM methylglyoxal in 0.2 M K,Na-phosphate buffer, pH 7.8; *2*) the same as (*1*) + 400 SOD units.

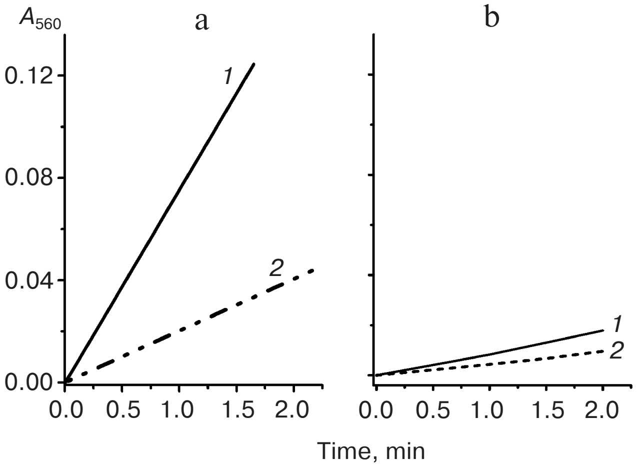

Fig. 3. Effect of SOD on kinetics of formazan formation during the reaction of L-lysine with methylglyoxal (a) or MDA (b). The reaction medium contained: *1*) 100 mM carbonate buffer, pH 9.5, 10 mM L-lysine, and 10 mM methylglyoxal or MDA; *2*) the same as (*1*) + 120 SOD units.

superoxide radical generated in the tested model system. Indeed, the data obtained in work [12] indicate that $O_2^{\dot{-}}$ is formed by single-electron oxygen reduction by methylglyoxal semidione in accordance with the reaction:

$$MG^{\dot{-}} + O_2 \rightarrow MG + O_2^{\dot{-}}. \qquad (1)$$

Our model system has also demonstrated that $O_2^{\dot{-}}$ is intensively generated on the interaction of L-lysine with methylglyoxal in carbonate buffer, pH 9.5. Superoxide formation was assessed by the accumulation of formazan on NBT reduction (Fig. 3a). The accumulation of formazan under these conditions might not depend on $O_2^{\dot{-}}$, since it is probable that NBT is reduced by other intermediates of L-lysine reaction with methylglyoxal. Nevertheless, reasoning from the fact that SOD significantly (more than 4 times) inhibited the formation of formazan under the above conditions, one can state that the most part of NBT is reduced under the action of $O_2^{\dot{-}}$ (Fig. 3a). However, only insignificant generation of superoxide radical was observed on the interaction of L-lysine with MDA (Fig. 3b). The rate of reaction of amino groups with methylglyoxal becomes lower on increasing acidity of the medium [16, 17]. The application of chemiluminescence as a method more sensitive than NBT reduction [18] revealed the formation of $O_2^{\dot{-}}$ in the mixture of methylglyoxal with L-lysine at pH 7.8 (Fig. 4), i.e. under conditions close to physiological. SOD under these conditions almost completely inhibits the chemiluminescence of lucigenin, which is evidence of the dependence of this process on the presence of superoxide anion radical (Fig. 4, curve *2*).

The decrease in concentration of free radicals recorded by EPR in aerated reaction medium is probably

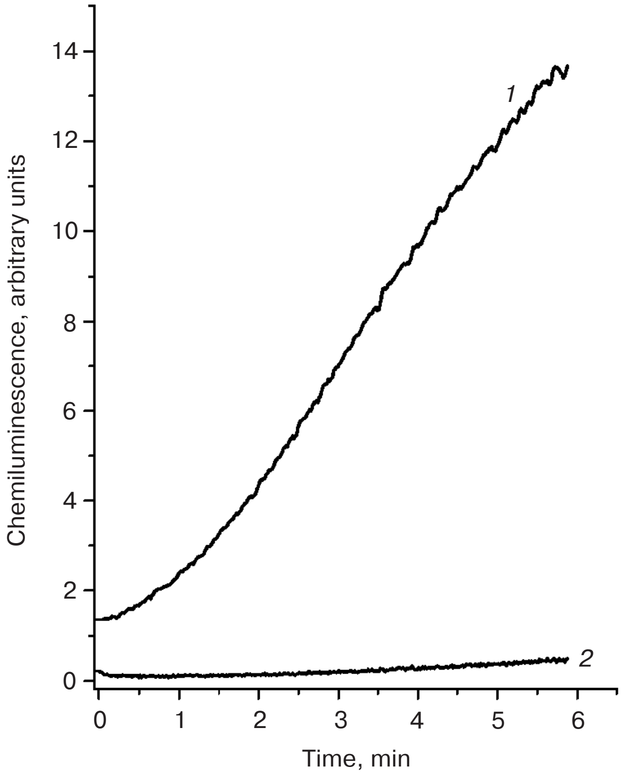

Fig. 4. Effect of SOD on superoxide-dependent chemiluminescence of lucigenin. The reaction medium contained: *1*) 100 mM K,Na-phosphate buffer, pH 7.8, 20 µM lucigenin, 15 mM L-lysine, 15 mM methylglyoxal; *2*) the same as (*1*) + 120 SOD units.

not associated with inhibition of their formation. Indeed, with nitrogen purging the content of free radical intermediates reaches its maximum in 8 min after the mixing of reaction components; but after the gas medium is replaced by air the level of EPR-revealed free radicals quickly drops (Fig. 5a). Under these experimental conditions SOD reliably reduced the rate of decline of EPR signal intensity during aeration (Fig. 5a, curve *2*). In 2 min after the increase in oxygen concentration in the medium containing L-lysine and methylglyoxal, it is impossible to reveal there free radical intermediates (Fig. 5a, curve *1*). Nevertheless, the EPR spectrum containing five components of hyperfine structure and a g-factor equal to 2.0042 were recorded on aeration of the reaction medium in the presence of SOD (Fig. 5b, spectrum *2*). According to the literature data, the characteristics of the EPR spectrum presented in Fig. 5b (spectrum *2*) correspond to the signal of the *cis*-form of methylglyoxal anion radical [19, 20]. This fact confirms the above assumption that the free radical intermediates of L-lysine reaction with methylglyoxal are $MG^{\dot{-}}$ and the cation radical of dialkylimine. Thus, molecular oxygen seems to interact directly with the free radical derivatives of methylglyoxal and dialkylimine, and the products formed in this reaction are not registered by EPR (Fig. 5a). However, SOD protects the anion radical of methylglyoxal under aerobic conditions, which points to the possibility of $MG^{\dot{-}}$ elimination under the effect of superoxide. Indeed, it has been established that in aqueous media $O_2^{\dot{-}}$ reduces some organic radicals [19, 20] and catalyzes protonation and disproportionation of nitrobenzene anion radical [21]. By analogy, it can be supposed that superoxide radical interacts with the protonated semidione of methylglyoxal, reducing it in accordance with the reaction:

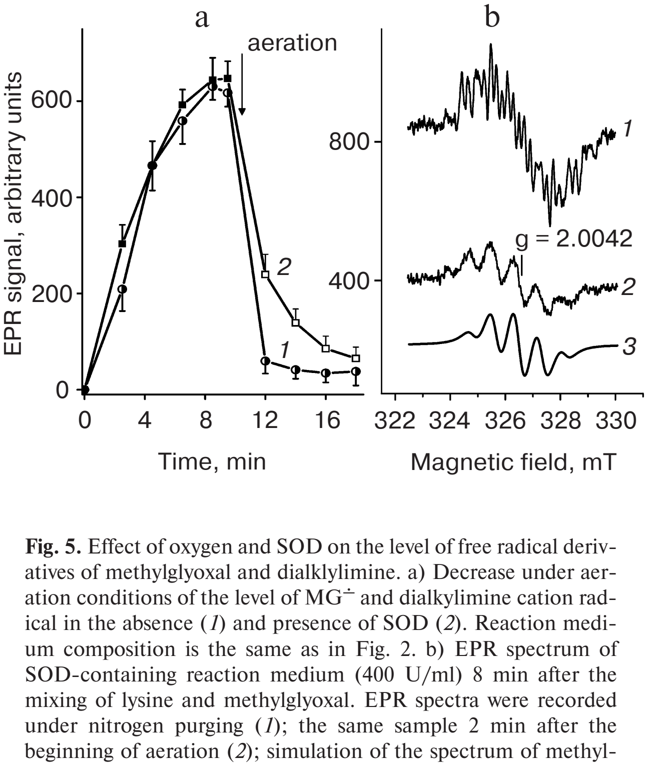

Fig. 5. Effect of oxygen and SOD on the level of free radical derivatives of methylglyoxal and dialklylimine. a) Decrease under aeration conditions of the level of $MG^{\dot{-}}$ and dialkylimine cation radical in the absence (*1*) and presence of SOD (*2*). Reaction medium composition is the same as in Fig. 2. b) EPR spectrum of SOD-containing reaction medium (400 U/ml) 8 min after the mixing of lysine and methylglyoxal. EPR spectra were recorded under nitrogen purging (*1*); the same sample 2 min after the beginning of aeration (*2*); simulation of the spectrum of methylglyoxal anion radical (*3*). Closed squares on curve *2* (panel (a)) correspond to EPR signals analogous to spectrum *1* (panel (b)); open squares correspond to the signal analogous to spectrum *2* (panel (b)).

$$MG^{\cdot}(H) + O_2^{\dot{-}} \rightarrow \text{hydroxyacetone} + O_2. \qquad (2)$$

Analysis of the literature data [12, 13, 15] suggests a sequence of reactions resulting in the formation of free radicals on the interaction of amino acids with carbonyl compounds (Fig. 6). The presented scheme shows that dialkylimine is a Schiff base, which is a product of interaction of methylglyoxal carbonyl groups with two L-lysine molecules. As a result of reaction of dialkylimine with one more molecule of α-ketoaldehyde, the Schiff base cation radical and methylglyoxal semidione ($MG^{\dot{-}}$) are formed, respectively (Fig. 6). The absence of noticeable quantities of free radical products in the medium with L-lysine and MDA might be due to low reactivity of Schiff bases formed on their interaction. In fact, in contrast to the dialkylimine formed by methylglyoxal and L-lysine, the dialkylimine being an MDA derivative has no coupled double bonds (Fig. 6). This peculiarity of chemical structure seems to reduce the ability of dialkylimines to participate in reactions of single-electron oxidation/reduction.

Since SOD less effectively prevents the dissipation of the cation radical of dialkylimine of methylglyoxal and L-lysine than that of methylglyoxal anion radical (Fig. 5b), one can suggest that dialkylimine radical is mainly used up in reaction with molecular oxygen. It is well known that the C-centered free radicals of amino acids and lipids interact with molecular oxygen with the formation of peroxyl radicals, which are then transformed into non-radical products [5, 22]. However, in contrast to the C-centered allyl radicals, the free electron in the dialkylimine cation radical is localized in the system of coupled double bonds between the Schiff base nitrogen atoms and methylglyoxal carbons (Fig. 6). At the same time, such system of coupled double bonds is present in the molecule of methyl viologen (paraquat). It is also known that the reaction of single-electron reduction of oxygen by the cation radical of methyl viologen results in the formation of methyl viologen dication and superoxide anion radical [23, 24]. It is probable that the analogous reaction between O_2 and the dialkylimine cation radical occurs under the conditions of our experiments (Fig. 7).

The findings make it possible to substantially supplement the previously proposed mechanism of superoxide generation on modification of amino acids under the action of methylglyoxal [12]. According to this mechanism, the reactions presented in Fig. 6 result in the formation of $MG^{\dot{-}}$, which further reduces oxygen to $O_2^{\dot{-}}$

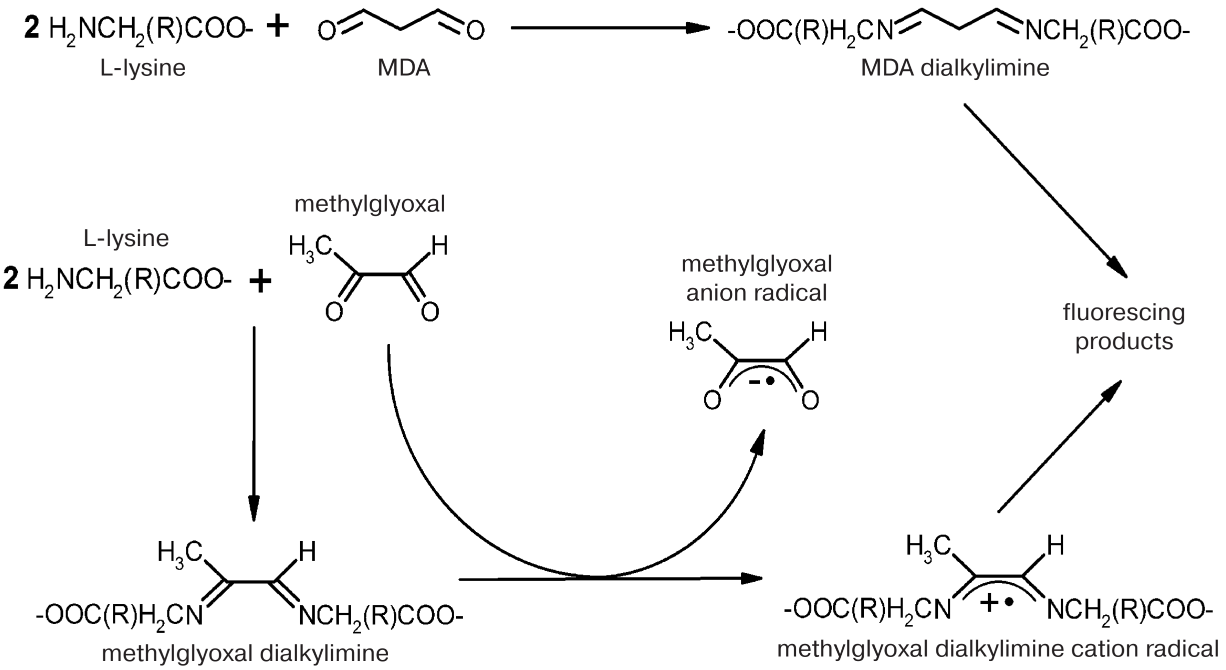

Fig. 6. Scheme of possible reactions of L-lysine with methylglyoxal and malondialdehyde.

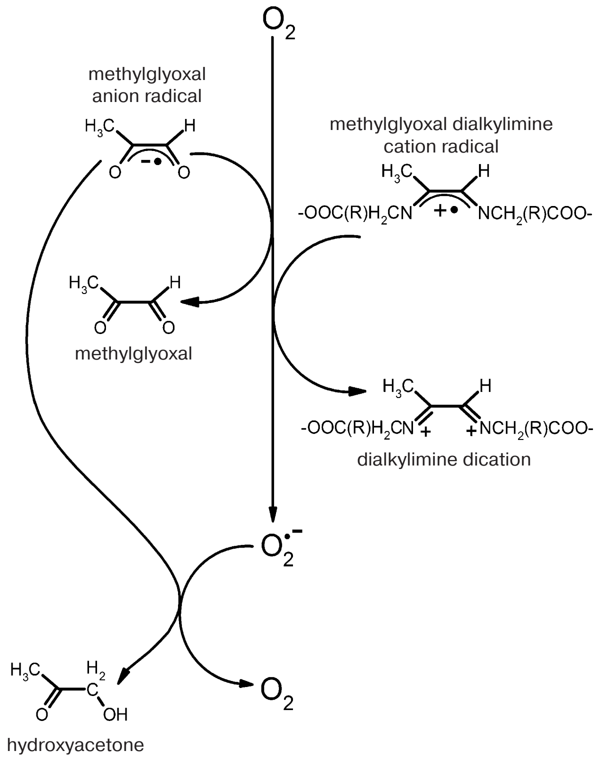

Fig. 7. Hypothetical scheme of superoxide generation on the interaction of molecular oxygen with free radical intermediates of methylglyoxal reactions with L-lysine.

(reaction (1)). We assume that $O_2^{\cdot-}$ is not only formed but also seems to be utilized in reactions with the involvement of $MG^{\cdot-}$ (Fig. 7). Besides, superoxide is probably produced on oxidation by oxygen of both methylglyoxal semidiones and dialkylimine cation radicals (free radicals of the Schiff bases of lysine and methylglyoxal) (Fig. 7).

These assumptions explain the effect of oxygen on the kinetics of accumulation and dissipation of free radicals emerging in the mixture of L-lysine and methylglyoxal. The work [12] has shown that accumulation of methylglyoxal semidione and Schiff base cation radical depends only insignificantly on oxygen concentration. According to our data, this fact may be associated with application of L-alanine as amino acid in the cited work and by the high pH value of the reaction medium (carbonate buffer, pH 9.5). It should be noted that our model system (phosphate buffer, pH 7.8) is much closer to physiological conditions. Thus, it is most likely that oxidative modification of proteins and other biomolecules might be the consequence of local generation of superoxide on the interaction of the residues of L-lysine (and probably other amino acids) with α-ketoaldehydes. This phenomenon of non-fermentative superoxide generation might be an element of autocatalytic intensification of pathophysiological action of carbonyl stress.

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