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Original Contribution

PHOTOREACTIVATION OF SUPEROXIDE DISMUTASE BY INTENSIVE RED (LASER) LIGHT

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Abstract— The effects of helium-neon laser (HNL) on activity, absorption spectra, and ESR signals of superoxide dismutase (SOD; E Cul.15.1.1) from bovine erythrocytes in acid medium were investigated. It was found that incubation during 2 hours at pH 5.9 led to inactivation of the enzyme. The subsequent illumination of SOD by HNL brought about the enzyme reactivation. Both absorption and ESR spectra were changed after incubation at pH 5.9 and restored after laser irradiation. In a model system, copper-histidine complex, absorption maximum was shifted from 632–633 nm at pH 5.8 to 639–640 nm at pH 8.5–9.0. The similar shift of the maximum was observed after illumination by HNL at pH 5.8. It may be postulated that the photoreactivation of SOD consists essentially in deprotonation of His-61 residue in the enzyme active site and subsequent recovery of imidasol bridge between copper and zinc which had been destroyed at low pH.

Since many other enzymes possess similar histidine-copper structures in their active sites, one may expect diverse effects of red (laser) light on the enzyme activity. Heme-containing enzyme, catalase was also found to be photoreactivated by HNL after inactivation at pH 6.0.

Keywords—Superoxide dismutase, SOD inactivation by low pH, Photoreactivation of SOD, Laser action on SOD, ESR spectra of SOD, Free radical

INTRODUCTION

Free radicals including oxygen radicals (superoxide and hydroxyl radicals) are generally recognized as agents of tissue damage. They have been implicated in many diseases associated with inflammation, hypoxia, hyperoxia, effects of ionizing and ultraviolet radiation, side-effects of antibiotics, intoxications with heavy metal ions, "immune complex" diseases etc.[1–5] Coming into contact with cell and intracellular membranes, nucleus, and plasma constituents, active oxygen species ($\cdot O_2^-$, H_2O_2, $\cdot OH$ and occasionally singlet oxygen $^1O_2$) perform chemical modifications of the cell components. A complex of defences have been elaborated by living organisms in the course of evolution including protective enzymes such as superoxide dismutase, glutathione peroxidase, catalase, and antioxidant compounds such as vitamin E, β-carotine and uric acid. Unfortunately, protective compounds, in particular enzymes, are not very resistant against the same radicals that they are aimed to remove. Like other proteins, protective enzymes are damaged by oxygen free radicals,[3] as well as under action of different agents concomitant pathology, for example, acidification of the medium. This effect may account for decrease of superoxide dismutase (SOD) and catalase activity detected in the blood and tissues of experimental animals and patients in many diseases (rheumatoid arthritis, miocarditis, heart infraction etc.).[8–11] The increased level of lipid peroxidation and decreased activity of protective enzymes in the site of injury appeared to be a reason of application of exogeneously introduced SOD and catalase in therapy of a number of diseases[8,9,12,14] and as potent radioprotectors.[11,13]

On the other hand, it has been demonstrated in many clinical observations and experimental works that intensive red light emitted by helium-neon laser (HNL) acts similarly to injection of protective enzymes.[15–18] Positive effects of laser therapy in the case of heavy rheumatoid arthritis,[15] suppurating wounds and trophic ulcers have been communicated for the cases where chemical drugs and physiotherapy were ineffective.[12,13]

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A particularly pronounced therapeutic effect was observed under condition of HNL application in therapy of diseases associated with inflammatory process,[14-16] the effect of laser illumination being comparable with the action of antioxidant compounds.[21] Both HNL and SOD were effective as radioprotectors when being applied after irradiation.[22,23]

The question arises in this connection if the therapeutic effects of intensive red laser light relate to its ability to reactivate metal-containing enzymes, in particular SOD and catalase, that had been inhibited under conditions of pathological process. Indeed, these enzymes have absorption maxima in the spectral region of HNL emission. The stimulating action of red laser light on catalase was described earlier[24] along with the inhibition of free radical production during the decomposition of hydrogen peroxide, as estimated by means of chemiluminescence measurements.[24] An attempt to activate native SOD was, however, unsuccessful.[25] One may speculate that laser light may not be effective in case of native, very active protective enzymes in healthy tissues, but it reactivates the enzymes, inhibited under action of unfavorable surrounding conditions in damaged tissues. Among those, low pH is, in our opinion, one most common and probable candidate, since acidification is observed at earlier stages of diversed pathological processes associated with hypoxia, ishemia and inflammation.[26]

Low pH modify SOD significantly in several respects.[27-29] The loss of 450 nm shoulder is observed in its absorption spectrum. Circular dichroism and electron spin resonance (ESR) spectra are modified also.[27,28] The changes are thought to be a result of protonation of His-61 residue which exists in the form of imidasol anion and binds Cu and Zn in the active site of the enzyme. The protonation of His-61 causes rupture of the bond between Zn and Cu-His-61 complex.[29] A reversible protonation of His-61 with subsequent splitting of Cu-His-61 bond were postulated to occur during the catalytic reaction.[30] Protonation-deprotonation of His-61 is important in two aspects:

1) Protonation changes a ligand sphere of Cu and the active site architecture is altered in going from Cu^{2+} to Cu^{+}.

2) Deprotonation at the step of reoxidation liberates proton which is necessary for formation of peroxide anion HO_2^- at the end of reaction.[30] We can speculate that protonation of His-61, bound to Cu, makes the change of metal valency during dismutation reaction difficult to occure and hence leads to SOD inhibition.

Acidic environment may produce inactivation of catalase as well. Apparently, acid-inactivated catalase is able to degrade hydrogen peroxide molecules, but free radicals, · OH are formed in this case rather than water and dioxygen.[31]

It was the purpose of this work to answer the question whether SOD will be inactivated at low pH and reactivated by helium-neon laser.

MATERIALS AND METHODS

Superoxide dismutase (SOD;ECu1.15.1.1) isolated from bovine erythrocytes ("Boehringer Mannheim") was dissolved in 10 mM Tris-HCl buffer, pH 7.4. After 30 min incubation the sample was divided into three portions and in two of them pH was adjusted to 5.9 and 8.2. The samples were kept at room temperature for at least two hours. The SOD concentration of 2 mg/ml was used in ESR experiments and 0.2 mg/ml in absorption spectroscopy and enzyme activity measurements. Bovine liver catalase ("Serva") was prepared similarly, but enzyme concentration was 0.05 mg/ml and pH was adjusted to 6.0 and 7.4.

The copper-histidine complex was prepared by dissolving 1 mM $CuCl_2$ and 4 mM histidine ("Reachim") in 10 mM Tris-HCl buffer and adjusting pH to 5.9, 7.4, and 8.2. The samples were kept at room temperature during 4–6 hours.

SOD activity was measured by the modified method.[32] The inhibition by SOD of the rate of nitroblue tetrazolium (NBT) reduction to phormazane by superoxide radicals was followed spectrophotometrically at 560 nm; NADH: phenazine methosulphate system was used. The activity of SOD incubated at pH 7.4 was taken for 100%.

The catalase activity was measured by monitoring the absorption of H_2O_2 at 240 nm.[33] The absorption spectra of SOD were measured in the region between 460 and 700 nm at room temperature.

ESR spectra were recorded on a radiospectrometer "Varian" E-4 with the 100 kHz modulation, amplitude 10 Gauss, magnetic field scan 2000 Gauss, scanning rate 250 Gauss/min at the incident microwave power of 10 mW. The sample of 150 μl in volume was frozen at 77 K and was allowed to be stabilized during 1 min. The illumination of the sample was performed at room temperature by the laser LG-78 (Soviet) producing radiation with wave length 632.8 nm with output power 2 mW and beam diameter 3 mm. The sample was placed at the distance of 30 cm from the light source, with the measurements of paramagnetic, optical and catalytic parameters being fulfilled immediately after irradiation.

RESULTS AND DISCUSSION

It has been found that the incubation of SOD in acidic medium brings about the enzyme inactivation. The SOD activity after two hours of incubation at pH 5.9 was found to be as low as 2–5% of initial value

(Fig. 1, left). The irradiation of solution gave rise to the enzyme reactivation: the activity was restored up to 82% of control level in 30 s of irradiation while in 60 s of the activity was even higher (107.5% of that for the control solution incubated at 7.4) (Fig. 1, right).

To elucidate the mechanism of photoreactivation of SOD in acid medium we measured the enzyme absorption spectra at different pH before and after irradiation by laser light beam. The results obtained are presented in Figure 2. It is seen that the incubation of enzyme at pH 5.9 leads to changes in its absorption spectrum, namely some changes in the region of the maximum at 680 nm were detected along with decrease of absorption around 450 nm. This is in agreement with the earlier data[27] obtained in experiments with SOD incubated during shorter period but at lower pH (below 5.0). The changes were reversible. When pH was adjusted to moderate alcaline values, then absorption spectra restored the feature typical of intact enzyme at these pH (8.2 for experiments in Fig. 2). The illumination of the enzyme in acid medium (pH 5.9) by red laser light resulted in the same changes in the spectra as the alcalinization of the solution. Indeed, it is seen in Figure 2 that the absorption spectrum of SOD measured immediately after irradiation by laser beam at pH 5.9 virtually coincides with that of SOD stored at pH 8.2 from the very beginning. The effect of irradiation was reversible: in several minutes after irradiation the absorption spectrum acquired the shape characteristic of the enzyme at low pH.

Additional information concerning ligand surrounding of metal in enzyme active site in different environmental conditions may be obtained by measuring ESR spectra. In Figure 3 ESR spectra of SOD are shown at pH 8.2 (1) and 5.9 (2). The incubation of the enzyme for 2 h at pH 5.9 is followed by certain

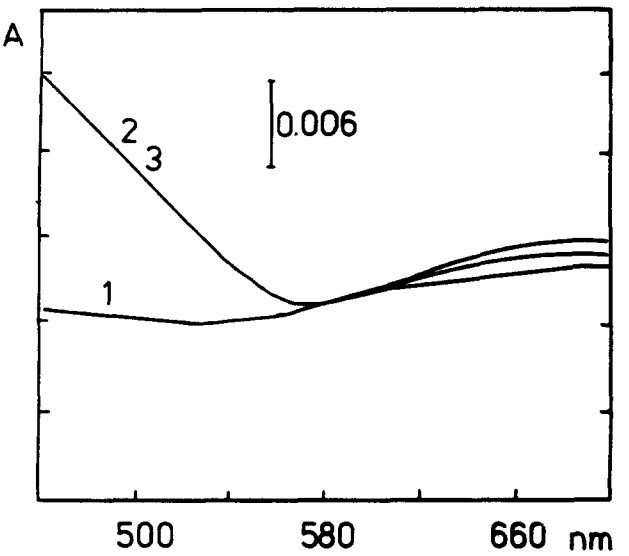

Fig. 2. Absorption spectrum of Cu-Zn-SOD at different pH before and after laser irradiation. 1- SOD in 10 mM Tris-HCl buffer, pH 5.9.; 2- The same after 60 s of irradiation; 3- SOD at pH 8.2.

changes in ESR spectra: the parameter A increased from 150 Gauss which is characteristic of SOD at pH 8.2 to 175 Gauss, along with a decrease of the parameter g_m. These changes were described earlier and may be attributed to some changes in coordination sphere of copper in active site of SOD as a result of dissociation of the bond between copper-histidine complex and zinc due to protonation of His-61.[27,28]

The illumination of SOD solution at pH 5.9 during 1 min by HNL light led to recovery of ESR spectrum parameters characteristic of the enzyme at pH 8.2 (Fig. 3, spectrum 3). The alcalinization of the solution to pH 8.2 led to the same changes in the enzyme ESR signal (Fig. 3, spectrum 4). The similarity between

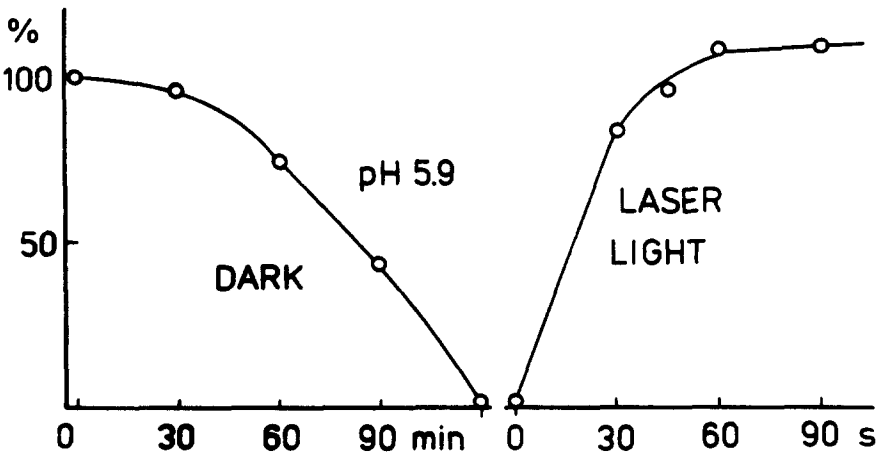

Fig. 1. The changes in superoxide dismutase activity during the incubation at pH 5.9 (left) and subsequent irradiation with red laser light (right). Ordinate activity of SOD, percentage of control level at pH 7,4. Abscissa-the time of incubation at pH 5.9 min (left), and of laser irradiation, s (right).

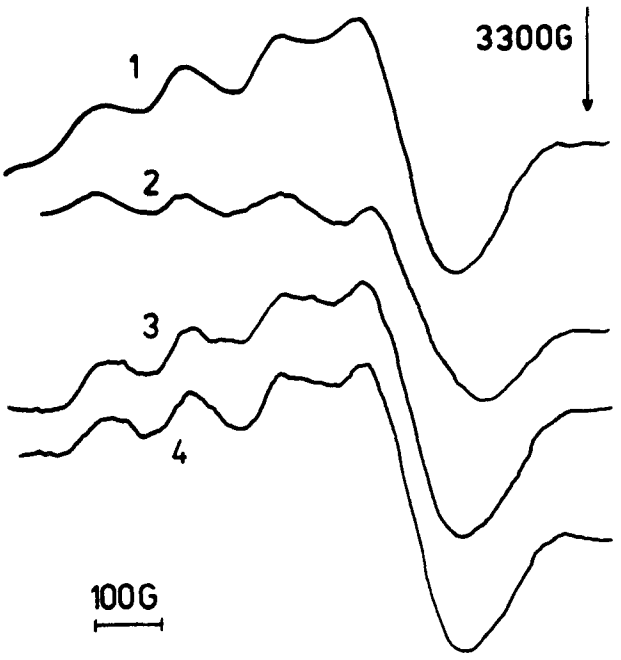

Fig. 3. ESR spectra of Cu-Zn-SOD at different pH before and after laser irradiation. 1-SOD at pH 8.2; 2-after incubation during 2 h at pH 5.9; 3-the sample 2 after 60 s of irradiation; 4-as in 2, but immediately after alkalinisation pH to 8.2.

effects of red (laser) light and increased pH makes it possible to assume that the mechanism of photoreactivation of SOD, previously inactivated at lower pH, consists of deprotonation of His-61 residue following the absorbtion of a photon by copper-histidine complex.

The absorbancy of Zn-Cu-SOD at 600–700 nm may be attributed to d-d transitions in Cu^{2+} -ion surrounded by four histidine residues (His-61, -44, -46, and -118) to whose nitrogen atoms copper ion is tightly linked. This complex in protonated state may be thought to serve as the acceptor of red light quantum. The experiments with a model system, copper-histidine complexes provided further evidence for this concept. The absorption of the complexes in long-wavelength region is pH dependent, with absorption maximum being situated at 632–633 nm at pH 5.8–6.0 and shifting to longer wave length at higher pH. In Figure 4 dependence of the absorption maximum position on pH and irradiation time (at pH 5.8) are presented. One can see that the alcalinization of the solution as well as irradiation by red laser light cause the same result: a shift of absorption maximum to longer wave lengths. It is noteworthy that the effect of illumination in copper-histidine complexes is reversible like that for the enzyme SOD: the incubation of irradiated solution in the dark was followed by the recovery of spectral properties peculiar to nonirradiated sample.

We may propose the following interpretation of our results with SOD and Cu-His complexes. The complex of Cu^{2+} with four histidine residues (His-61, -44, -46, and -118) is responsible for both absorption and ESR spectra of the enzyme SOD. In native enzyme (at neutral or mild alcaline pH) the histidine residue His-61 exists in deprotonated state and a bond between His-61 and Zn^{2+} is formed, necessary for the enzyme to be active. The mild acidification of the medium (to pH 5.9) brings about protonation of histidine and loss of the catalytic ability; definite changes in absorption and ESR spectra occur at the same time.

The absorption of a photon cause the transition of Cu-histidine complex from ground to the exited state. Many chromophores including aromatic amino acids are known to change their dissociation properties in excited states, the dissociation constant index pK in exited state being lower than that for ground state by one to several units.[34] One may postulate that the pK value of Cu-His complex in excited state is lower than 5.9, so that complex would be deprotonated in excited state at this pH. The changes in absorption spectra and ESR signals in this case are the same as after pH increase. The most important is the fact that catalytic activity of SOD would be restored both at pH shift to higher values (pH 8.2) and as a result of intensive (laser) light illumination at low pH (5.9 in our experiments).

A great amount of different enzymes containing organic complexes of copper in their active sites are known at present.[35] We may speculate that they all may have similar mechanism of photochemical modification of their functions by red (laser) light. On the other hand, a heme-containing enzyme catalase was also reported to increase its activity after irradiation by laser light.[24,36] Apparently, the enzyme had been altered in that case. The incubation of the enzyme solution at pH 6.2 led in our experiments to decrease in the enzyme activity to 88 ± 2% of that for the enzyme stored at pH 7.4 (control). At the same time an increase in absorption was observed at 407 and 630 nm. The subsequent illumination of the enzyme by red light at pH 6.2 caused complete reactivation (105 ± 2% of control). Our preliminary observations show that in the case of catalase a modification of catalytic pathway may occur along with decrease in orthodox enzyme activity. Catalase is a heme-containing oxidoreductase with iron-protoporphirin IX in its active cite.[37] In the vicinity of iron atom a water molecule is situated, connecting via hydrogen bonds with amino acids including histidine residue, distal to the heme.[37] The histidine participates in interaction of the active site with reactive substrate intermediates.[37] It seems likely that in acid medium the protonation of the histidine residue occurs like that observed in hemoglobin molecule[38] and

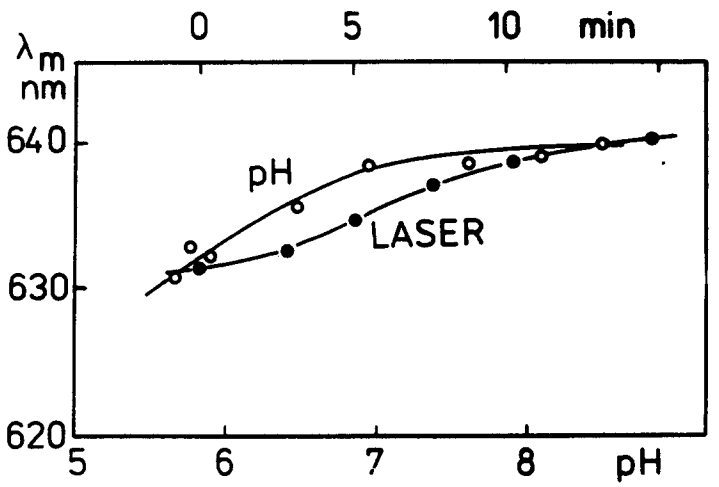

Fig. 4. The dependence of absorbtion maximum position (λ_m) of model copper-histidine complexes on pH (○, lower scale) and on the laser irradiation time (●, upper scale).

resulting in enzyme inactivation. The modified enzyme is probably still capable of destroying H_2O_2 but free radicals are produced in this case rather than neutral molecules of water and O_2 as by native enzyme.[31] The formation of oxygen free radicals, in particular · OH, may be detected by means of chemiluminescence.[39] The chemiluminescence in the mixture of catalase and H_2O_2 was described as early as in 1962.[39] In wound exudates, where pH may fall to the level pH 6.0 and lower,[26] the chemiluminescence in the presence of H_2O_2 was increased, while it was decreased after irradiation by red laser light. The irradiation of solutions of catalase also decreased significantly the emission of light in the course of H_2O_2 decomposition.[36] The precise mechanism action of red laser light on modified catalase molecule demands further investigation.

The recovery of initial activity of SOD and, possibly, catalase and other metal-histidine enzymes under illumination with intensive (laser) light may underlie the therapeutic effects of red laser irradiation. Tissue acidification is a typical consequence of pathological processes and may modify metal-containing enzymes, in particular, SOD. The protonation of the active sites in these enzymes may, however, be reversed under irradiation with intensive (laser) light. From this point of view, it is easy to understand why laser illumination does not affect normal tissues, cells or enzymes and exhibits pronounced stimulating effect when being applied to objects under extremal and pathological conditions.[18]

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