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ESR EVIDENCE OF SUPEROXIDE RADICAL DISMUTATION BY HUMAN CERULOPLASMIN

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SUMMARY: The formation of the paramagnetic complex between human ceruloplasmin and radiation produced superoxide radicals was observed by the ESR method at low temperatures. The disappearance of the complex without changes in the oxidation state of copper give the direct evidence that ceruloplasmin, the major antioxidant in serum, is able to dismutate superoxide radicals.

Ceruloplasmin, Cp, (EC 1.16.3.1) the multifunctional blue copper containing enzyme, shows oxidase activity directed towards aromatic amines and ferrous ions (1-5).

Recent report of Goldstein et al. (6) that the native human Cp, the major antioxidant in serum (7), inhibits in concentration-dependent fashion the O_2^- mediated reactions prompted us to show the ESR evidence of the formation of paramagnetic intermediate complex between Cp and HO_2 radicals. The ESR signal of this complex, recorded at 120 - 190 K for the frozen aqueous solutions of human Cp γ-irradiated at 77 K, is very similar to that registered for superoxide dismutase under identical experimental conditions (8). The disappearance of this complex above 200 K does not affect quan-

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titatively the ESR signal of human Cp. All this indicates that human Cp is able to dismutate HO_2/O_2^- radicals.

MATERIALS AND METHODS

Human Cp was obtained from Biomed Serum and Vaccine Manufactures, Warsaw (Poland), and purified before use by preparative polyacrylamide gel electrophoresis. The purity of Cp was tested by disc electrophoresis in 7% polyacrylamide gel at pH = 8.3, according to Marceau and Aspin (9), and at pH = 10.2 according to Ryden (10). In both cases gels showed electrophoretic homogenity of Cp preparation.

Chromophoric properties were determined by measuring the absorbance at 610 and 280 nm and only the samples with the absorbance ratio 0.044 (purity of about 97%) were used. Concentrations of Cp were calculated from the absorbance coefficients at 280 and 610 nm (11), and checked by the biuret method, Layne (12), with bovine serum albumin as a standard. The copper content determined according to the method of Gubler et al. (13) was equal to 2.68 μg per mg of protein.

Ceruloplasmin oxidase activity before and after irradiation was determined by the method of Ravin (14) with p-phenylenediamine dihydrochloride as a substrate. No marked changes were observed.

To prepare the samples for γ-irradiation the solutions of human Cp in 0.85% NaCl (pH = 6.8, protein content 55 mg/ml) were dropped into the liquid nitrogen to form solid spheres of diameter equal to about 3 mm. The samples were irradiated at 77 K in Co-60 source to the dose of 10 kGy at the dose rate of 6 kGy/hr. Under these conditions (8) the concentration of radiation produced HO_2 radicals was approximately equal to enzyme concentration. The irradiated samples were transferred into the liquid nitrogen ESR dewar or into a cold nitrogen gas flow system for thermal annealing.

Before and after the irradiation, as well as each heat treatment, the ESR spectra were recorded at 77 K with X-band microwave spectrometer SE-X/20 (Poland) provided with TE_{104} cavity. DPPH was used as standard.

RESULTS

Fig. 1 presents the ESR spectra recorded for the frozen solution of human Cp before (A) and after γ-irradiation at 77 K (B), and after subsequent thermal annealing at 210 (C), 230 (D), 250 (E) and 270 K (F). The spectrum of native human Cp, Fig. 1A, is very similar to that reported previously (15,16). It consists of two components: one with a three hyperfine peaks at about 2800 - 3010 Gs (type 1 Cu^{2+}) and the

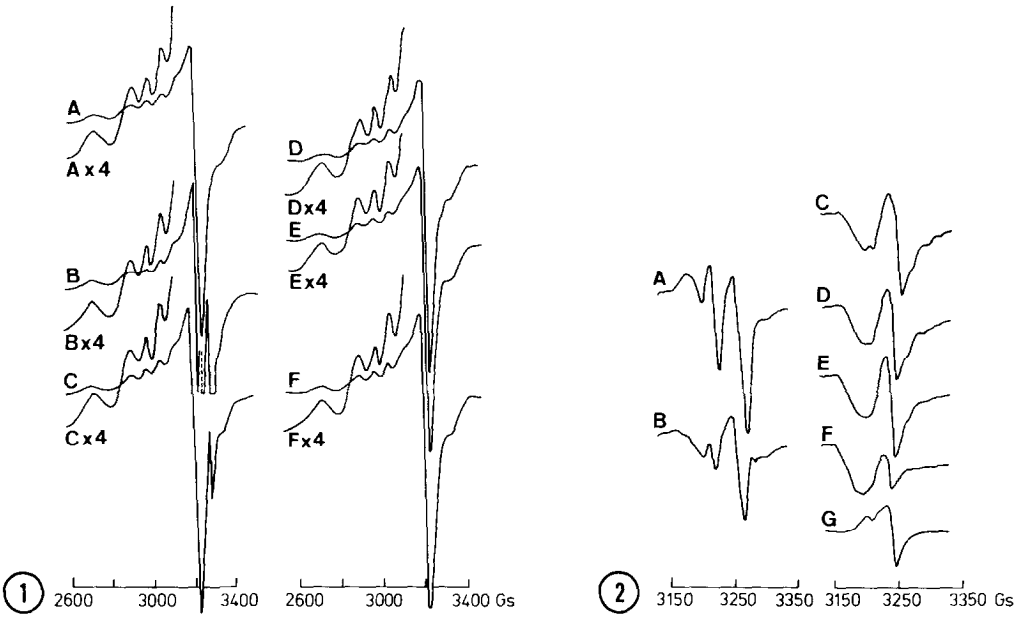

Fig. 1. ESR spectra recorded at 77 K (frequency 9.1 GHz, microwave power 3 mW, modulation amplitude 10 Gs) for frozen solution of human ceruloplasmin in 0.85% NaCl (pH = 6.8, protein content 55 mg/ml): unirradiated sample (A); sample γ-irradiated at 77 K (10 kGy, 6 kGy/hr) (B); γ-irradiated sample annealed for 10 min. at 210 (C), 230 (D), 250 (E), and 270 K (F).

Fig. 2. Radiation induced signals for frozen solution of human ceruloplasmin in 0.85% NaCl (pH = 6.8, protein content 55 mg/ml): sample γ-irradiated at 77 (10 kGy, 6 kGy/hr) (A); spectrum obtained by subtraction of OH radical signal from A (B); γ-irradiated sample annealed for 10 min. at 120 (C), 150 (D), 170 (E), 190 K (F); spectrum obtained by subtraction of E from F (G).

second one with a broade-hyperfine splitting at about 2650 Gs (type 2 Cu^{2+}). The signal generated by γ-irradiation, shown in detail in Fig.2, is superimposed on the unchanged spectrum of Cp, cf. Fig. 1B. It disappears completely above 200 K, cf. Fig. 1C and 1D. Its decay is not accompanied by the change of signal integral intensity of copper ions, Fig. 1D, E and F. Also the hyperfine structure of the low-field part of the spectrum is identical for unirradiated, Fig. 1A, and γ-irradiated Cp annealed to 270 K, Fig. 1F.

The details of the signals generated by γ-irradiation are shown in Fig. 2. After γ-irradiation at 77 K, Fig. 2A, one sees mainly the OH radicals, asymmetric doublet at $g = 2.009$ and a broad hump at $g = 2.048$ (17). Subtracting, however, the spectrum of OH radicals recorded for polycrystalline ice without Cp from that for the frozen solution of Cp one obtains the signal which is due to the presence of Cp, Fig. 2B. It could be also seen upon the thermal annealing of the samples above 120 K, Fig. 2C-F, after the decay of OH radicals. Subtracting e. g. spectrum depicted in Fig. 2F from that in Fig. 2E one obtains the asymmetric singlet, Fig. 2G, with $g_{\perp} = 2.01$ and $g_{\parallel} = 2.04$. The features of this signal do not resamable the sulfur radicals produced by γ-irradiation of proteins containing disulphide bonds (18), they are very similar to the complex $[E\text{-}Cu^{2+}...HO_2]$ observed for superoxide dismutase under identical experimental conditions (8).

DISCUSSION

In polycrystalline ice γ-irradiated at 77 K the OH and HO_2 radicals are trapped. They do decay upon thermal annealing at about 120 and 160 K, resp. (8,19). In the presence of human Cp, at the concentration approximately equal to that of HO_2 radicals, the asymmetric singlet, Fig. 2G, with $g_{\perp} \sim 2.01$ and $g_{\parallel} \sim 2.04$, is observed up to about 200 K. It decays above 200 K without changes in the ESR spectrum of Cp.

The spectral parameters of the asymmetric singlet are close to this for HO_2 radicals trapped in polycrystalline ice ($g_{\perp} = 2.0044$ and $g_{\parallel} = 2.039$) (20) and to that for the loose complex $[E\text{-}Cu^{2+}...HO_2]$ ($g_{\perp} = 2.008$ and $g_{\parallel} = 2.039$) observed under similar experimental conditions for superoxide dismuta-

se containing type 2 Cu^{2+} ions (8,25). Because of this we do assume that the asymmetric singlet signal observed in the presence of Cp is due to the similar complex formed by trapping HO_2 radicals. It involves the entrance of HO_2 radicals into the coordination sphere of copper ions, acting as the trapping agent (21), and replacement of water ligand (22) without subsequent spin exchange (8).

In Cp only type 2 Cu^{2+} ions have the water ligands (3,23). Regarding the structure and function of these copper sites (24) one would expect the decay of the complex $[E\text{-}Cu^{2+}...HO_2]$ by electron transfer onto Cu^{2+}. It is not the case as the decay of the complex above 200 K proceeds without changes in the ESR spectrum of Cp.

The obtained results, i. e. the formation of the complex $[E\text{-}Cu^{2+}...HO_2]$ and its decay without changes of copper oxidation state, do imply strongly that reaction of human Cp with HO_2 radicals proceeds according to the dismutation mechanism developed recently for superoxide dismutase (25).

The essential features of this mechanism (25), written e. g. for HO_2 radicals as:

$$E^{o} + 2\ HO_2 = E^{o} + O_2 + H_2O_2 \quad ,$$

where E^{o} denotes the native superoxide dismutase in which two copper ions are oxidized, consists in electron transfer between radicals proceeding without changes of the oxidation state of copper ions. They are fully consistent with recent findings (26) for $Cu(ClO_4)_2$ solutions: the HO_2/O_2^- radicals first form copper complexes:

$$Cu^{2+} + O_2^- \longrightarrow CuO_2^+ \quad ,$$

$$Cu^{2+} + HO_2 \longrightarrow Cu(OOH)^{2+} \quad ,$$

in which the Cu^{2+} cation plays the same role as the proton in HO_2, then dismutation without changes of copper oxidation state proceeds:

$$Cu(OOH)^{2+} + O_2^- + H_2O \longrightarrow Cu^{2+} + H_2O_2 + O_2 + OH^- \quad ,$$

$$CuO_2^+ + O_2^- + 2\ H_2O \longrightarrow Cu^{2+} + H_2O_2 + O_2 + 2\ OH^- \quad .$$

Thus the model proposed for superoxide dismutase (25) may be also valid for blue oxidases containing type 2 Cu^{2+} ions which share most of ligand properties with superoxide dismutase (3,23,27). As far as Cp is concerned, one of its subunits (27), containing type 2 Cu^{2+} ions, is likely to play the protective role for the molecule by scavenging the superoxide radicals which are formed in one-electron transfer reaction under the physiological conditions.

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