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ESR STUDIES ON OXIDATION STATE CHANGES OF COPPER IN SUPEROXIDE DISMUTASE DURING REACTIONS WITH WATER RADIOLYSIS PRODUCTS AT CRYOGENIC TEMPERATURES

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Summary

Irradiating the aqueous solutions of native and reduced superoxide dismutase with ^{60}Co γ-rays at 77 K and recording the ESR spectra during thermal annealing the formation and decay of the complexes $E\text{-}Cu^{2+}...HO_2$ and $E\text{-}Cu^{+}...HO_2$ have been observed. Decay of ESR signals corresponding to HO_2 in these complexes is not accompanied by immediate changes of the oxidation state of copper. The delayed changes of copper oxidation state are probably due to the reactions of dismutation products with superoxide dismutase.

Quenching of the dilute aqueous solution of superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) in liquid nitrogen and subsequent γ-irradiation at that temperature leads to the enzyme-substrate system trapped in the fairly rigid matrix of polycrystalline ice [1] in which all reactions involving molecular motion are effectively suppressed. During thermal annealing of polycrystalline ice from 77 K to the melting point the system passes through at least three widely separated relaxation regions, at 115 ± 5, 160 ± 30, and 230 ± 30 K [2], in which due to microscopic displacement of solvent molecules the trapped species could be made mobile and/or reactive. In the second relaxation region in which in pure polycrystalline ice the hydroperoxy radicals decay completely [1] we have observed in the presence of native superoxide dismutase a loose complex, $E\text{-}Cu^{2+}...HO_2$ [3].

Below we report the attempts to follow the next steps of dismutation reaction which start with tightening of the complex at temperatures of about 200 K. The decay of the ESR signal of superoxide radicals is not accompanied

by immediate changes of the oxidation state of copper in the enzyme. There is a marked delay of the changes of the oxidation state of copper both for native and reduced superoxide dismutase. This may indicate, in our opinion, that dismutation of superoxide radicals proceeds without changes in the oxidation state of copper in superoxide dismutase and contradicts the reaction scheme involving alternate use of two active centres present in superoxide dismutase [4,5]. The delayed changes in the oxidation state of copper in enzyme are due to the reactions with products of dismutation reaction leading to partial reduction of native superoxide dismutase or to partial oxidation of reduced superoxide dismutase, respectively.

Freeze-dried powder of superoxide dismutase isolated from bovine erythrocytes (12 300 units/mg) was kindly supplied by Miles Laboratories (PTY) Ltd. It was dissolved in 0.005 M potassium phosphate buffer, (pH 7.4) to form a solution of a concentration of 10 mg/ml. Part of this solution was treated with 25 M excess of H_2O_2 for 10 s under argon and dropped into liquid nitrogen to form spheres of about 2—3 mm diameter. Samples of reduced superoxide dismutase formed in this way, and similarly prepared samples of native superoxide dismutase, with or without HCOONa added, were γ-irradiated in ^{60}Co source at 77 K. The irradiated samples were transferred into liquid nitrogen ESR dewar vessels for measurements at 77 K or into a cold nitrogen gas flow system for annealing at successively higher temperatures.

Activity of superoxide dismutase in γ-irradiated and annealed samples was checked after melting according to the method of Misra and Fridovich [6] and no marked changes were observed.

The ESR spectra were recorded at 100 kHz magnetic field modulation with an X-band microwave spectrometer SE-X/20 (Poland) provided with TE_{104} cavity. 1,1′-Diphenyl-2-picrylhydrazyl radical and Mn^{2+} were used as standards.

Irradiating the aqueous solutions of superoxide dismutase with ^{60}Co γ-rays at 77 K and recording the ESR spectra during thermal annealing we were able to observe [3] the first step of enzymatic dismutation of superoxide radicals: substrate entrance into the first coordination sphere of Cu^{2+} with formation of so-called 'loose complex'. Its ESR signal consisted of this due to HO_2 radicals, $g_\perp = 2.008$, $g_\parallel = 2.039$, superimposed on that due to Cu^{2+} in the native superoxide dismutase, $g_\perp = 2.062$, $g_\parallel = 2.263$, $A_\parallel = 152$ G. In the third relaxation region the ESR signal of HO_2 radicals decayed completely and a small decrease (about 10—12%) of the signal due to Cu^{2+} was observed.

To explore the details of this step we have carried out experiments on polycrystalline ice containing native superoxide dismutase and 0.1 M HCOONa which acting as a scavenger towards OH radicals substantially increases the concentration of HO_2 radicals under the same irradiation conditions [1]. Typical results obtained in several runs are depicted in Fig. 1.

When irradiating native superoxide dismutase at 77 K one obtains the signals of OH and HO_2 radicals superimposed on the signal of Cu^{2+} in native superoxide dismutase (Fig. 1B) [3]. Upon thermal annealing to the temperature of the first relaxation region the OH radicals decay and the remaining ESR signal consists of this due to HO_2 radicals superimposed on that of Cu^{2+}. In pure polycrystalline ice the signal of HO_2 radicals decays in the second relaxa-

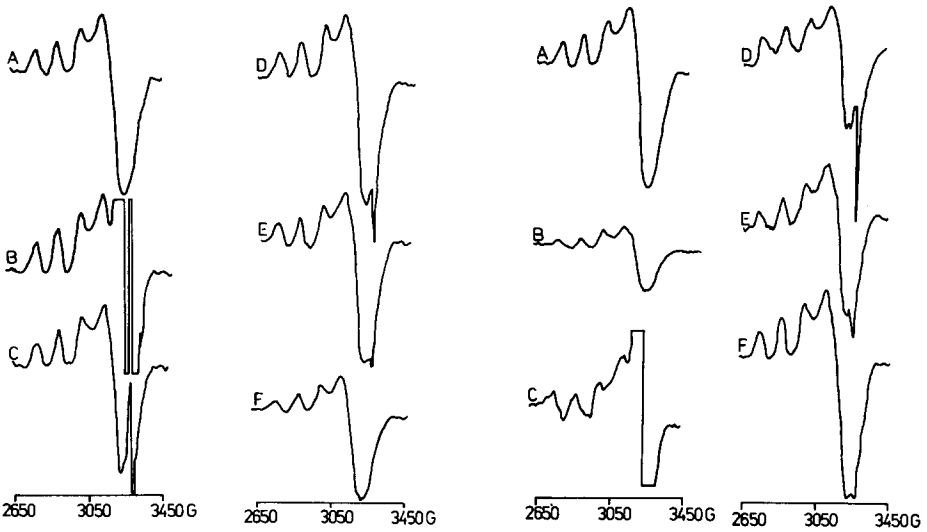

Fig. 1. ESR spectra (frequency 9.48 GHz, microwave power 3 mW, modulation amplitude 3G) recorded at 77 K for polycrystalline ice prepared from native superoxide dismutase in air saturated 0.005 M potassium phosphate buffer with 0.1 M sodium formate added (pH 7.4, protein content 10 mg/ml, enzymatic activity 12 300 units/mg): (A) unirradiated sample, (B) sample γ-irradiated ($8 \cdot 10^3$ gray/h, 1 h) at 77 K, (C) irradiated sample annealed for 10 min at 210 K, (D) irradiated sample annealed for 10 min at 230 K, (E) irradiated sample annealed for 10 min at 250 K, and (F) irradiated sample annealed for 10 min at 270 K.

Fig. 2. ESR spectra (frequency 9.48 GHz, microwave power 3 mW, modulation amplitude 3G) recorded at 77 K for polycrystalline ice prepared from superoxide dismutase in 0.005 M potassium phosphate buffer (pH 7.4, protein content 10 mg/ml, enzymatic activity 12 300 units/mg): (A) unirradiated sample of native superoxide dismutase in air saturated buffer, (B) unirradiated sample of superoxide dismutase reduced at room temperature for 10 s with 25 M excess of H_2O_2 in argon saturated buffer, (C) sample of reduced superoxide dismutase γ-irradiated ($8 \cdot 10^3$ gray/h, 1 h) at 77 K, (D) irradiated sample annealed for 10 min at 210 K, (E) irradiated sample annealed for 10 min at 230 K, (F) irradiated sample annealed for 10 min at 250 K.

tion region. Addition of water complexes of Cu^{2+} is without effect on the stability of HO_2 radicals [7]. Presence of superoxide dismutase stabilizes the signal of HO_2 radicals effectively, presumably due to formation of a 'loose complex' [3]. Under the present experimental conditions, i.e. with 0.1 M HCOONa, the signal of HO_2 radicals is 10—12 times greater than that observed for the same system without HCOONa. The signal of HO_2 radicals starts to decay in the third relaxation region at about 200 K. Its decay is not accompanied by immediate changes of the oxidation state of copper in the enzyme (cf. Fig. 1C, D and E). A marked reduction of Cu^{2+} (30—38%) proceeds upon annealing at about 270 K (cf. Fig. 1).

Reducing native superoxide dismutase (Fig. 2A) at room temperature for 10 s with 25 M excess H_2O_2 under argon, we obtained reduced superoxide dismutase (Fig. 2B) with the enzymatic activity preserved. Upon γ-irradiation some reoxidation of superoxide dismutase proceeds as may be inferred from the signal of Cu^{2+} (cf. Fig. 2C) on which, as before, the signals of OH and HO_2 radicals are superimposed. The OH radicals decay in the first relaxation region, the HO_2 radicals start to decay in the third relaxation region (cf. Fig. 2D and E). The decay of HO_2 radicals is not accompanied by immediate changes of

the oxidation state of copper in the enzyme. As before, there is a marked delay of copper oxidation which proceeds upon annealing above 210 K (cf. Fig. 3). Annealing to slightly higher temperatures of about 270 K causes reduction of Cu^{2+} due to reaction with excess H_2O_2 not removed, and the nonreducible fraction, presented in Fig. 2B, remains [5].

Using the technique of rapid-freezing ESR, Fielden et al. [5] have observed the reduction of copper in native superoxide dismutase and oxidation of copper in reduced superoxide dismutase in reaction with O_2^- generated in liquid solution. These experiments failed to detect the formation of an intermediate enzyme-O_2^- complex since, as we believe, only late events were observed (cf. below). Based on these experiments and on pulse radiolysis data [5,8] it has been suggested that copper in superoxide dismutase undergoes a cyclic reduction and oxidation during catalysis:

$$E\text{-}Cu^{2+} + O_2^- \rightarrow E\text{-}Cu^{+} + O_2 \quad (1)$$

$$E\text{-}Cu^{+} + O_2^- + 2H^+ \rightarrow E\text{-}Cu^{2+} + H_2O_2 \quad (2)$$

There is no support for this reaction scheme in our experimental results. Producing more than equimolar amounts of HO_2 radicals in the presence of HCOONa acting as OH radical scavenger it is highly probable that both active sites in superoxide dismutase molecules were occupied. An increased intensity

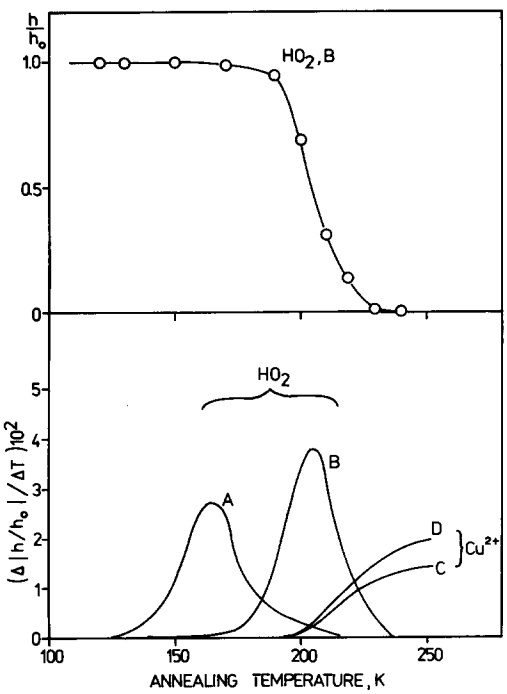

Fig. 3. Effect of annealing temperature on ESR signal of HO_2 radicals (A, B) and Cu^{2+} (C, D) in γ-irradiated (at 77 K) polycrystalline samples of water (A) and aqueous solutions of native (B, C) and reduced superoxide dismutase (D) with HCOONa added. Upper panel, illustrative depiction of thermal annealing curve, i.e. signal amplitude of HO_2 radicals in sample B (relative units) vs. annealing temperature Lower panel, derivative presentation of annealing curves.

of the ESR signal of the 'loose complex', mentioned above, is in full agreement with this assumption. Fig. 3 shows the temperature-dependent changes intensity of ESR signals for the HO_2 radicals and Cu^{2+}. First of all it is seen from this figure that the temperature range of the decay of HO_2 radicals in pure polycrystalline ice does not greatly overlap that of the HO_2 radicals in polycrystalline sample containing superoxide dismutase. The increased thermal stability of HO_2 radicals in the latter case indicates a more effective trapping of the radicals when bound to the enzyme molecules in the form of 'loose complexes'. There are also two other pertinent points in the results, presented in Fig. 3: (a) decay of the 'loose complexes' proceeds without changes in the oxidation state of copper; and (b) changes in the oxidation state of copper in the enzyme molecules are observed with a marked delay.

Due to this, rather than to the mechanism involving successive copper reduction and oxidation according to Reactions 1 and 2, we are inclined to Reaction 3 for native superoxide dismutase and Reaction 4 for reduced superoxide dismutase:

$$E^{o} + 2HO_2 = E^{o} + O_2 + H_2O_2 \quad (3)$$

$$E^{2-} + 2HO_2 = E^{2-} + O_2 + H_2O_2 \quad (4)$$

where E^{o}, as in Ref. 8, denotes the native enzyme in which both copper atoms are oxidized while E^{2-} denotes the doubly reduced enzyme. These schemes involve two active centres present in superoxide dismutase to explain dismutation of superoxide radicals. In order to realize the dismutation Reaction 3 and 4, any redox reaction at one particular active centre is required to be followed by an immediate transfer of the redox equivalent from the second site, as it was originally suggested by Lazdunski et al. [4]. Although the second redox reaction might be brought about by the transfer of the HO_2 radical initially trapped in the 'loose complex', the more probable process would be the reaction of 2 molecules of superoxide being in intimate contact in the transient state. The reaction mechanism requires deprotonization of one HO_2 radical in the bimolecular complex to yield superoxide ion, since it is known that O_2^- must react with its conjugate acid HO_2 [9]. This specific acid-base catalysis of dismutation reaction may be realized at the active site of superoxide dismutase, because it contains a water molecule bound to the copper ion [10].

The delayed changes in the oxidation state of copper in superoxide dismutase observed under the present experimental conditions, i.e. in the closed enzymatic system, are probably due to the reactions of superoxide dismutase with the products of HO_2 dismutation.

In the case of native superoxide dismutase it would be [8,11—13]:

$$E^{o} + H_2O_2 \rightarrow E^{2-} + O_2 + 2H^{+} \quad (5)$$

and in the case of reduced superoxide dismutase [13,14]:

$$E^{2-} + O_2 + 2H^{+} \rightarrow E^{o} + H_2O_2 \quad (6)$$

We hope that the above presented results, complementary to those obtained by Fielden et al. [5] by rapid-freezing ESR (Fig. 9 in Ref. 5), may be

of some use in a situation when experimental results forming the basis of the half-sites mechanism of superoxide dismutase action seem to be doubtful (Bray, R.C., personal communication).

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