THE EFFECT OF OXYGEN ON ALKALINE SOLUTIONS OF SUPEROXIDE DISMUTASE

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SUMMARY

The blowing of oxygen through strongly alkaline solutions of SOD leads to the drop of pH by more than 3 units. The rate of the process depends linearly on the concentration of SOD. The effect of oxygen on the modification of the shape and the decrease of the intensity of EPR signal of SOD were observed. The incubation of strongly alkaline solutions of SOD under vacuum leads to the reduction of the protein copper. The data obtained suggest, that the reduced copper may be at least partially reoxidized by oxygen. It is suggested that at pH 12.5 and higher in the presence of SOD the reaction of electron transfer from hydroxyl anion to the oxygen takes place:

$$OH^- + O_2 \longrightarrow \cdot OH + O_2^{\cdot -}$$

INTRODUCTION

Superoxide dismutases (SOD) are known to catalyze the reaction of dismutation of oxygen anion radicals as well as the reverse process of generation of superoxide radicals from hydrogen peroxide and molecular oxygen (1,2):

$$2O_2^{\cdot -} + 2H^+ \rightleftharpoons H_2O_2 + O_2$$

In the dismutation process two protons are involved and thus it is important to investigate the properties of the enzyme in the media of different acidity. In the recent work (3) it has been shown that in weakly acid media the enzyme undergoes a change in the environment of the copper and some conformational modifications take place. On the other hand, the data

available (4,5) indicate that no essential changes in the properties of the enzyme occur in alkaline solutions up to pH 12.0. Moreover, the observed changes in the EPR and optical spectra of SOD at pH 12.5 are still reversible. These facts suggest the remarkable stability of SOD in the alkaline media. Such stability of the protein must have a certain biological significance.

In the present communication we report effects of oxygen and vacuum on the properties of SOD alkaline solutions and pay attention to a reaction possibly catalyzed by SOD in strongly basic media.

MATERIAL AND METHODS

The preparation of SOD from bovine erythrocytes was obtained by the method of McCord and Fridovich (6) and it was further purified as described in the work (7). The final preparation was electrophoretically homogeneous and had spectral purity index (A_{260}/A_{680}) of 26. Concentrations of the protein were estimated by using molar extinction of 300 $M^{-1}cm^{-1}$ for the band at 680 nm. The apoprotein was prepared by the method of McCord and Fridovich (6). The pH was adjusted by adding 0.5 M KOH to the protein solution in 0.005 M phosphate buffer, pH 7.4. EPR spectra were recorded on a Varian E-4 instrument at -160°C. Optical spectra were obtained at 22°C in 10 mm cells. The incubation of the protein under vacuum (10^{-1} mm of Hg) was lasted for several hours.

RESULTS

The aeration of strongly basic solutions of SOD with oxygen was found to lead the drop of their pH. This phenomenon is clearly observed if initial pH of the protein solution was 12.5 or higher. In various control experiments, when oxygen was blowed through alkaline solutions containing no proteins or in the presence of the apoprotein, no decrease of their pH was observed. Also, no changes of pH of alkaline protein solution were noted when the solution was bubbled with nitrogen, but not with oxygen. From obtained kinetic curves (Fig. 1) the following conclusions may be drawn.

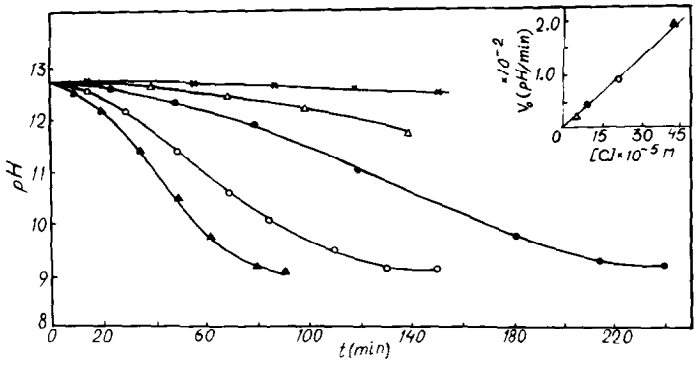


Fig. 1. Time course of pH drop at different concentrations of SOD at blowing of oxygen. The inserted part shows the dependence of the initial rate of pH drop on concentration of SOD. Oxygen pressure in all cases was 20 mm of Hg. x - data in the absence of the protein.

The final decrease in pH (ΔpH$\sim$3.5) does not depend on the concentration of SOD. Similar results were obtained when pH of the protein solution before aeration with oxygen was adjusted to 13.2. Whereas, after several hours of aeration no remarkable drop of pH was observed when the initial pH of the solution was 11.2. Thus it has been established that oxygen leads to the drop of pH in SOD solutions only when their pH were sufficiently high with pH of 12.5 being as the critical point. In the second line of experiments the effect of vacuum on the properties of SOD solutions was studied. No changes in pH as well as in optical and EPR spectra were observed when the protein solutions with initial pH of 7.4 or 11.7 were incubated under vacuum for 4 hours. However, when initial pH of SOD solutions was 12.7 or higher the progressive decrease of the integral intensity of EPR spectra was observed in the course of incubation under vacuum, indicating the reduction of the protein copper. Thus, "self-reduction"

of the copper took place when strongly basic solutions of SOD were subjected to vacuum-storation. It has been found that this reduced protein might be, at least, partially reoxidized by the blowing of oxygen through the solution which pH was decreased from 12.7 to 9.3 in the course of the blowing. The results of these experiments are shown in Fig. 2.

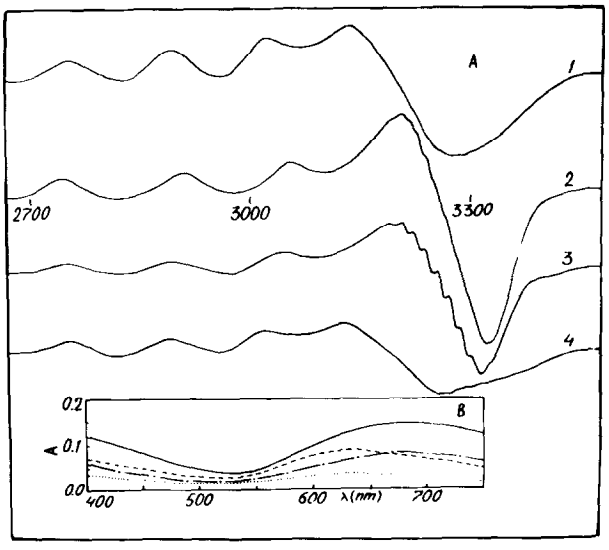


Fig. 2. Changes in EPR (A) and optical (B) spectra of SOD (4.10^{-4}M) in the course of oxygen blowing. 1- (——) SOD was aerated at pH 7.4 during 4 hours. 2- (– – –) SOD immediately after the alkalyzation to pH 12.7. 3- (.–.–.) sample 2 after 20 min aeration by oxygen. 4- (....) sample 2 after 90 min aeration by oxygen. EPR spectra were taken under the following conditions: frequency 9.38 GHz, power 10 mW, modulation amplitude 6.3 gauss, time constant 0.3 sec.

Fig. 3 represents the optical and EPR spectra of SOD before and after the aeration of the solutions. As may be concluded the short-time blowing of oxygen results in 30% decrease of integral intensity of the EPR spectrum and in the essential intensity increase of the nitrogen superhyperfine struc-

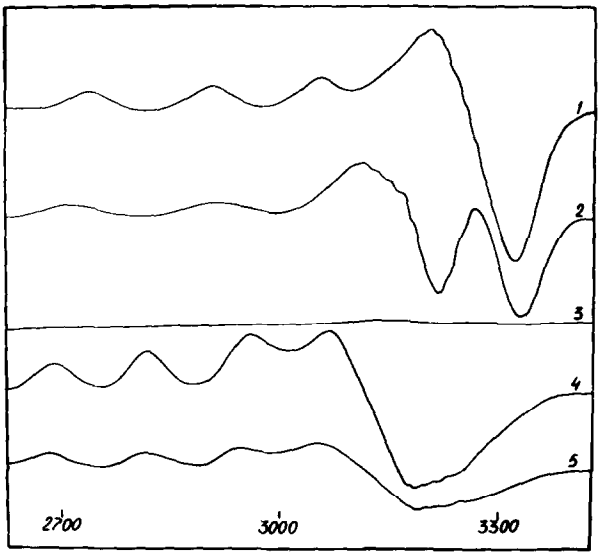


Fig. 3. Effect of vacuum on EPR spectra of SOD. 1- SOD alkalyzed to pH 12.7, 2- SOD alkalyzed to pH 13.5, 3- samples 1 or 2 after incubation under vacuum for 4 hours (pH was not changed). 4- SOD at pH 7.4 after incubation under vacuum for 20 hours (the shape and intensity were not changed). 5- sample 3 after aeration by oxygen for 5 hours, (pH became 9.3). Conditions of EPR spectroscopy were similar to those of Fig. 2.

ture. Although more prolonged aeration of the solution results in the EPR spectrum which have the shape characteristic for neutral and basic media, however its integral intensity remains 30% lower than that of the initial spectrum. It should be noted that the changes in optical spectra completely correlate with EPR data. In control experiments when the neutral or mildly basic solutions of SOD were aerated by oxygen no changes in optical and EPR spectra of these solutions were observed. Thus, oxygen leads to the partial reduction of the SOD copper in strongly basic (pH 12.5) solutions and to the drop by more than 3 units in pH of such solutions. On the other hand, the incubation of strongly basic solutions of SOD

under vacuum results in partial reduction of copper, but no changes in the pH of the solutions were noted.

DISCUSSION

The phenomenon of the reduction of blue copper-containing proteins in weakly basic media was observed in several works (8,9). It has been suggested that in these proteins there is a reducing group which is dissociated at alkaline pH. However the properties of SOD are essentially different from those of the blue copper-containing proteins (e.g. laccase or ceruloplasmin). The optical and EPR spectra of SOD were not changed in alkaline media with pH about 12.0. However at pH 12.5 and higher, certain changes were observed (4). At this pH the deprotonization of some functional groups (in particular, imidazol (10)) of the protein takes place and the substitution of water molecule, coordinated to the copper by hydroxyl anion occurs (11). The effect of oxygen on pH of alkaline solutions of SOD described here should be connected with the loss of hydroxyl groups from the medium rather than with the rise of proton concentration in the medium. Therefore it may be suggested that in basic media in the presence of SOD the reaction of electron transfer from hydroxyl anion to oxygen takes place:

$$OH^- + O_2 \longrightarrow \cdot OH + O_2^{\cdot -} \quad (1)$$

In the absence of the protein this reaction does not proceed the copper being essential for the effectivity of this process. Fatiadi (12) proposed that such a reaction is responsible for the formation of superoxide radicals in alkaline solutions of some low-molecular weight complexes generally used for the oxidation of carbohydrates. By means of the reaction (1) it is possible to explain the partial reduction of the protein copper

in the course of the aeration of strongly basic solutions, because generated superoxide radicals possess reducing properties:

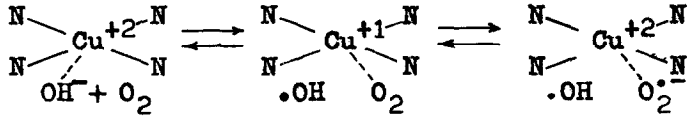
$$Cu^{+2} + O_2^{\bullet -} \longrightarrow Cu^{+1} + O_2$$

On the other hand, the "self-reduction" of the copper under vacuum which, in contrast to the reduction by aeration, was not accompanied by the drop of pH, gives us the possibility to imagine that in the last reaction there is the equilibrium:

$$Cu^{+2} + O_2^{\bullet -} \rightleftharpoons Cu^{+1} + O_2$$

Actually, in accordance with this scheme the removal of oxygen under vacuum should result in the reduction of the copper, if there were superoxide radicals in the solution.

It is known that these radicals are stabilized in rather alkaline media, or in some organic solvents (13). In connection with the obtained results it should be noted that the EPR spectrum of the so-called pink copper-containing enzyme, galactose oxidase, is similar to that of SOD at pH 12.5 (14). Galactose oxidase was shown to have superoxide dismutase activity (15,16). In this enzyme the copper atom is also linked with the nitrogen atoms. Besides, the superoxide radical is coordinated to the copper atom of the enzyme. It is known (17,18) that in SOD the copper atom is linked with 4 atoms of imidazol nitrogens. In the first coordination sphere of the copper of SOD the water molecule which is involved in the process at in strongly alkaline media, is substituted by hydroxyl ion (11). Therefore it may be suggested that at pH 12.5 and higher in the coordination sphere the electron transfer takes place such as:



Thus, from the above data it is possible to think that certain similarities exist between the mechanisms of galactose oxidase, the low-molecular weight copper complexes, oxidizing of carbohydrates and copper-containing SOD.

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