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ON THE MECHANISM OF SUPEROXIDE DISMUTASE REACTION OF THE BOVINE ENZYME WITH HYDROGEN PEROXIDE AND FERROCYANIDE

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SUMMARY

1. The cupric copper at the active site of bovine superoxide dismutase is reduced at a relatively fast rate by ferrocyanide and sulfide in the ratio of one molecule of reducing agent per copper atom.

2. The ferrocyanide-reduced product is stable in air, and is reoxidized by ferricyanide at approximately the same rate as the reduction reaction. Reoxidation by ferricyanide is much faster if anions such as CN^- and N^-_3 are added to the ferrocyanide-reduced protein.

3. The addition of H_2O_2 leads to an almost complete disappearance of absorbance and of the electron paramagnetic resonance spectra of the enzyme copper. Evidence is presented that this is a true reduction by the H_2O_2/O_2 couple, and not a coupling between two Cu^{2+}. The rate of reduction is comparable to that by ferrocyanide and sulfide, as it is the rate of reoxidation of the H_2O_2-reduced protein by ferricyanide. On the other hand, reoxidation by oxygen is very slow, as in the case of the sulfide-reduced protein.

4. From these data, E'_0 for the enzyme copper is approximately calculated at +0.40 V, which is appropriate for a dismutation mechanism involving alternate reduction and reoxidation of copper for the O^-_2/O_2 and O^-_2/H_2O_2 couples. The kinetics of these reactions indicate stability of the reduced protein toward oxygen and facilitation of reoxidation by anion binding. These results support the same type of mechanism.

INTRODUCTION

Superoxide anion radicals (O^-_2), produced by univalent reduction of oxygen during biological oxidations, dismutate spontaneously according to the following reaction:

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

This reaction is catalyzed by the enzyme superoxide dismutase. The mammalian enzyme contains two zinc ions and two cupric ions per molecule of protein (mol. wt 33 000). Copper appears to be directly involved in the dismutase reaction on the basis of recombination studies[1,2]. However, in certain experimental conditions removal of zinc causes irreversible denaturation[2]. This could be due to a structural role of the Zn^{2+} in maintaining the protein conformation necessary for enzyme activity. This active conformation seems to be related to a peculiar geometry of the copper site, which is significantly distorted from the square-planar coordination usually found in Cu(II) complexes and in copper proteins[3]. Most recently, pulse radiolysis studies of the bovine superoxide dismutase[4,5] gave extensive information on the steady state parameters of the reaction; moreover, experiments performed at high enzyme concentrations in the presence of approximately stoichiometric amounts of O^-_2 showed a significant decrease of the absorption band of the enzyme copper without any apparent recovery within 1 ms. Since this time exceeds the expected turnover time, the bleaching seemed unrelated to the catalytic mechanism unless it is assumed that a stable reduced intermediate is formed under those conditions. In such a case a plausible reaction mechanism would be

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2$$
$$Cu^+ + O_2^- + 2H^+ \rightarrow Cu^{2+} + H_2O_2$$

It can be envisaged as a ping-pong mechanism where the Cu^{2+}–enzyme is reduced by substrate acting as a reductant, and the Cu^+–enzyme is oxidized by substrate acting as an oxidant. In this case both copper sites would be equivalent. While further pulse radiolysis work is in progress to definitely prove this mechanism, it seemed to us to be interesting to investigate in detail the oxidation–reduction reactions of the enzyme copper, as an independent approach to the understanding of a dismutase reaction mediated by a valence change of the one-electron acceptor metal ion.

MATERIALS AND METHODS

All chemicals used were reagent grade. Bovine superoxidase dismutase was purified according to McCord and Fridovich[1]. Protein concentrations were determined from the absorbance at 680 nm (see ref. 1) or from the intensity of the electron paramagnetic resonance (EPR) signal of copper (protein concentration is half the copper concentration). Absorbance and EPR spectra were recorded as previously described[6].

RESULTS

Reaction with ferrocyanide

Fig. 1 shows a spectrophotometric titration of superoxide dismutase (water solution, 25 °C) with ferrocyanide. The copper optical absorption band, with a maximum at 680 nm, decreases on addition of ferrocyanide, with concomitant absorbance increase at 420 nm due to the ferricyanide formed. A parallel decrease in the intensity of the Cu^{2+} EPR signal of the frozen solutions was also observed. No appreciable differences were found in the absence of oxygen. Overnight dialysis against water did not reverse the spectral change completely, whereas dialysis against phosphate buffer resulted in a complete restoration of the original absorbance. This

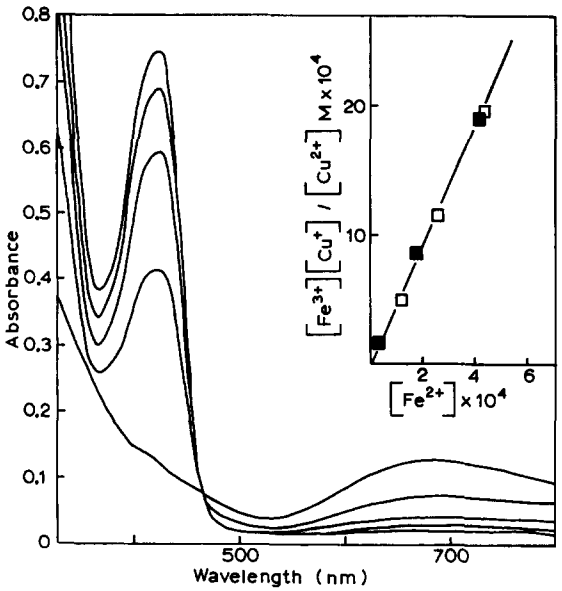

Fig. 1. Absorbance spectra of the reaction of bovine superoxide dismutase with ferrocyanide. The isosbestic series of curves refers to the reaction of $5 \cdot 10^{-4}$ M enzyme with $6 \cdot 10^{-4}$, $9 \cdot 10^{-4}$ and $12 \cdot 10^{-4}$ M $K_4[Fe(CN)_6]$ in water solution at 25 °C. In the inset ■ refers to the same series, □ to another similar experiment.

could be due to the fact that raising of the ionic strength facilitates dialysis of anions through the dialysis membranes, which usually bear negative charges. However, the possibility that buffer anions displace ferricyanide from a ferricyanide binding site on the protein can not be ruled out.

From the optical titration $K \simeq 5$ was calculated for the equilibrium $[Fe^{3+}][Cu^{+}]/[Fe^{2+}][Cu^{2+}]$ (Fig. 1, inset), assuming identical extinction coefficients for free and protein-bound ferricyanide. The same approximate value was obtained by adding ferricyanide to the enzyme + ferrocyanide reaction mixture and observing the resulting optical changes.

Kinetic measurements carried out in the standard spectrophotometer used for optical spectra gave comparable rates for reduction and reoxidation with half times of a few seconds in pseudo-first-order conditions in the presence of 10^{-3} M enzyme.

Effects of azide and cyanide on ferrocyanide-reduced protein

Figs 2A and 2B show the effects of the addition of azide and cyanide respectively to ferrocyanide-reduced samples. They cause reoxidation of the copper moiety at the expense of the ferricyanide present at equilibrium, as demonstrated by the reappearance of the absorbance bands of the oxidized protein between 500 and 750 nm, with a parallel decrease of the ferricyanide peak. It is interesting to note that, while the absorbance band which arises on addition of cyanide corresponds to that of the cyanide complex previously described[3,6], the absorbance maximum of the azide-treated protein moves, on standing, toward shorter wavelengths. Nevertheless the low temperature EPR spectrum of this sample (Fig. 2C) is typical of the azide complex of the protein copper[3].

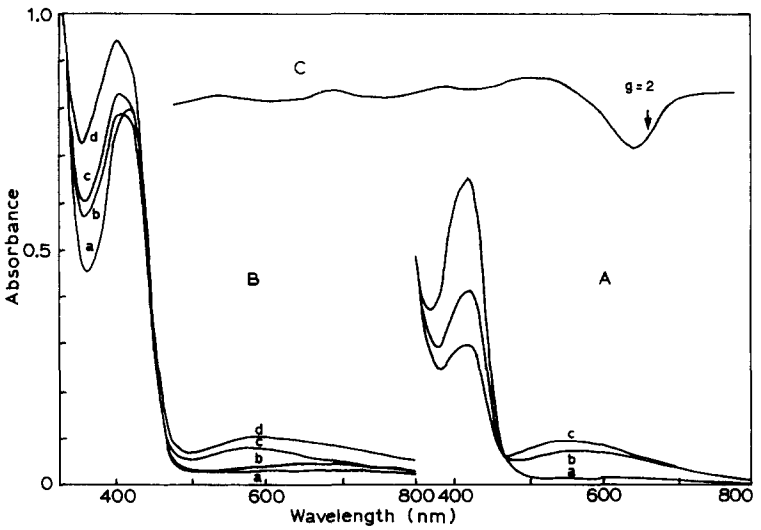

Fig. 2. The reaction of ferrocyanide-reduced superoxide dismutase with cyanide and azide in water solution at 25 °C. (A) absorbance spectra of $4 \cdot 10^{-4}$ M enzyme, treated with a 2-fold excess of ferrocyanide (a) and then reacted with $2 \cdot 10^{-3}$ M (b) and $4 \cdot 10^{-3}$ M (c) NaCN. (B). Absorbance spectra of $5 \cdot 10^{-4}$ M enzyme, treated with a 2-fold excess of ferrocyanide (a) and then reacted with $2 \cdot 10^{-2}$ M NaN_3 (b). Curves c and d refer to the same sample as in b, after 30 min and 4 h, respectively. (C). EPR spectrum at 9.15 GHz of the sample of Curve B, d. Modulation amplitude, 10 G microwave power; 20 mW; temperature, −150 °C.

The rate of reoxidation by ferricyanide in the presence of anions is much higher than that of reduction by ferrocyanide, and is not measurable by standard spectrophotometric techniques.

Reaction with hydrogen peroxide

When stoichiometric amounts of hydrogen peroxide are added to superoxide dismutase in the absence of oxygen, the EPR signal decreases in a linear fashion to reach a minimum which cannot be abolished by excess peroxide. The stoichiometry of this reaction did not change in experiments performed in diluted buffer at pH 7.4 and pH 10.1. The signal decrease is complete within 2 min after each addition. The residual signal appears to be slightly different from the original one and could be attributed to a different copper species, possibly from denatured molecules. When the anaerobic EPR cell is open to air, the signal intensity slowly increases to a value very near to the original. The original signal shape is substantially recovered when nearly stoichiometric amounts of H_2O_2 are used, while in the presence of higher excesses the shape of the recovered signal is slightly modified. Fig. 3 illustrates some of these results.

The copper absorbance around 680 nm is bleached by the addition of increasing amounts of H_2O_2, with a titration curve completely superimposable to corresponding EPR experiments.

Kinetic measurements, carried out by following the optical changes at 680 nm after the addition of a 3-fold excess of reactant with respect to the protein copper showed that the H_2O_2 bleaching occurs at approximately the same rate as the ferrocyanide reaction, and that it is partially reversed (about 60%) by the addition of ferri-

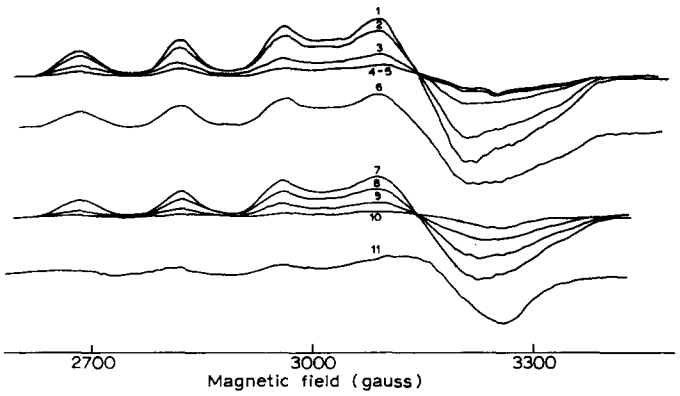

Fig. 3. EPR spectra at 9.15 GHz of the reaction of bovine superoxide dismutase with H_2O_2. To an anaerobic EPR cell with $5 \cdot 10^{-4}$ M enzyme in 0.05 M carbonate buffer, pH 10.1 (1), $2.5 \cdot 10^{-4}$ M (2), $7.5 \cdot 10^{-4}$ M (3), 10^{-3} M (4), $1.5 \cdot 10^{-3}$ M (5) H_2O_2 was added. The cell was then open to the air for 30 min (6). To an anaerobic EPR cell with $5 \cdot 10^{-4}$ M enzyme in 0.03 M phosphate buffer pH 7.4 (7), $5 \cdot 10^{-4}$ M (8), 10^{-3} M (9), and $8 \cdot 10^{-3}$ M (10) H_2O_2 was added. The cell was then open to the air for 1 h (11). Instrument settings as in Fig. 2C.

cyanide at the same rate as in the ferri–ferrocyanide redox reactions. On the other hand only 30% recovery of the original absorbance is obtained after 2 h incubation in the presence of air.

Effect of cyanide on the H_2O_2-treated enzyme

It has already been reported that H_2O_2 has no effect on the cyanide complex of the enzyme[3]. Similarly, cyanide added to the H_2O_2-treated enzyme (Fig. 4) reacts only with that part of the copper which is detectable by EPR. In fact, double integration of the EPR signals recorded before and after cyanide addition (Fig. 4, Curves c and d) gave $4.5 \cdot 10^{-4}$ and $4.8 \cdot 10^{-4}$ M Cu^{2+} respectively.

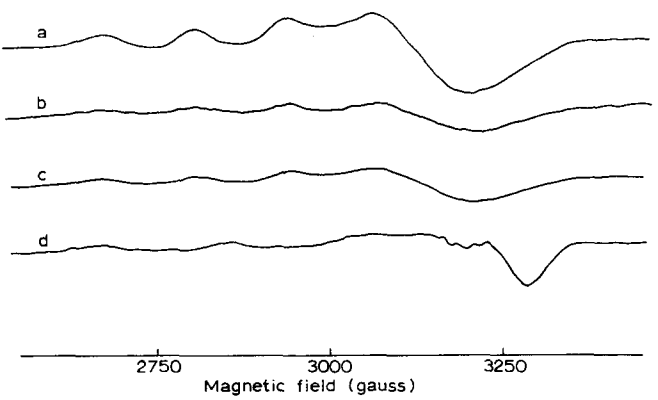

Fig. 4. EPR spectra at 9.15 GHz of bovine superoxide dismutase treated with H_2O_2 and cyanide. To $5 \cdot 10^{-4}$ M enzyme (water solution, Curve a) was added 10^{-3} M H_2O_2 (Curve b). The sample was then exposed to fluxing oxygen for 5 min (Curve c) and treated with $2 \cdot 10^{-3}$ M NaCN (Curve d). The last spectrum does not change on prolonged incubation. Instrument settings as in Fig. 2C.

Reaction with sodium sulfide

On the addition of sodium sulfide in the absence of oxygen, the optical and EPR signals of the protein copper disappear at a rate which is comparable to that of the ferrocyanide reaction. Reoxidation by air or oxygen is as slow as after treatment with H_2O_2.

DISCUSSION

The results presented above show that the copper of superoxide dismutase, which is part of the active site of the enzyme[1-5], reacts with ferrocyanide, hydrogen peroxide and sulfide. All these reactions produce a decrease in the intensity of the copper EPR signal and a bleaching of the copper optical absorption. We want to stress and discuss here some points which may be relevant to the catalytic mechanism.

(a) These are the first data reported in detail on the redox activity of the superoxide dismutase copper. Ferrocyanide reduces the copper, as indicated by the formation of ferricyanide. The effects of sulfide can only be interpreted in terms of reduction. It is our belief that the hydrogen peroxide reaction is also a true reduction. However this statement deserves some comment, since it ensues from considerations which are not as straightforward as in the other two cases. In fact, the enzyme contains two copper atoms per molecule. On the basis of the spectral properties they are considered independent and equivalent. However, it cannot be *a priori* ruled out that they might be sufficiently close to each other so as to permit the formation of a peroxo-bridge between them. In this case, one molecule of hydrogen peroxide could react with both copper atoms and bring about a superexchange coupling[7] through the O–O bridge. This possibility could be explored by magnetic susceptibility techniques and if it exists it would permit speculation on the distance between the two copper sites in the protein. Nevertheless, some facts seem to conflict with this hypothesis. The bleaching of optical absorption which occurs on the addition of hydrogen peroxide points to a reduction of copper rather than to a coupling, as the optical spectrum of copper complexes is not bleached by coupling[7]. Reversal of bleaching by ferricyanide and oxygen also favors a redox reaction.

(b) All the reducing agents tested reacted in the ratio of one molecule of reductant per atom of copper. Such a stoichiometry fits in with one-electron oxidation reduction in the case of ferri-ferrocyanide couple, but is rather intriguing for H_2O_2. If the peroxide gives one electron per copper atom, the product is superoxide anion (O^-_2). However, a peroxide–superoxide couple has too high an E'_0 (+0.98 V; see ref. 8) to be reasonably involved in copper reduction as it is, for instance, in the case of periodate or permanganate. In the case of two-electron donation, the product will be O_2; this redox couple has $E'_0 = +0.27$ V (see ref. 8), near to the ferri–ferrocyanide value of +0.36 V (see ref. 9), as should be expected from the similar behavior of the two redox systems. On the other hand, it is evident that two-electron donation by hydrogen peroxide per copper atom implies the presence of another one-electron acceptor of sufficiently high redox potential in the protein. The nature of such an acceptor challenges future investigation.

(c) The assumed redox potentials of the systems involved in ferrocyanide and hydrogen peroxide reactions, and the low value of the calculated equilibrium constant in the case of ferrocyanide, gives a preliminary E'_0 for the superoxide dismutase

copper near +0.40 V. Such a value is well suited for effective involvement in a dismutation reaction requiring alternate reductions and reoxidations, being intermediate between the O^-_2/O_2 couple ($E'_0 = -0.45$ V; see ref. 8) and the O^-_2/H_2O_2 couple ($E'_0 = +0.98$ V). However, it should be pointed out that E'_0 for ferri–ferrocyanide could change on binding to the protein, so that the E'_0 for the enzyme becomes a very approximate value.

(d) Reoxidation by oxygen of the samples previously reduced by peroxide or sulfide is very slow, but it occurs with a half time of a few seconds in the presence of ferricyanide. This confirms the supposition that the reduced enzyme is kinetically stable toward oxygen, as already suggested by pulse radiolysis experiments[4]. Oxygen seems to escape from the equilibrium with H_2O_2, otherwise it would reoxidize copper, no matter how slowly, in the presence of cyanide, as ferricyanide does when the ferrocyanide-reduced protein is reacted with cyanide (see Fig. 4). This could mean that the protein does not bind oxygen with an appreciable affinity, whereas it binds ferricyanide.

Fast reoxidation of the ferrocyanide-reduced protein by the addition of anions is another suggestive result, as an "alternate reduction–reoxidation mechanism" of the superoxide dismutase activity involves, as the second step, binding of an anion (O^-_2) to the reduced copper, besides reoxidation by the same molecule. It has been shown[3] that anions bring about a drastic symmetry change of the enzyme copper site; this change may affect the redox properties of the enzyme copper, stabilizing the oxidized state.

The results described in this paper strongly support the idea that anions may significantly affect the mechanism of action of superoxide dismutase.

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REFERENCES

1 McCord, J. M. and Fridovich, I. (1969) *J. Biol. Chem.* 244, 6049–6055
2 Rotilio, G., Calabrese, L., Bossa, F., Barra, D., Finazzi Agrò, A. and Mondovì, B. (1972) *Biochemistry* 11, 2182–2187
3 Rotilio, G., Finazzi Agrò, A., Calabrese, L., Bossa, F., Guerrieri, P. and Mondovì, B. (1971) *Biochemistry* 10, 616–621
4 Rotilio, G., Bray, R. C. and Fielden, E. M. (1972) *Biochim. Biophys. Acta* 268, 605–609
5 Klug, D., Rabani, J. and Fridovich, I. (1972) *J. Biol. Chem.* 247, 4839–4842
6 Rotilio, G., Morpurgo, L., Giovagnoli, C., Calabrese, L. and Mondovì, B. (1972) *Biochemistry* 11, 2187–2192
7 Katoh, M., Jonassen, H. B. and Fanning, J. C. (1964) *Chem. Rev.* 64, 99–128
8 George, P. (1965) in *Oxidases and Related Redox Systems*, (King, T. E., Mason, H. S. and Morrison, M. eds), Vol. 1, pp. 3–36, Wiley, New York
9 Mahler, H. R. and Cordes, E. H. (1966) *Biological Chemistry* p. 207, Harper Intern. edn, London and Tokyo