Oxidation of reduced Cu,Zn superoxide dismutase by molecular oxygen

A kinetic study

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The rate of oxidation of reduced bovine Cu,Zn superoxide dismutase [ECu(I)] by molecular O₂ was studied by magnetic-resonance techniques and was found to be low under physiological conditions. The analysis of the kinetic data and of the experiments carried out in the presence of tetranitromethane confirms that O₂^{•−} is a product of the oxidation process. At [ECu(I)]/[ECu(I)] + [ECu(II)] > 0.5 the O₂^{•−} produced reacts mainly with ECu(I), increasing the oxidation rate of the enzyme, whereas at [ECu(I)]/[ECu(I)] + [ECu(II)] < 0.5 it reacts mainly with ECu(II), decreasing the oxidation rate, the kinetics, at constant O₂ concentration, being an apparent second-order process. The oxidation rate increased linearly with both O₂ and OH[−] concentration, indicating that only a deprotonated form of the ECu(I) reacts with O₂^{•−}.

INTRODUCTION

Cu,Zn superoxide dismutases are enzymes made of two identical subunits each containing a catalytically active copper ion, which disproportionates O₂^{•−} according to the redox cycle:

where $k_1 = k_2 \sim 2 \times 10^9 \text{ M} \cdot \text{s}^{-1}$ (Klug-Roth *et al.*, 1973; Fielden *et al.*, 1974).

In spite of the great number of studies carried out on the structure and mechanism of this enzyme, the kinetic behaviour of its reduced form, ECu(I), is poorly described. In particular, scattered observations (Mann & Keilin, 1939; Fee & DiCorleto, 1973) indicate that the rate of reoxidation by molecular O₂ is quite low, but no quantitative study under different conditions has been reported.

Experiments carried out in the presence of H₂O₂, as ECu(II)-reducing agent, and tetranitromethane, as O₂^{•−} scavenger, indicated that superoxide dismutase may catalyse, at high pH, the reverse reaction (Hodgson & Fridovich, 1975):

Both oxidation of H₂O₂ by ECu(II) and reduction of O₂ by ECu(I) were suggested as the source of O₂^{•−}. The aim of the present paper is to study the kinetics of the oxidation by molecular O₂ of Cu,Zn superoxide dismutase reduced by NaBH₄, which, in contrast with H₂O₂, was shown to be a non-denaturing reductant of the enzyme (Viglino *et al.*, 1985).

EXPERIMENTAL

Analytical-grade reagents were used. Cu,Zn superoxide dismutase was prepared from bovine erythrocytes (McCord & Fridovich, 1969). High-purity mixtures of O₂ and N₂ were from S.I.O. (Milan, Italy). ¹H- and ¹⁹F-n.m.r. relaxation measurements were made with a pulse n.m.r. spectrometer operating at 16 MHz as previously reported (Viglino *et al.*, 1979). The T₁ values were measured by using a π/2–τ–π/2 sequence. Room-temperature X-band e.p.r. spectra were obtained with a Bruker 200 D spectrometer. Superoxide dismutase activity was assayed by the polarographic method of catalytic currents (Rigo *et al.*, 1975). Reduction of Cu,Zn superoxide dismutase by NaBH₄ and reduction of the enzyme to the steady state, ECu(I)/ECu(II) ~ 1, were performed as previously described (Viglino *et al.*, 1981, 1985). The oxidation of reduced superoxide dismutase by molecular O₂ was studied in the pH range 4.8–8.6, usually at ionic strength 0.1. The pH values were obtained by suitable additions of concentrated solutions of sodium acetate or K₂HPO₄ or Tris. The change of oxidation state of the enzyme from ECu(I) to ECu(II) was followed by monitoring the re-appearance of the 680 nm optical absorption band or the e.p.r. spectrum or the enhancement of the water protons or ¹⁹F[−] longitudinal relaxation rate (T₁^{−1}) (Rigo *et al.*, 1979); the magnitude of all these parameters is in fact proportional to the ECu(II) concentration. The reoxidation experiments were always carried out in the presence of 0.1 μM-catalase to eliminate H₂O₂, a possible reaction product, which may interfere by both reducing and denaturing the enzyme (Bray *et al.*, 1974; Hodgson & Fridovich, 1975). The oxidation kinetics of the reduced enzyme were more conveniently followed by measuring the T₁ of the solution, and no difference was found in the time course

Abbreviations used: ECu(II), oxidized form of the active site of Cu,Zn superoxide dismutase; ECu(I), reduced form of the active site of Cu,Zn superoxide dismutase.

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when less-sensitive methods such as e.p.r. or visible absorption were used. The frequency of the T_1 measurements was about $\frac{1}{3}T_1$, which, under our experimental conditions, corresponds to at least five measurements each minute. Under these conditions the precision of a single T_1 measurement was about 3%. The average of three T_1 measurements was taken when the ratio $\Delta T_1/\Delta t$ was lower than 10^{-3} . To simplify the analysis of the kinetic data all the measurements were carried out at constant O_2 concentration. During the n.m.r. experiments, in order to achieve a fast equilibrium of the O_2 between the gaseous and the aqueous phase the n.m.r. tube, filled with the appropriate O_2/N_2 mixture, was inclined at 45° and was rotated slowly. In the spectrophotometric and e.p.r. experiments, the O_2 concentration was kept constant by intermittent bubbling of the appropriate O_2/N_2 mixture. Under these conditions the O_2 concentration was found to be constant by a polarographic method. At the end of the oxidation process both the relaxation rate and the activity of the enzyme corresponded, within 2–3%, to the values typical of the native Cu,Zn superoxide dismutase. All measurements were carried out at $22 \pm 0.2^\circ C$.

RESULTS

Plots A and B in Fig. 1 are the time courses of the ECu(II) formation when solutions of ECu(I), equilibrated with air, were adjusted to pH 5.50 and 7.35 respectively. At constant O_2 concentration a dependence of ECu(II) concentration on time was found (see the straight lines C and D in Fig. 1), described by the equation:

$$1/(1 - [ECu(II)]/C) = 1 + 2k't \quad (3)$$

where $2k'$ is an apparent kinetic rate constant and C is equal to $[ECu(II)] + [ECu(I)]$. Eqn. (3) fits all the enzyme oxidation kinetic studies that we carried out in the pH range 4.8–8.6 and at O_2 concentrations from 0.26 to 1.2 mM, the regression coefficient being always higher than 0.99. No kinetic study could be carried out at pH > 8.6 because the oxidation rate of the enzyme became too great to be followed by n.m.r. The apparent kinetic rate constant k' was found to be independent of the concentration of the reduced superoxide dismutase. In fact, oxidation experiments carried out at different enzyme concentrations showed that variations of ECu(I) concentration by a factor of 50-fold had no effect on the k' values. Although it was reported that superoxide dismutase activity is partially inhibited by phosphate ion (Mota de Freitas & Valentine, 1984), no change in the k' values was observed when phosphate was substituted for acetate at pH 6.3 and when Tris was substituted for phosphate at pH 7.9.

The dependence of the oxidation rate on O_2 concentration was studied in the O_2 concentration range 0.26–1.2 mM at pH 6.1 and 7.4. The k' values were always found to increase linearly with the O_2 concentration, the correlation coefficient being higher than 0.99 (see also Fig. 1 inset). In accordance with these findings eqn. (3) can be rewritten in the form:

$$1/(1 - [ECu(II)]/C) = 1 + 2k[O_2]t \quad (4)$$

where the k dimensions are $M^{-1} \cdot s^{-1}$. Since it has been shown (Scarpa *et al.*, 1984) that in erythrocytes, where $O_2^{\cdot -}$ is continuously generated, the redox state of the enzyme is $ECu(I)/ECu(II) = 1$, the behaviour of the

Fig. 1. Time course of the oxidation of reduced Cu,Zn superoxide dismutase by molecular O_2

Enzyme (0.2 mM) was reduced by $NaBH_4$ (see the text) and its oxidation was followed by measuring the n.m.r. relaxation rate of the water protons. The oxidation experiments were carried out in acetate buffer, pH 5.50 (curve A), and in phosphate buffer, pH 7.35 (curve B). The straight lines C and D are the correspondent linearizations according to eqn. (3) (see the text). The time units are hours for plots A and D and minutes for plots B and C. Inset: plot of k' versus O_2 concentration.

Fig. 2. Dependence on the pH of the kinetic rate constant of the autoxidation of reduced Cu,Zn superoxide dismutase

The measurements were carried out at $22^\circ C$ as described in the text. The ionic strength was 0.1. The dimensions of k are $M^{-1} \cdot s^{-1}$.

Fig. 3. Influence of tetranitromethane on the oxidation of reduced Cu,Zn superoxide dismutase

The plot A (left-hand scale) was obtained according to eqn. (12) in the presence of 4 mM-tetranitromethane, whereas plot B (right-hand scale) was obtained according to eqn. (10) in the absence of tetranitromethane. Other conditions were as follows: 0.2 mM-Cu,Zn superoxide dismutase, 0.26 mM-O₂, ionic strength 0.1, pH 8.4. Inset: plot of k'' versus O₂ concentration.

enzyme half-reduced by O₂^{•-} (Viglino *et al.*, 1981) was tested. The kinetics of reoxidation by O₂ were found to be described by eqn. (4) in this case also, and the relative k values were, within the experimental error, similar to those measured with the enzyme fully reduced by NaBH₄. This result rules out possible interactions between subunits during the oxidation process.

From measurements carried out at different pH values, $\log k$ was found to be a linear function of pH in the pH range 6.2–8.6 (Fig. 2). Since the slope of this straight line is practically 1, there is direct relationship between the k values and the OH⁻ concentration.

In order to check that O₂^{•-} was generated in the autoxidation of the NaBH₄-reduced enzyme, as in the case of the system ECu(II)/H₂O₂/O₂ (Hodgson & Fridovich, 1975), we repeated the experiments in the presence of tetranitromethane (TMN). Measurements carried out at [TMN]/[ECu(I)] > 10 showed that the nitroform ion, the one-electron-reduction product of tetranitromethane characterized by $\epsilon_{366} = 1.02 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Rabani *et al.*, 1965), was formed when ECu(I) was added to tetranitromethane solutions containing molecular O₂. Taking into account the results of blank experiments, which showed a small

increase of the absorbance at 350 nm, probably due to trace impurities, it resulted that one nitroform ion is produced for each ECu(I) oxidized. It should be observed that under our experimental conditions H₂O₂ did not react with tetranitromethane.

Experiments carried out at pH 8.4 in the presence of 0.26 mM-O₂ and at [TMN]/[ECu(I)] > 10 showed that both the oxidation of ECu(I) and the reduction of tetranitromethane occurred as a first-order process with respect to ECu(I), with a kinetic rate constant, k'' , of $1.53 \times 10^{-3} \text{ s}^{-1}$ (see Fig. 3, curve A). The plot of k'' versus the O₂ concentration was found to be linear (see Fig. 3 inset).

DISCUSSION

It was demonstrated (Stevanato *et al.*, 1984) that the oxidation of low- M_r complexes of Cu(I) by molecular O₂ usually occurs with high rates, through the formation of a Cu(I)-O₂ adduct that reacts with another Cu(I) with the direct formation of H₂O₂. Since the Cu,Zn superoxide dismutase contains two copper ions, the reoxidation of the reduced enzyme, according to the above mechanism, should occur by either an intra- or an inter-molecular transfer of two electrons to the O₂ molecule. However, since the intramolecular distance of the two copper ions of superoxide dismutase is approx. 3 nm (30 Å) (Richardson *et al.*, 1975), the direct reduction of O₂ to H₂O₂ is very unlikely. The analysis of the experimental data presented above is in agreement with these considerations. In fact, the independence of the k value of the concentration of the enzyme and the lack of a first-order dependence with respect to the ECu(I) seem to exclude both inter- and intra-molecular bielectronic transfer from the reduced enzyme to O₂. On the other hand, according to the results of experiments with tetranitromethane, which demonstrate the production of O₂^{•-} in the oxidation of superoxide dismutase, we can write:

Since in our experiments the concentration of superoxide dismutase was always higher than 5 μM , and the O₂^{•-} generated in reaction (5) will react with the superoxide dismutase even at pH 4.8, where the spontaneous dismutation of O₂^{•-} occurs at the highest rate (Czapski & Dorfman, 1964). Furthermore, the kinetic rate constants of the O₂^{•-} reactions with ECu(I) and ECu(II) being the same (Klug-Roth *et al.*, 1973; Fielden *et al.*, 1974), it appears that the O₂^{•-} generated by the ECu(I) autoxidation will react, at [ECu(I)]/C > 0.5, mainly with ECu(I), with the formation of H₂O₂ [see eqn. (2)], thus increasing the oxidation rate of the enzyme. On the other hand, at [ECu(I)]/C < 0.5 the O₂^{•-} will mainly react with ECu(II) according to reaction (1), decreasing the oxidation rate of the enzyme. This behaviour is in agreement with the time course reported in Fig. 1, and we can write:

$$\frac{d[\text{O}_2^{\bullet -}]}{dt} = k_5[\text{O}_2][\text{ECu(I)}] - k_2[\text{ECu(I)}][\text{O}_2^{\bullet -}] - k_1[\text{ECu(II)}][\text{O}_2^{\bullet -}] \quad (6)$$

$$\frac{d[\text{ECu(II)}]}{dt} = k_5[\text{O}_2][\text{ECu(I)}] + k_2[\text{ECu(I)}][\text{O}_2^{\bullet -}] + k_1[\text{ECu(II)}][\text{O}_2^{\bullet -}] \quad (7)$$

Taking into account that $k_1 = k_2$ and introducing into eqn. (6) the steady-state approximation for $[O_2^{\cdot-}]$, this gives:

$$[O_2^{\cdot-}]_{ss} = k_5[O_2][ECu(I)]/k_1C \quad (8)$$

Substitution of eqn. (8) into eqn. (7) gives:

$$\frac{d[ECu(II)]}{dt} = 2k_5[O_2](C - [ECu(II)])^2/C \quad (9)$$

From the integration of eqn. (9) we obtain:

$$1/(1 - [ECu(II)]/C) = 1 + 2k_5[O_2]t \quad (10)$$

which is the same as eqn. (4), k_5 being equal to k .

Under our experimental conditions and in the presence of tetranitromethane, the $O_2^{\cdot-}$ generated in reaction (5) should react almost quantitatively with tetranitromethane, and eqn. (7) can be rewritten as:

$$\frac{d[ECu(II)]}{dt} = -\frac{d[ECu(I)]}{dt} = k_5[O_2][ECu(I)] \quad (11)$$

which, at constant $[O_2]$, is a pseudo-first-order process with respect to $ECu(I)$.

Integration of the above equation gives:

$$\ln\left(\frac{C}{[ECu(II)]}\right) = k_5[O_2]t \quad (12)$$

Since in the presence of tetranitromethane and at constant $[O_2]$ the oxidation of $ECu(I)$ was first-order with respect to $ECu(I)$ itself (see curve A in Fig. 3), according eqn. (12), the experimentally measured k'' value must be identified with the product $k_5/[O_2]$, from which a k_5 value of 5.9 can be calculated. This value agrees quite well with the k_5 value of 5.4 calculated according to eqn. (10), from curve B in Fig. 3, obtained under the same experimental conditions but in the absence of tetranitromethane.

The direct relationship between the k_5 values and the OH^- concentration (Fig. 2) seems to indicate that a deprotonated form of the enzyme reacts with O_2 much faster than does the protonated one, its pK_a being greater than 8.6. This is in accordance with previous findings (Fee & DiCorleto, 1973) on the pH-dependence of the oxidation-reduction potentials of Cu,Zn superoxide dismutase. These results could be explained by the equilibrium:

the pK of the reduced form being greater than 9. The deviation from linearity of the plot of $\log k_5$ versus pH observed below pH 6 (Fig. 2) may be correlated with previous observations indicating a modification of the enzyme behaviour at these pH values, because of the altered binding of Cu(II) to the enzyme (Rigo *et al.*, 1978).

Finally, it appears that, under physiological conditions, the reoxidation of the reduced enzyme by molecular O_2 is slow. In fact, it can be calculated from eqn. (10) that, at $[O_2] = 0.26$ mM and in the absence of $O_2^{\cdot-}$ generation by reactions other than the superoxide dismutase oxidation, the decrease of the $ECu(I)$ fraction from 0.50 to 0.25 or from 0.50 to 0.05 requires more than about 2 h.

However, the $ECu(I)$ oxidation by O_2 may explain a ratio $[ECu(II)]/[ECu(I) + [ECu(II)]]$ slightly higher than the expected value of 0.50 that we observed when turnover experiments were performed with $O_2^{\cdot-}$ fluxes of the order of $10^{-8} \text{ M} \cdot \text{s}^{-1}$ and Cu,Zn superoxide dismutase concentrations in the micromolar range. The low rate of reaction of the reduced enzyme with O_2 is likely to be related to the electrostatic facilitation of the binding of anionic ligands to the copper of superoxide dismutase by properly located positively charged amino acid chains (Getroff *et al.*, 1983). The kinetic unreactivity of the enzyme towards O_2 and neutral H_2O_2 is an essential part of its absolute specificity as a superoxide dismutase under physiological conditions.

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