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A pulse radiolysis study of superoxide dismutase

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SUMMARY

The superoxide dismutase activity of bovine erythrocuprein has been studied by pulse radiolysis under steady-state and pre-steady-state conditions. Enzyme activity is independent of pH in the range 5.3 to 9.5. Cyanide inhibits the enzyme, indicating involvement of copper in the catalytic process. Enzyme turnover is governed by a second-order rate constant for reaction between enzyme and superoxide of $1.8 \cdot 10^9$ $M^{-1} \cdot s^{-1}$. If a Michaelis complex is formed, K_m must be greater than 0.5 mM. Increasing viscosity of the medium by addition of glycerol slowed the reaction, confirming that enzyme turnover is effectively diffusion-limited.

Bovine erythrocuprein has been extensively investigated since its superoxide dismutase activity was discovered[1–8]. However, results mostly concern structural, rather than kinetic or mechanistic aspects of the enzyme, since routine activity measurements rely on reduction of cytochrome c[1,9] or nitro blue tetrazolium[10] by superoxide produced by the xanthine oxidase/xanthine/oxygen system. With such methods, quantitative evaluation of any catalytic constant, as well as pH or inhibitor effects, is clearly too complicated. Furthermore, the steady-state concentration of superoxide radical (O_2^-) is too low to attempt a titration of enzyme with its substrate or pre-steady-state studies. Ballou *et al.*[8] tried chemical production of O_2^- in conjunction with rapid freezing and electron paramagnetic resonance spectroscopy (EPR) but found the time resolution of the method inadequate for detailed kinetic studies on superoxide dismutase. However, pulse radiolysis is an efficient tool for obtaining O_2^- (refs 11–13) in the relatively high concentrations

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which are required for such work. The method has a time resolution of about 1 μs, and we have now applied it to studies on the kinetics and mechanism of the enzyme.

Superoxide dismutase was purified from ox blood according to McCord and Fridovich[1]. O_2^- radicals were produced by irradiation of oxygen-saturated solutions by 4-MeV electrons in the presence of 0.085 M ethanol as scavenger for OH radicals (*cf.* ref. 11). The reaction between superoxide dismutase and its substrate, at about 25 °C, was followed by observing both the decay of O_2^- at 250 nm and absorbance changes at 650 nm, where the copper of superoxide dismutase has maximum absorption. EPR spectra of superoxide dismutase, after reacting with O_2^-, were obtained using a technique which combines pulse radiolysis and rapid freezing[13]. The yield of O_2^- (in the range 2–50 μM) was controlled by varying the intensity or the duration of the pulse. Concentrations of O_2^- were calculated from its extinction coefficient[12,14,15]; these values agreed satisfactorily with concentrations calculated from the radiation dose. 2 mM phosphate or pyrophosphate buffer solutions were used, the pH being measured before and after irradiation.

Fig. 1 shows oscilloscope traces of the reaction between superoxide dismutase and O_2^-. It is evident that the presence of catalytic amounts of enzyme greatly affects the rate of O_2^- decay and that denaturation of the protein by heat treatment abolishes this effect. Initial rates of O_2^- decay were proportional to enzyme concentration. Variations in ethanol concentration were without effect on the rate. Enzyme degradation by radiation, even at the lowest enzyme concentrations was not a problem, since activity was lost only very slowly when pulsing was repeated, *e.g.* 30% activity loss after 10 pulses, $\Sigma O_2^- \simeq$ 300 μM. Even this small loss was probably due mainly to ultraviolet radiation from the optical system, since the presence of albumin (70 μg/ml) diminished it still further.

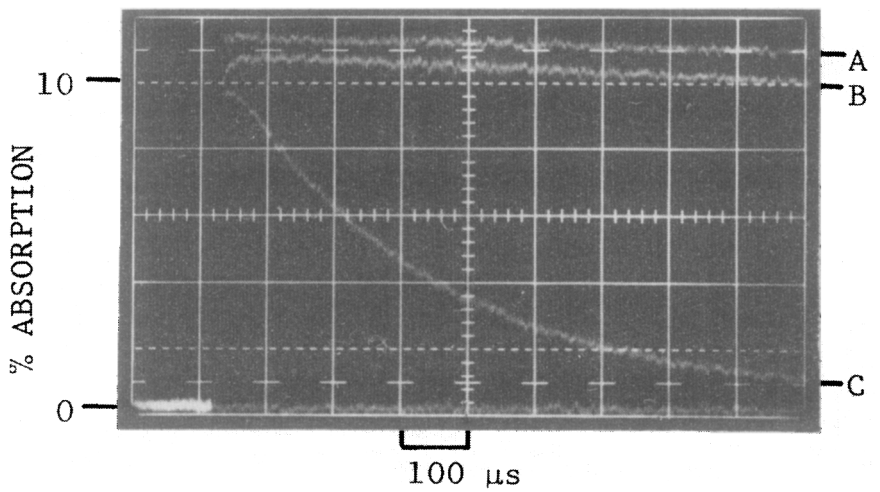


Fig. 1. Oscilliscope traces showing decay at pH 8.8 of superoxide (initial concn 32 μM), followed at 250 nm. Trace A: spontaneous reaction; Trace C: in the presence of 2.0 μM superoxide dismutase; Trace B: in presence of enzyme (2.0 μM), boiled for 5 min.

Fig. 2 shows a plot of initial velocities of the enzyme-catalysed reaction *versus* pH.

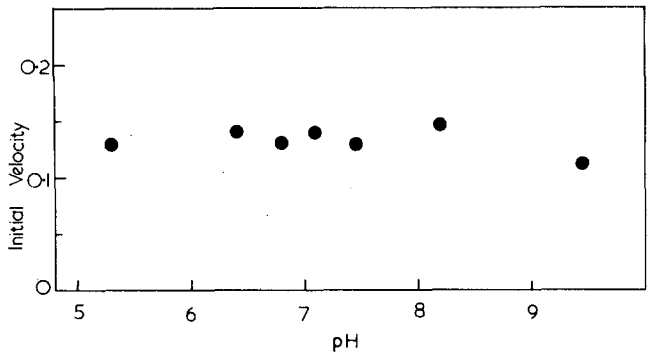
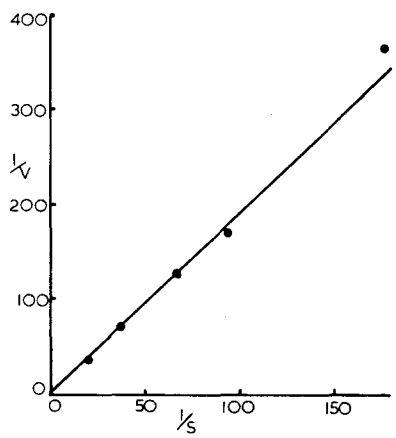


Fig. 2. pH dependence of initial velocity of the reaction between superoxide dismutase (1.6 μM) and O_2^- (50 μM). Initial velocities are expressed as ΔA_{250} nm/ms, and have been corrected by subtracting blanks obtained in the absence of enzyme.

While the rate of spontaneous dismutation decreases rapidly with increasing pH (ref. 12), the superoxide dismutase activity of erythrocuprein is relatively independent of pH in the range between pH 5.3 and pH 9.5. A double-reciprocal plot of initial velocities against substrate concentration is presented in Fig. 3. It is a straight line passing through the origin. Hence, under the experimental conditions, there is no evidence that the enzyme can become saturated with O_2^-. If any Michaelis complex is formed, K_m must be greater than 0.5 mM. The slope of the graph yields a second-order rate constant for reaction between O_2^- and superoxide dismutase of $(1.6 \pm 0.3) \cdot 10^9$ $M^{-1} \cdot s^{-1}$. No limiting turnover number can of course be given but the highest value observed was in fact about $8 \cdot 10^4$ s^{-1} for 50 μM O_2^-. Cyanide was found to be a potent inhibitor of the enzyme (Fig. 4). With



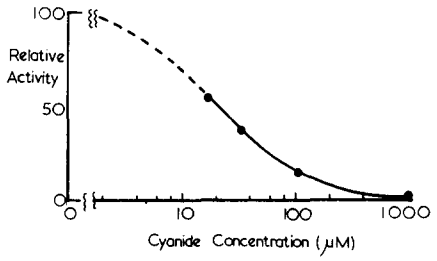


Fig. 3. Double-reciprocal plot of initial velocities of the enzyme-catalysed reaction (v) *versus* O_2^- concentration (S), with 0.16 μM superoxide dismutase, at pH 8.2. Velocities are expressed as $\Delta A^{\frac{1}{2}\,cm}_{250\ nm}$/ms and O_2^- concentrations as millimolarities.

Fig. 4. Inhibition of superoxide dismutase by CN^- at pH 8.8. Activity is expressed as percent of that in a control without CN^-. Enzyme was 2.0 μM and O_2^- 32 μM.

32 μM O_2^- and 2 μM enzyme at pH 8.8, 50% inhibition was obtained with 20 μM CN^-, rising to 99% inhibition at 1 mM CN^-. On the other hand, 1 mM azide had little effect on the enzyme's activity.

Experiments were also carried out with the enzyme (6–50 μM) present in an excess (up to 7-fold on a molar basis) over its substrate. Under these conditions the second-order rate constant, measured at 250 nm, was $(1.9 \pm 0.3) \cdot 10^9$ $M^{-1} \cdot s^{-1}$. Measurements at 650 nm indicated a small decrease in absorbance occurring at about the same rate but corresponding to only about 40% of the maximum possible absorbance change (on the basis of one O_2^- reducing one Cu^{2+}). However, recovery of 650 nm absorbance was not observed when all the substrate was consumed, and further investigations on the nature of this spectral change are in progress.

EPR spectra were recorded after mixing 50 μM superoxide dismutase with 200 μM O_2^-, followed by rapid freezing 6 ms later. In this time all the substrate was consumed (as shown by disappearance of the O_2^- EPR signal[13]). Nevertheless a significant decrease (about 40%) in amplitude of the Cu^{2+} signal was found. This is possibly due to effects of the reaction product, H_2O_2 (ref. 16) on the enzyme (G. Rotilio *et al.*, manuscript in preparation). Additional rapid freezing experiments with approximately equimolar superoxide dismutase and O_2^- provided no evidence for further decrease in the Cu^{2+} signals, such as might perhaps have been anticipated if the enzyme mechanism involved alternate reduction and re-oxidation of copper by successive O_2^- molecules.

The above results emphasise the usefulness of the pulse radiolysis method in this field, revealing important features of enzymic superoxide dismutation. Whereas spontaneous dismutation is strongly dependent on pH, the enzyme-catalysed reaction is independent of this variable. A similar pH independence has been found for catalase[17], which like superoxide dismutase has a high turnover number. These features would appear to make involvement of acid–base catalysis in the mechanism improbable[18]. On the other hand, inhibition by cyanide is clearly related to the formation of a copper–cyanide complex in the enzyme[8,19] and confirms direct involvement of the metal in catalysis[1,16]. However, no evidence was obtained either by pulse radiolysis or by EPR for substantial copper reduction as part of the turnover process. Further work on the mechanism is therefore required.

The exceptionally high rate of the catalytic reaction of superoxide dismutase has now been given a definite value, expressed as a second-order rate constant for reaction between enzyme and substrate of $(1.9 \pm 0.3) \cdot 10^9$ $M^{-1} \cdot s^{-1}$. In agreement with the initial second-order step being rate-limiting in enzyme turnover, the same value was obtained both under catalytic conditions and with enzyme in excess. Though our rate constant is much lower than the one suggested by McCord and Fridovich[1] of $5 \cdot 10^{11}$ $M^{-1} \cdot s^{-1}$, it is nevertheless near to the upper limit expected for a diffusion-controlled enzyme–substrate reaction[18]. In agreement with diffusion control of the overall catalytic process, further studies showed that when the viscosity of the medium was increased by addition of glycerol, then enzyme turnover was slowed. Activities were 70% and 37%, respectively, of that of a control, for solutions with viscosities (relative to buffer) of 2.1 and 5.3. We

conclude that turnover of superoxide dismutase is effectively diffusion-limited and that dismutation of O_2^- in its presence is the fastest known enzyme-catalysed reaction.

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