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The Mechanism of Action of Superoxide Dismutase from Pulse Radiolysis and Electron Paramagnetic Resonance

EVIDENCE THAT ONLY HALF THE ACTIVE SITES FUNCTION IN CATALYSIS

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1. Detailed studies on the mechanism of the enzymic reaction of bovine superoxide dismutase were carried out by using pulse radiolysis and electron paramagnetic resonance (e.p.r.). 2. The second-order rate constant for reaction between superoxide dismutase and the superoxide ion was redetermined as $(2.37 \pm 0.18) \times 10^9 M^{-1} \cdot s^{-1}$ at 25°C. This reaction governs the turnover, and any first-order steps must have rate constants higher than about $10^6 s^{-1}$. Turnover has a low activation energy and is slowed substantially when the viscosity is increased with glycerol, confirming that the reaction rate is near the limit for diffusion control. In water a reversible conformation change to a less active form appears to take place above about 40°C. 3. Pre-steady-state rates of reduction and reoxidation of copper in the enzyme are consistent with these processes being rate-limiting in enzyme turnover. 4. Examination, with the help of computer simulation, of the e.p.r. spectra at 9 and 35 GHz of native superoxide dismutase indicated that, apart from 10–20% of impurities, only one species of Cu^{2+} is distinguishable. Further, the specific activity of our enzyme preparations, measured by pulse radiolysis, is at least as high as that obtained by other workers. 5. Nevertheless, measurement of the proportion of copper present as Cu^{2+} (determined both optically and by e.p.r. spectroscopy) in the steady states approached from both the oxidized and the reduced forms of the enzyme, indicates (after allowing for the impurities) that only half of the copper atoms participate in turnover. E.p.r. spectroscopy provided no evidence for differences between functioning and non-functioning Cu^{2+} atoms. 6. It is suggested that the results may be best interpreted in terms of an allosteric type of mechanism, with two initially indistinguishable copper atoms in the enzyme. Reaction of one of these with a superoxide ion then renders the other, at least transiently, unreactive.

Superoxide dismutase has been intensively studied since the discovery of this enzymic activity by McCord & Fridovich (1969). The enzyme molecule has two probably equivalent subunits (Keele *et al.*, 1971) and contains two atoms each of copper and zinc [see Fee (1973) for recent work on the metal content of the enzyme]. The turnover rate is exceptionally high and hence pulse radiolysis has proved the most satisfactory method of generating the substrate for kinetic and mechanistic studies on the enzyme (Rotilio *et al.*, 1972*a*; Klug *et al.*, 1972; Fielden *et al.*, 1973; Klug-Roth *et al.*, 1973).

We now present an extension of earlier pulse-radiolysis work on the enzyme by Rotilio *et al.* (1972*a*). It was necessary to take stringent precautions throughout the work to avoid inactivation of the enzyme by H_2O_2, one of the products of the catalytic reaction, a process described in the preceding paper (Bray *et al.*, 1974). As will be discussed in detail below, a failure to take such precautions appears to have led Klug-Roth *et al.* (1973) into serious errors, though one of the basic conclusions from their work is the same as one of ours. This conclusion (noted in a preliminary publication by Fielden *et al.*, 1973) is that the enzyme functions by having its copper alternately reduced by one superoxide ion, then reoxidized by the next.

Materials and Methods

General

All superoxide dismutase samples, buffers and reagents were prepared and standardized as described by Bray *et al.* (1974). E.p.r. measurements were made as described in the same paper.

Pulse-radiolysis experiments

Irradiation of dilute aqueous solutions above neutrality by high-energy electrons yields approximately equal amounts of the hydroxyl radical ($OH^{\bullet}$) and the hydrated electron ($e^-_{aq.}$) as primary species. Minor products include H_2O_2, which is produced in about one-fifth the yield of $OH^{\bullet}$ and $e^-_{aq.}$. Dissolved O_2 reacts with $e^-_{aq.}$ to produce O_2^- and, in experiments where O_2^- was required, care was taken to avoid excessive amounts of other compounds that are reactive towards $e^-_{aq.}$ (e.g. superoxide dismutase). In experiments with O_2^-, either 85mM-ethanol or 100mM-sodium formate was used as an hydroxyl scavenger. Thus $OH^{\bullet}$, a potential inactivating species for the enzyme and a source of undesirable secondary radicals, was removed and simultaneously converted into extra O_2^- (Czapski & Dorfman, 1964). Experiments in which $e^-_{aq.}$ was the desired product were carried out anaerobically with t-butyl alcohol used as hydroxyl scavenger. Radicals produced from the scavenging reaction of t-butyl alcohol, which decay by dimerization, are less reactive than the equivalent ethanol radicals and therefore less prone to side reactions.

In the pulse-radiolysis apparatus at Sutton, 4.3 MeV electron pulses of 1.6 or 0.16μs duration are produced by a linear accelerator. Pulses of 1.6μs were generally used and generated either singly, for conventional pulse radiolysis, or in a burst of predetermined number at repetition rates of 50, 100, 200 or 400 pulses/s. Short bursts of pulses were used to produce the steady state in enzyme turnover. Extra-high concentrations of O_2^- (210μM) were obtained rapidly by using a special double-pulse facility in which two radiation pulses only were produced with a separation of about 5μs. This separation is acceptable provided that it is short compared with O_2^- disappearance.

Samples were contained in high-purity silica cells with a light-path of either 0.7cm, which was used for routine observation of O_2^- kinetics at 250nm ($\varepsilon = 2000 M^{-1} \cdot cm^{-1}$; Rabani & Nielsen, 1969), or of 7.5cm, which were used for the observation of the copper absorption band at 650 or 680nm (ε at 680nm $= 300 M^{-1} \cdot cm^{-1}$, see Bray *et al.*, 1974) and for the kinetics of low O_2^- concentrations reacting with excess of enzyme. The cells formed part of a single-beam spectrophotometer system capable of recording photographically the time-dependence of absorbance changes (>0.001) in the region 200–800nm, between 1μs and 1min after irradiation. A double monochromator was used to decrease scattered light to negligible proportions at the wavelengths used. In work at 650 or 680nm, a heat filter and a filter absorbing below 350nm were incorporated to protect the enzyme against heat or u.v. inactivation. Similarly, in work at 250nm, a mechanical shutter was opened only about 5s before irradiation to minimize exposure to u.v.

Except in the experiments involving rapid freezing (see below), O_2^- or $e^-_{aq.}$ were generated directly in solutions containing the enzyme. Simple assays of superoxide dismutase activity were carried out as described by Bray *et al.* (1974).

Many buffers were unsuitable, because of reactions with $e^-_{aq.}$ or $OH^{\bullet}$ which decrease the yield of O_2^- and lead to radicals which may cause undesirable absorption changes or undergo side reactions with the enzyme. The buffers and reagents used in this work were checked for possible inhibition of the enzymic activity. Most work was carried out in the standard assay medium (Bray *et al.*, 1974), i.e. 2mM-sodium pyrophosphate, pH9.0–9.5, containing 85mM-ethanol. Replacement of ethanol by 100mM-sodium formate in this buffer had no effect on activity, although some inhibition was found at higher formate concentrations (about 50% for 1M-formate). Moderate concentrations of pyrophosphate also had a definite inhibitory action on the enzyme. It is probable that at least part of the inhibition by high salt concentrations is a result of the effect of ionic strength on the reaction. Inhibition in our usual medium (i.e. 2mM-sodium pyrophosphate) appeared to be only of the order of 5%.

Combination of rapid freezing and pulse radiolysis

The apparatus and techniques were as described by Bray *et al.* (1973). In these experiments, O_2^- was generated separately from the enzyme by repetitive pulsing of a glass coil containing flowing O_2-saturated solutions. Conditions were such that each element of solution received about 10–20 pulses. To maximize O_2^- yields, formate was preferred to ethanol as the $OH^{\bullet}$ scavenger and a high pH (11.0) was used. O_2^-, which has a long life-time at such pH values (Rabani & Nielsen, 1969), was then made to react with the enzyme via the flow system detailed in Fig. 1. The reaction mixture was frozen rapidly by squirting into cold isopentane (Bray *et al.*, 1973) and subsequent examination of the frozen samples by e.p.r. spectroscopy (Bray *et al.*, 1974) was carried out at Brighton.

The e.p.r. experiments summarized in Table 2, for superoxide dismutase sample 1, were carried out with the flow system of Fig. 1, as detailed in the legend to this figure. O_2^- concentrations in such experiments were determined by e.p.r. since its spectrum in samples prepared by rapid freezing is well known (Knowles *et al.*, 1969; Ballou *et al.*, 1969; Nilsson *et al.*, 1969). O_2^- controls were obtained, for the above experiments, by replacing superoxide dismutase in syringe B by buffer. Double integration of the e.p.r. spectrum then gave O_2^- concentrations under the radiation

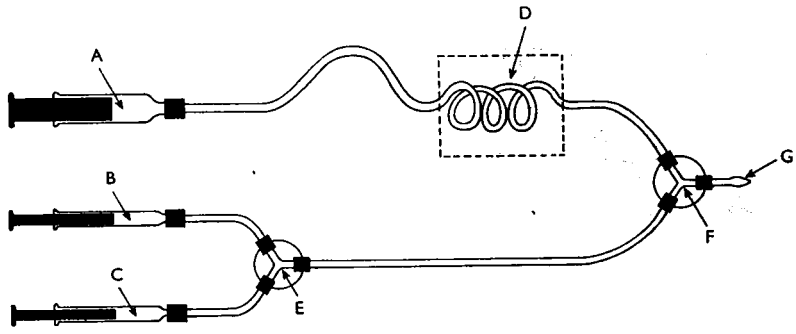


Fig. 1. *Apparatus for generating steady-state mixtures from native or reduced superoxide dismutase for rapid-freezing e.p.r.*

This apparatus (cf. Bray *et al.*, 1973) was used for all the e.p.r. experiments on superoxide dismutase sample 1, which are summarized in Table 2. The syringes (A, B and C), the mixing chambers (E and F), the glass irradiation coil (D), and the jet (G) are interconnected, as shown, by nylon tubing. The syringes are driven mechanically and solution leaving the jet is squirted into cold isopentane to quench the reaction for subsequent e.p.r. measurement. Syringe A contained alkaline sodium formate saturated with O_2, B contained enzyme in carbonate–bicarbonate buffer and C contained either H_2O_2 or water. Control samples of native enzyme were first obtained by using water in syringe C and without irradiation. Similar samples, but with 4 MeV irradiation, corresponded to native enzyme reduced to a steady state with O_2^-. When syringe C contained H_2O_2 and samples were obtained in the absence of irradiation, they corresponded to the H_2O_2-reduced enzyme. With H_2O_2 and irradiation, they corresponded to reduced enzyme, after reoxidation to a steady state with O_2^-. O_2^- concentrations were determined as described in the Materials and Methods section. Concentrations, flow rates and dead spaces were such that reaction conditions (at about 25°C) were as follows. Reaction between native enzyme and O_2^- was for 6ms at pH9.9 with 38μM-enzyme and 120μM-O_2^-; reduction of enzyme with H_2O_2 was for 450ms at pH9.0 with 133μM-enzyme and 23mM-H_2O_2. When enzyme reduced in this manner was further treated with O_2^-, the reaction time was again 6ms at pH9.9 with 38μM-enzyme, but [O_2^-] was now only 100μM and 6mM-H_2O_2 was also present.

conditions of the experiment. In view of the importance of knowing precisely the molar ratios of O_2^- to superoxide dismutase, the enzyme, shot under the same conditions but without irradiation, was used as integration standard, thereby eliminating uncertainties in the isopentane packing correction. Controls without superoxide dismutase, with H_2O_2 in place of the water in syringe C, gave slightly decreased nett yields of O_2^-. This may have been due to trace metal impurities introduced with the H_2O_2.

Computer simulation of e.p.r. spectra

The computer program used for simulating the 9 and 35 GHz spectra shown in Fig. 6 used a procedure similar to that of Venable (1967), with second-order hyperfine interactions and correction of intensities for *g*-factor anisotropy as described by Toy *et al.* (1971). The symmetry of the copper was assumed to be orthorhombic, i.e. the principal axes of *A*, *g* and the line-width 'tensors' were assumed to be parallel. Individual line-shape functions were taken as Gaussian. The program was written in FORTRAN IV and run on a CDC 7600 computer. The required spectra were scaled and drawn by using a Calcomp plotter in conjunction with an ICL 1904A computer.

Results and Discussion

Disappearance of substrate under catalytic conditions

The present experiments have been confined to the pH range 9.0–9.9. In this range, non-enzymic decay of O_2^- is conveniently slow. The superoxide dismutase reaction rate is independent of pH over a wide range (Rotilio *et al.*, 1972*a*; Klug *et al.*, 1972) but begins to decline rapidly at pH values above 10.0 (P. B. Roberts & E. M. Fielden, unpublished work). We first carried out more precise and detailed measurements than those reported by Rotilio *et al.* (1972*a*) on the kinetics of superoxide decay in the presence of catalytic quantities of the enzyme, as followed by changes in E_{250}.

Superoxide dismutase has proved to be an exceptionally stable enzyme in our hands, even in the dilute solution used for activity measurements. Thus no loss of activity was observed in 1–2μM solutions either on heating to 55°C for 5min, or on bubbling the solution with O_2 for some minutes or on storage at −20°C for some weeks. Full stability of the enzyme in the assay procedure itself is illustrated in Fig. 2 (top). On the other hand, the reaction product, H_2O_2, inactivates the enzyme quite rapidly (Bray *et al.*, 1974).

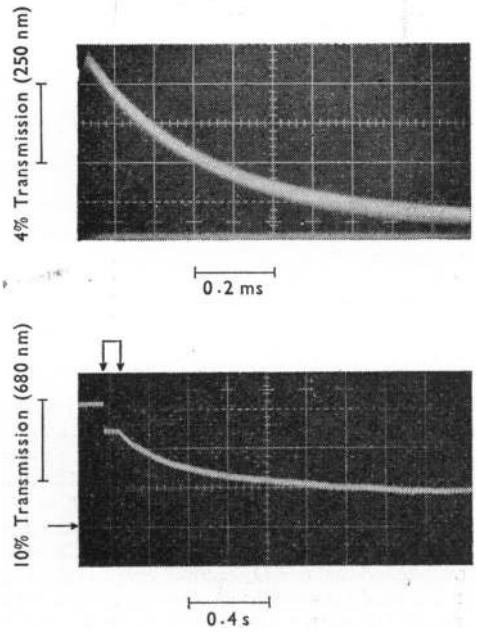


Fig. 2. *Oscilloscope traces from pulse-radiolysis experiments*

The top trace shows repeated assays carried out on an enzyme sample at 10ms intervals. O_2^- was introduced by each of 31 successive pulses of high-energy electrons and was essentially completely decomposed by the enzyme before the next pulse. The 31 decay curves (followed at 250nm) are almost completely superimposed, indicating full stability of the enzyme in the assay procedure. Conditions, in the standard assay medium at about 25°C were: approx. 1.5 μM-enzyme; 31 pulses (1.6 μs duration yielding 26 μM-O_2^- per pulse) at 10ms intervals; light-path 0.7cm. The bottom trace, at 680nm, shows reduction of the enzyme to a steady state by O_2^- followed by slower and incomplete reduction by H_2O_2 after the steady state. Between the vertical arrows, 35 pulses (1.6 μs duration, yielding 34 μM-O_2^- per pulse) were applied at 2.5ms intervals so as to reach and maintain a steady state. The horizontal arrow corresponds to the transmission expected for complete bleaching of the enzyme. The standard assay medium at about 25°C was used, with 32 μM-enzyme and a light-path of 7.5cm.

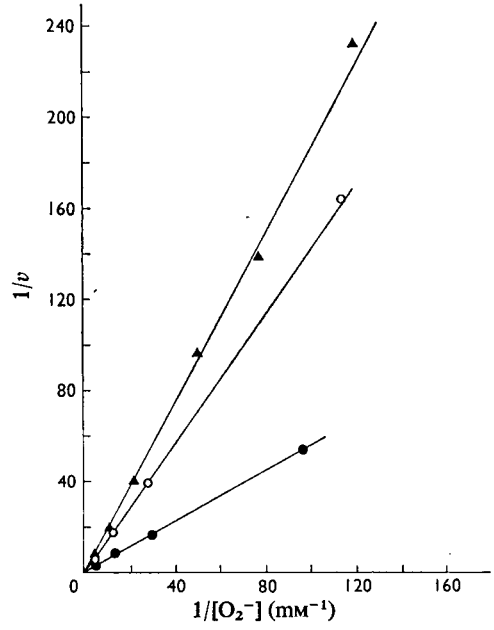


Fig. 3. *Effect of O_2^- concentration on the activity of superoxide dismutase alone and in the presence of cyanide (double-reciprocal plots)*

Activity measurements were made in the standard medium at about 25°C by pulse radiolysis and the observed half-time for O_2^- decay ($1/v$), in arbitary units, is plotted against the reciprocal of the O_2^- concentration (mM). ▲, 0.3 μM-enzyme; ●, 1 μM-enzyme; ○, 1 μM-enzyme in the presence of 20 μM-cyanide.

O_2^- decay curves in the presence of the enzyme were always first-order (cf. Fig. 2, top). The rate of decay was strictly proportional to enzyme concentration, over the range measured (0.03–16 μM). A double-reciprocal plot of activity against substrate concentration is presented in Fig. 3. There are no indications of saturation at the highest O_2^- concentration used, which has now been raised to 210 μM. A similar result was obtained when the enzyme was partially inhibited by addition of cyanide (cf. Rotilio *et al.*, 1972*a*). Thus the overall reaction between enzyme and substrate is governed by second-order kinetics under all turnover conditions used. Our value for the second-order rate constant, determined in turnover experiments, is $(2.37 \pm 0.18) \times 10^9\,M^{-1} \cdot s^{-1}$ (see Table 1). From this and the data of Fig. 3, a lower limit of about $10^6\,s^{-1}$ can be set for any first-order steps in the catalytic cycle.

Effects of temperature and viscosity on the catalytic reaction

Since the second-order rate constant, given above, for enzyme turnover is close to the theoretical limit for a diffusion-controlled reaction between an enzyme and a small substrate molecule (Eigen & Hammes, 1963), the effects of temperature and of increasing the viscosity of the medium on the rate of the reaction were of interest. Fig. 4 shows an Arrhenius-type of plot for the enzyme turnover rate constant, measured as a function of temperature, in water and in aqueous gylcerol, at two different glycerol concentrations. Viscosity data on the solutions are plotted in a similar manner for comparison. A

Table 1. *Second-order rate constants for superoxide dismutase reaction with O_2^- during turnover and in the presence of excess of native or reduced enzyme*

Reaction between enzyme and O_2^- was followed at 250nm. Values given are averages for the number of enzyme samples indicated, with average deviations. Enzyme concentrations were determined from E_{680} (see Bray *et al.*, 1974). All measurements were carried out in the standard medium at about 25°C.

Conditions	Initial state of enzyme	Mol of O_2^-/ mol of enzyme	$10^{-9} \times k$ ($M^{-1} \cdot s^{-1}$)	No. of samples
Turnover	Native	10–30*	2.37 ± 0.18	3
Turnover	Reduced (H_2O_2)§	17*	2.56 ± 0.30	2
No turnover	Native	0.1†	2.45 ± 0.18	3
No turnover	Reduced (H_2O_2)‖	0.1†	2.40 ± 0.07	3
No turnover	Reduced ($e^-_{aq.}$)	0.1‡	2.23	1

* O_2^- concentration 32 μM.
† O_2^- concentration varied from 1 to 5 μM.
‡ O_2^- concentration 1 μM.
§ Enzyme reduced with 120 μM-H_2O_2 for 30–40s.
‖ Enzyme reduced with 350 μM-H_2O_2 for 30–40s.

number of interesting conclusions may be drawn. First, activation energies are low under all conditions, e.g. in water the value at 0°C is about 19.2kJ/mol (4.6kcal/mol). Secondly, at low temperatures (5°C and below) the rate-constant curve for each solution becomes approximately parallel to the corresponding viscosity curve. Thus under these conditions, activation energy of the turnover reaction appears to be controlled solely by the effect of temperature on viscosity and hence on the diffusion coefficient. Both the above findings point to a true diffusion-limited reaction. Contrary to this simple picture, it is equally clear that glycerol has a greater effect on the rate constant than on viscosity, and therefore factors other than viscosity must substantially influence the reaction rate at a given temperature.

The flattening of the rate-constant curves at high temperatures, particularly in water alone, calls for comment. This graph flattens completely around 40°C and the rate even falls slightly when the temperature is raised to 55°C. Since this effect was clearly not due to irreversible destruction of the enzyme at the highest temperature, the most reasonable interpretation would appear to be a reversible conformational change (hindered by the presence of glycerol) to a less active form at high temperatures.

Oxidation state of copper in the enzyme during and after catalysis

Preliminary work by Rotilio *et al.* (1972*a*) showed rapid partial reduction of superoxide dismutase by O_2^- (as followed at 650nm). Fig. 2 (bottom) illustrates changes which take place in E_{680} of the enzyme during and after catalysis. The application of repeated pulses yielding O_2^- at 2.5ms intervals caused the E_{680} to decrease to a constant value, which is presumably closely related to, if not identical with, the absorption value in true steady-state turnover. When pulsing ceased, E_{680} decreased from the plateau value, but at a relatively slow rate. This post-steady-state bleaching was due to reduction by H_2O_2 generated in the enzymic reaction (and to a lesser extent generated directly by the radiation), as was shown by further experiments (not illustrated), in which catalase was added. With catalase, after a similar steady state, the absorption increased towards the value for the native enzyme, instead of decreasing. In experiments similar to that of Fig. 2 (lower), both the rate and the ultimate extent of bleaching of superoxide dismutase by H_2O_2, were consistent with the peroxide-reduction kinetics reported by Bray *et al.* (1974).

The corresponding rate of post-steady-state reoxidation of the enzyme in the presence of catalase was also studied. Bray *et al.* (1974) were unable to obtain reproducible rates for reoxidation of reduced enzyme by O_2 alone. However, in the presence of catalase we found that post-steady-state reoxidation rates were roughly proportional to the catalase concentration, with half-times as low as 13s at catalase concentrations in the region of 5 μg/ml. Such results might be best explained by assuming that catalase here is acting as a peroxidase, with reduced superoxide dismutase as electron donor. Klug-Roth *et al.* (1973), however, report only slow reoxidation in the presence of catalase. Since they used concentrations of catalase similar to ours (though their enzyme was of unstated purity and origin) their result is entirely in conflict with our work and clarification is obviously required.

Rate of reaction of O_2^- with excess of native and reduced enzyme

Enzyme turnover was shown above to be governed by a second-order rate constant of $2.37 \times 10^9 M^{-1} \cdot s^{-1}$.

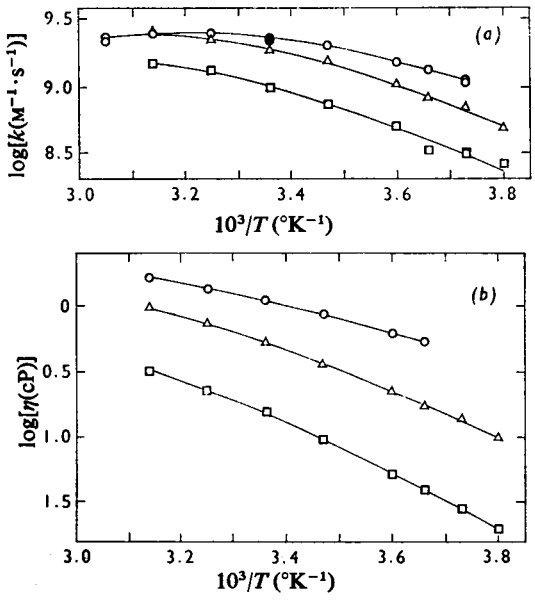


Fig. 4. *Effect on superoxide dismutase activity of increasing the viscosity of the medium by addition of glycerol*

Assays were carried out at various temperatures by pulse radiolysis with a constant enzyme concentration (about 1 μM), in 2 mM-sodium pyrophosphate with 85 mM-ethanol, either alone (○) or containing glycerol at approx. 25% (v/v) (△) or 50% (v/v) (□). The log of the turnover rate constant (k) is plotted against the reciprocal of the absolute temperature. The lower portion of the graph gives a similar plot of the log of the measured viscosity (in cP, where 1 cP = 10^{-3} Pa·s) of the three solutions, against 1/T. In following O_2^- decay at 250 nm in the presence of glycerol it was necessary to ignore the first 0.1–0.2 ms of the decay curve, which was due to transient glycerol radicals. The pH of the solution without glycerol was 9.4 at 20°C. Glycerol and temperature changed the pH values slightly, e.g. without glycerol, at 55°C, the pH was 9.2. A fresh enzyme sample was used for each point on the graph. Stability of the enzyme in the assay procedure at the highest temperature was checked by reassaying one sample at 25°C (●) after a previous assay at 55°C.

Preliminary measurements, at 650 nm, of the oxidation state of enzyme copper after exposure to substrate (Rotilio *et al.*, 1972*a*; Fielden *et al.*, 1973) indicated an enzyme mechanism involving alternate copper reduction and reoxidation:

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

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

It was therefore thought useful to measure the rates of reduction and reoxidation individually. Excess of enzyme was required to avoid turnover, that is a combination of reactions (1) and (2). Since O_2^- at 250 nm has a higher ε than Cu^{2+} at 680 nm, the experiments were carried out at 250 nm. The reduced enzyme, when required, was obtained by exposure of the native enzyme to $e^-_{aq.}$ or, more frequently, H_2O_2; in the latter case, the conditions were such as to obtain adequate reduction with minimum inactivation (see Bray *et al.*, 1974). Rate constants, obtained from the first-order decay of the E_{250} in the presence of excess of enzyme (at a known concentration), are listed in Table 1. The rate constants for the reduction of native enzyme and the reoxidation of reduced enzyme by O_2^- are the same (2.4×10^9 M^{-1}·s^{-1}) and agree closely with the turnover rates measured for native and reduced enzyme (see Table 1), as would be expected for near diffusion-limited reactions. This agreement shows that there are no slower steps in the turnover than the reduction and reoxidation and is consistent with the mechanism proposed above.

To ensure that the reaction of the enzyme in the above experiments did indeed involve reduction and reoxidation of the copper, we carried out confirmatory experiments at 650 nm. Precise measurements at 650 nm were hindered by the low ε of the bivalent copper, which meant that high enzyme concentrations were required, and hence a satisfactory excess of enzyme over O_2^- could not be obtained. Measurements of the rates of reduction and reoxidation were obtained from the traces in Fig. 5. Although the conditions were not ideal in that the steady state was approached, the rate constants obtained were consistent, within experimental error, with those found under the conditions of Table 1. This further confirms the participation of copper in the primary catalytic processes of the enzyme.

Environment of the copper atoms in native superoxide dismutase preparations

Fielden *et al.* (1973) noted that a fraction of the Cu^{2+} absorption was unaccounted for when O_2^- brought native or reduced enzyme to near steady-state conditions. Before studying the proportions of copper in the oxidized and reduced states during enzyme turnover in more detail, it seemed appropriate to try to find out whether or not all the copper atoms in native superoxide dismutase preparations are in the same chemical environment. Detailed examination of powder-type e.p.r. spectra, particularly when these are measured at two frequencies, at good signal-to-noise ratios and particularly when the help of computer simulation is available, can be a valuable tool in deciding whether or not more than one type of paramagnetic centre is present in a protein sample. We therefore submitted the spectra of native superoxide dismutase to such an examination. Experimental and simulated spectra at 9 and 35 GHz

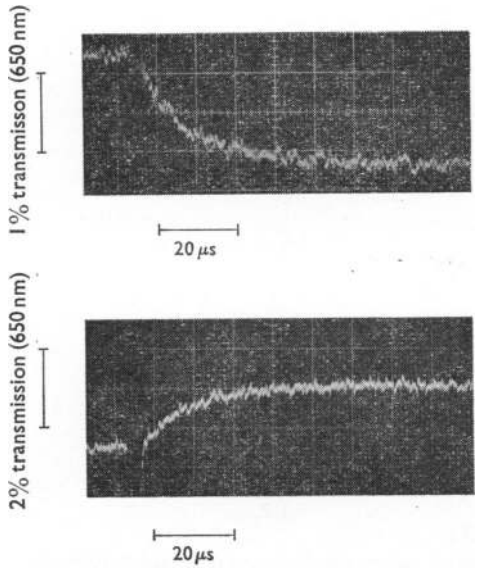


Fig. 5. *Oscilloscope traces showing the time-course of changes at 650 nm as native or reduced enzyme approaches the steady state*

The top trace shows reduction of native enzyme and the bottom trace oxidation of the reduced form by O_2^-. A single pulse (1.6 μs duration, yielding 28 μM-O_2^-) was used. Native enzyme was about 17 μM. Reduced enzyme (about 19 μM) was obtained by treatment with 280 μM-H_2O_2 for about 40 s. The standard assay medium was used at about 25°C and the light-path was 7.5 cm. In this experiment, the excess of O_2^- over enzyme was sufficient to give only 80–90% of the changes observed in the steady state (cf. Fig. 8).

are presented in Fig. 6. Two simulations were attempted. In one it was assumed that the unusually large line-width in the g_2 and g_3 regions was due to unresolved copper hyperfine structure (cf. Rotilio *et al.*, 1972*b*). In the other this was assumed to be due to orientation-dependence of the relaxation rate. The fit obtained in the former case is slightly better than that in the latter, though still better fits could probably be obtained if both sets of parameters were allowed to vary.

For both simulations, the fit is generally quite good at both frequencies, except around the 'bottom' of the g_2 feature and, in the 35 GHz spectrum, in the region between g_2 and g_3. Some of the observed features in this region are clearly due to impurities, particularly since the exact structure varied somewhat from sample to sample (compare Fig. 6 with Fig. 5 of Bray *et al.*, 1974). Subtraction of the computed 35 GHz spectrum from the experimental one yielded a difference spectrum in the g_2 and g_3 region reminiscent of that of a typical copper compound with g_m about 2.05. Integration of this difference spectrum (with allowance for the g_1 contribution) indicated that this component corresponded to 10–20% of the total copper. It seems reasonable to equate this copper, at least in part, with copper in the enzyme which is not readily reducible (see Bray *et al.*, 1974) and in any case to ascribe it to impurities.

If we neglect this impurity and consider only the remainder, it is clear that agreement between the experimental and computed spectra is as good as that normally obtained in spectral simulations of other copper proteins (see e.g. Vänngård, 1972). We may therefore conclude that 80% or more of the copper atoms in our native superoxide dismutase samples are in not clearly distinguishable and therefore similar, if not identical, chemical environments.

Extent of changes in the oxidation state of copper in the steady state

We next made measurements of the extent to which copper in the enzyme was reduced in the steady state when starting from oxidized enzyme, and also of the extent to which it was reoxidized when starting from the reduced enzyme. Results are summarized in Table 2 and are discussed in detail below. We thought it particularly important to carry out measurements by both e.p.r. and optical spectroscopy. Owing to the rapidity of the catalytic reaction, only the optical-measurement procedure had the time-resolution required to observe a steady state in which the enzyme was actually turning over. Typical optical experiments are illustrated in Fig. 7. In the intervals between the pulses (2.5 ms in this experiment), the transmission at 680 nm showed no signs of changing. It was thus a simple matter to read off the change in transmission obtained when sufficient pulses had been applied to attain a steady state and hence to calculate the proportion of the E_{680} that changed.

Since such optical measurements indicated that steady-state mixtures of oxidized and reduced enzyme generated by turnover were generally stable for at least some milliseconds after all the substrate had been exhausted, we were encouraged to use the rapid-freezing method to trap enzyme samples some 6 ms after turnover had been initiated, for e.p.r. study. E.p.r. data obtained in this manner would be expected to complement that from the optical work in a number of important ways. First, e.p.r. detects quantitatively all Cu^{2+} atoms present in the system whatever their environments (ignoring the somewhat unlikely possibility that interacting Cu^{2+} atoms might be present). Secondly, e.p.r. might be able to give direct information on possible changes in the environment of some or all of the Cu^{2+} atoms during the catalytic reaction. Such considerations are of prime importance, since data given by Klug-Roth *et al.* (1973) on the extinction coefficients of superoxide dismutase after reduction by one and two electrons,

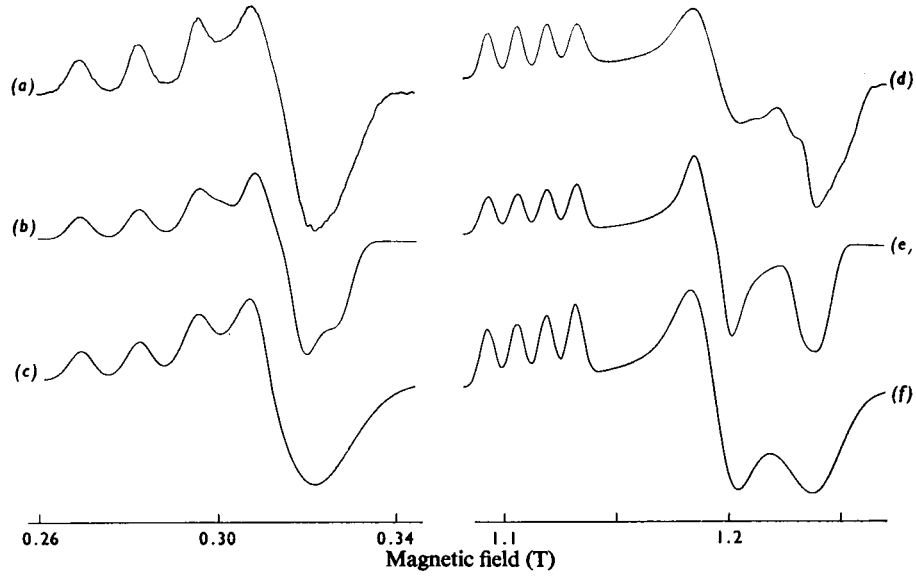


Fig. 6. *Experimental and computer-simulated e.p.r. spectra of native superoxide dismutase at 9 GHz and 35 GHz*

The experimental spectra (*a*) and (*d*) were of a sample of superoxide dismutase (1.3 mM in water) in a 3.0 mm external-diam. tube. (*a*) was recorded at 9.31 GHz at 122°K with 100 mW power and 5×10^{-4}T (5G) modulation. (*d*) was recorded at 35.14 GHz at about 120°K with about 30 mW power (10 decibels attenuation) and 1×10^{-3}T (10G) modulation. The computer-simulated e.p.r. spectra all used the principal g values g_x, 2.025, g_y, 2.103, g_z, 2.257. In (*b*) and (*e*) the corresponding principal A values were 0.00377 cm^{-1} [4×10^{-3}T (40G)], 0.00441 cm^{-1} [4.5×10^{-3}T (45G)] and 0.01388 cm^{-1} [1.32×10^{-2}T (132G)] respectively, and in (*c*) and (*f*) they were 0 cm^{-1}, 0 cm^{-1} and 0.01388 cm^{-1} [1.32×10^{-2}T (132G)]. (*b*) and (*e*) had isotropic line-widths of 2.5×10^{-3}T (25G) and 3.3×10^{-3}T (33G) respectively. In (*c*), anisotropic line-widths in the x, y, and z directions were 8.5×10^{-3}T (85G), 7.5×10^{-3}T (75G) and 2.5×10^{-3}T (25G) and in (*f*) 1.14×10^{-2}T (114G), 8.1×10^{-3}T (81G) and 3.3×10^{-3}T (33G). The field scale of (*b*) and (*c*) corresponds to a microwave operating frequency of 9.14 GHz and that of (*d*), (*e*) and (*f*) to one of 35.14 GHz. The line-shape functions were Gaussian.

Table 2. *Extent of Cu^{2+} reduction in two samples of superoxide dismutase during steady-state turnover of O_2^- and after treatment with reducing agents*

Estimates of the proportion of Cu^{2+} remaining in two different enzyme samples treated in various ways were made either by calculation from E_{680} changes or directly by e.p.r. from integrated signal intensities. Results are expressed relative to the Cu^{2+} content of native enzyme, which is taken as 100%. Cu^{2+} (%) was calculated from E_{680} changes by assuming all Cu^{2+} ions to have the same ε and Cu^+ ions to have zero extinction. The magnitude and direction of the E_{680} change observed on adding the reagent to the state of the enzyme indicated is given (ΔE%), E_{680} of native enzyme being taken as 100%. Conditions for carrying out the experiments are given in detail in the Materials and Methods section and also, in particular for the e.p.r. work, in Fig. 1. Reaction conditions with H_2O_2 varied and are detailed in footnotes. Errors quoted are average deviations, with the number of determinations given in parentheses.

		Sample 1			Sample 2	
		E_{680}			E_{680}	
State of enzyme	Reagent added	ΔE (%)	Cu^{2+} (%)	E.p.r. Cu^{2+} (%)	ΔE (%)	Cu^{2+} (%)
Native	O_2^-	−26 ±1 (3)	74	73 ±8 (3)	−18 ±1 (4)	82
Native	H_2O_2	−87† (1)	13	18*‡ ±2 (3)	—	—
Reduced (H_2O_2)	O_2^-	+17† ±2 (4)	30	30* ±3 (4)	+21‖ ±2 (4)	42//
Native	$e^-_{aq.}$	−87 (1)	13	—	−79 ±2 (2)	21

* Reduction with 23 mM-H_2O_2 for 450 ms.

† Reduction with 0.3 mM-H_2O_2 for 30–40 s.

‡ A value of 15 was obtained with 9 mM-H_2O_2 for 3 s.

‖ Reduction with 0.3 mM-H_2O_2 for 4 min. Some slight inactivation is possible under these conditions (see Bray *et al.*, 1974). In agreement with data of Klug-Roth *et al.* (1973) we found that exposure to H_2O_2 under more strongly inactivating conditions gave larger increases with O_2^-.

// Since the extent of reduction by H_2O_2 was not measured on this sample, this was assumed to be the same as that given by $e^-_{aq.}$ (cf. sample 1).

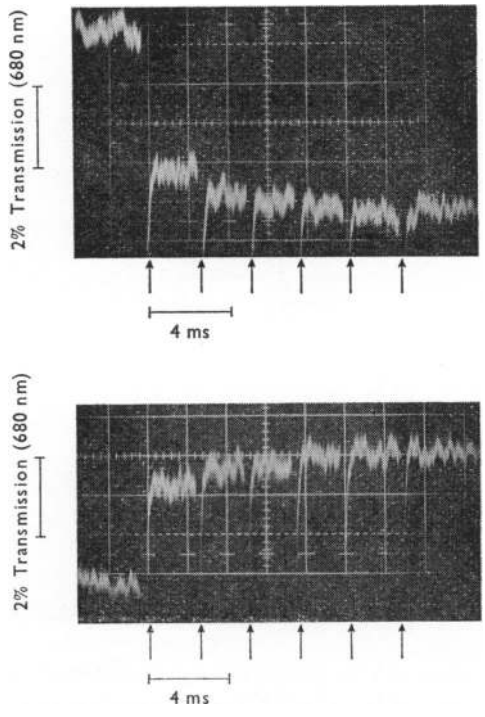


Fig. 7. *'Titration' of native and of reduced enzyme to the steady state with O_2^-, followed at 680 nm*

The top trace shows the oxidized enzyme and the bottom the reduced form. Six pulses (applied at the times indicated by the arrows) show as disturbances on the traces. In both cases the reaction to the steady-state value was brought to about 75% completion by the first pulse. Conditions, in the standard assay medium at about 25°C, were: six pulses (1.6μs duration yielding 34μM-O_2^- per pulse) at 2.5ms intervals, with a light-path of 7.5cm; 32μM-enzyme was used in both experiments. Reduced enzyme was prepared by treatment with 300μM-H_2O_2 for about 40s.

clearly imply that one of the two Cu^{2+} atoms in the enzyme has negligible E_{650}. If this were correct, it would greatly complicate interpretation of all absorption changes shown by the enzyme. The method of carrying out the e.p.r. experiments is detailed in Fig. 1 and its legend, and in the Materials and Methods section. It should be noted, particularly, that the flow system used gave the degree of control needed to reduce the enzyme completely with H_2O_2, without significant inactivation.

Results of our e.p.r. and optical measurements on two samples of the enzyme are fully presented in Table 2, and some of them are also shown diagrammatically in Fig. 8. The simple mechanism of eqns. (1) and (2), with both reactions diffusion-controlled, would be expected to lead to 50% bleaching of the copper in the steady-state turnover of O_2^-. Further, it would be expected that such a steady state could be approached from either direction, i.e. from the oxidized or from the reduced enzyme. Even if some 20% of non-reducible copper impurities, as discussed above, is corrected for, a 40% decrease in the Cu^{2+} concentration on treatment of native enzyme with O_2^- would still be expected, together with a similar increase on treatment of reduced enzyme with substrate. Observed changes were consistently smaller than this, whether measured optically or by e.p.r. Thus the decreases on treatment of oxidized enzyme with O_2^- ranged from 18 to 27% of the total Cu^{2+} concentration, but the corresponding increases for the reduced enzyme were slightly smaller, namely from 12 to 21%.

The agreement between optical and e.p.r. measurements on sample 1 makes it quite clear that the extinction coefficients of Klug-Roth *et al.* (1973) must be grossly in error. Our data show that the two copper atoms must be contributing equally to the enzyme E_{680}.

A strikingly constant feature of our experiments, illustrated in Fig. 8, is the magnitude of the 'gap' between the steady state, when approached from 'above' (i.e. native enzyme) and when approached from 'below' (i.e. reduced enzyme). This 'gap' corresponds to enzyme copper which is not responding to the presence of the substrate, and the magnitude of the gap always amounted to close to 50% of the readily reducible copper. Thus the e.p.r. measurements with sample 1 (Table 2), for example, gave a 'gap' of $(73-30)/(100-18) = 52\%$; the corresponding optical value on this sample was 51%, and that on sample 2 was also 51%.

It therefore seems clear that only about half of the readily reducible copper of the enzyme is responding to O_2^-. We next examined the steady-state e.p.r. spectra (Fig. 9) to see if we could find any evidence either for differences between the responding and the non-responding copper atoms or for the production of Cu^{2+} species in new environments in the turnover processes. Results, at the relatively low signal-to-noise ratios, at which the low concentrations of O_2^- available to us compelled us to work, were entirely negative. The differences among the spectra of Figs. 9(*a*), 9(*b*) and 9(*d*) are regarded as not significant. Only Fig. 9(*c*) is different, owing to the presence of a significant amount of the peroxide-non-reducible material.

Mechanism of the catalytic reaction and the number of active sites functioning in catalysis

All data are consistent with the enzyme functioning by having its copper alternately reduced by one O_2^- and then reoxidized by the next, according to eqns. (1) and (2). However, the apparent involvement of

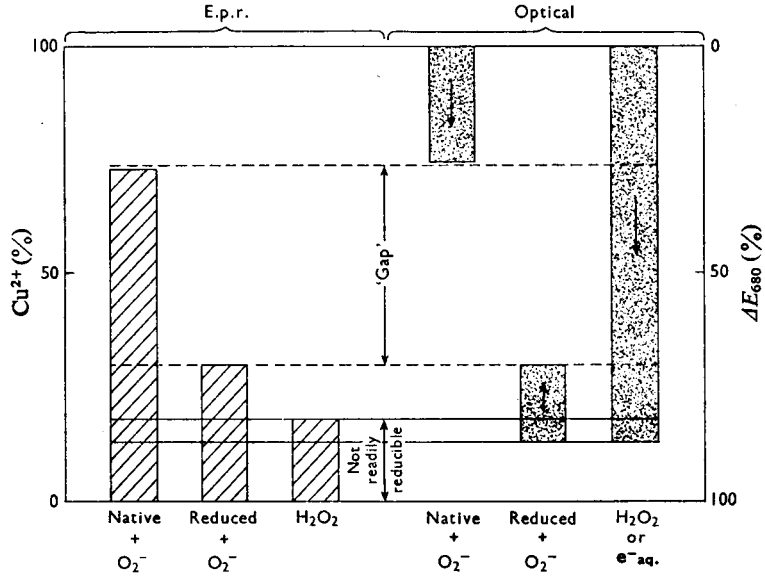


Fig. 8. *Extent of Cu^{2+} reduction of superoxide dismutase in steady-state turnover of O_2^- and after treatment with reducing agents*

Data from the e.p.r. and optical experiments of Table 2, sample 1, are presented in the form of a histogram to facilitate interpretation. Heights of cross-hatched areas represent e.p.r. signal intensities and those of stippled areas E_{680} changes. Arrows in the latter areas denote increasing or decreasing E_{680}. The solid horizontal lines cover the range of Cu^{2+} which was not reduced by H_2O_2 or $e^-_{aq.}$ in optical and e.p.r. experiments. The dashed horizontal lines show the extent of the 'gap', i.e. the distance between these lines corresponds to the proportion of the copper which does not respond to turnover.

only half the copper in the catalytic reaction, even when relatively minor impurities have been allowed for, might be interpreted in more than one way.

An obvious interpretation would be that the 'non-responding' copper is not part of the enzyme but is a further impurity, present in larger amounts. This seems unlikely because of the fairly exact stoicheiometry of one 'responding' to one 'non-responding' copper atom shown by two different enzyme preparations. The failure, with the help of computer simulations, to distinguish more than one type of copper as a major component in the e.p.r. spectrum of native superoxide dismutase, also argues against impurities. So, too, does the high specific activity of our enzyme (turnover second-order rate constant: $2.37\times10^9\,M^{-1}\cdot s^{-1}$). Klug *et al.* (1972) gave values of the rate constant, measured within the pH range at which we worked, ranging from 1.6×10^9 to $1.9\times10^9\,M^{-1}\cdot s^{-1}$, and in the later work of Klug-Roth *et al.* (1973) the rate constant appears to have been even lower. Thus it seems highly improbable that 'non-responding' copper in our samples is an impurity.

A second interpretation would be that the two copper atoms of resting enzyme are inherently different from one another, with only one performing a redox function in catalysis. This seems unlikely, since its two protein subunits are probably identical (Keele *et al.*, 1971), suggesting two identical active centres. Direct evidence against such gross non-equivalence of the two copper atoms of native enzyme comes from the e.p.r. spectra, as discussed above.

Accepting that the two copper atoms of native enzyme are identical, several types of mechanism may be envisaged. A single active centre containing two copper atoms seems highly improbable, first because this would presumably imply the unusual situation of having half of each active centre in one subunit and half in the other and secondly because the necessary close proximity of the two copper atoms to one another should then be detectable in the e.p.r. spectrum. Another interpretation involves reaction of a first copper atom with O_2^-, followed by very rapid intramolecular transfer of the reducing equivalent from one copper atom to the other, thereby leaving the second copper atom in a position to react with a second O_2^-, while the product dissociates from the first. Attractive though such a scheme may be, involving alternate use of two active centres (cf. Lazdunski *et al.*, 1971), we prefer a mechanism in

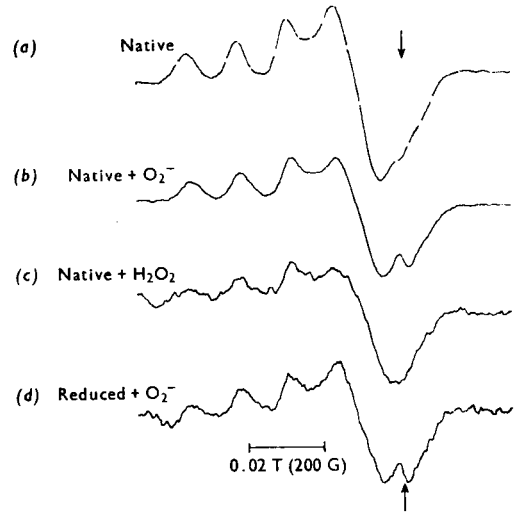
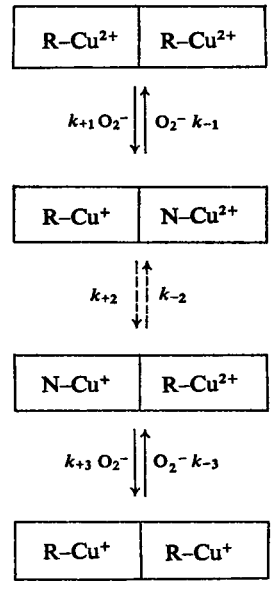


Fig. 9. *E.p.r. spectra of superoxide dismutase alone, after reduction with H_2O_2 and shortly after steady-state turnover of O_2^-, when starting from the native and from the reduced state*

The experiment is the one described in Table 2, for sample 1 (see also Fig. 1). (*a*) is untreated enzyme, (*b*) is enzyme reduced to the steady state by O_2^-, (*c*) is enzyme reduced by H_2O_2 and (*d*) is enzyme, first reduced by H_2O_2, then reoxidized to the steady state by O_2^-. Relative gain settings in (*a*), (*b*), (*c*) and (*d*) were ×1, ×1, ×4 and ×2.5 respectively. Spectra at 9 GHz were recorded at 122°K by using 100 mW power and 2×10^{-3} T (20 G) modulation. Computer averaging (up to 20 scans of 2 min duration) with subtraction of appropriate blank signals was used to obtain the spectra. The arrow corresponds to the resonance of diphenylpicrylhydrazyl. A weak free-radical signal is apparent in (*b*) and (*d*). This is presumably due to O_2^- and corresponds to about 0.2% of that initially present. Its presence may be due to incomplete mixing rather than incomplete reaction.



Scheme 1. *Proposed reaction scheme*

Each box represents one subunit of the enzyme. R denotes copper responding to O_2^-; N denotes copper not responding to O_2^-. k_{+1}, k_{-1}, k_{+3} and k_{-3} control the fast turnover reactions, and k_{+2} and k_{-2} represent much slower rates.

which reaction of one copper renders the other at least transiently unreactive and we tentatively propose Scheme 1. The enzyme turns over, when starting from native enzyme, by the reactions governed by k_{+1} and k_{-1}. On starting from reduced enzyme, k_{-3} and k_{+3} are the turnover reactions. It should be noted that the rates we have actually measured (Table 1) are k_{+1} and k_{-3}, but k_{-1} and k_{+3} remain unmeasured, though they cannot be less than the corresponding turnover rate constants. Scheme 1 involves postulating two singly reduced enzyme species, one of which has its Cu^{2+} atom reactive and its Cu^+ unreactive, whereas in the other this situation is reversed. Interconversions between these species (k_{+2} and k_{-2}), which might well go via the fully oxidized or fully reduced forms rather than directly as indicated in Scheme 1, must be relatively slow, since the steady states, approached from the two directions, were indeed steady for at least 20 turnovers of the enzyme.

E.p.r. work at 9 GHz (Fig. 9), at poor signal-to-noise ratios, failed to distinguish between the two singly reduced enzyme species of Scheme 1, but the possibility that more refined studies would do so is not excluded. Further, it seems unlikely that these species differ, e.g. in that one has a bound H_2O_2 molecule, since H_2O_2 is not an inhibitor of superoxide dismutase (Bray *et al.*, 1974). Though possible effects of bound O_2 cannot be excluded, it seems that an allosteric type of mechanism has to be invoked to interpret the scheme (cf. alkaline phosphatase: Simpson & Vallee, 1970; Reid & Wilson, 1971). It is necessary to assume that reaction of one copper atom, whether initially Cu^+ or Cu^{2+}, with O_2^- renders the other temporarily unreactive towards further O_2^- molecules. Thus the conformation change rendering a Cu^{2+} unresponsive has to be of a different type from the one rendering a Cu^+ unresponsive. The nature of the proposed conformation changes is entirely speculative, but it is of particular interest that they must apparently take place faster than any protein conformation changes reported previously,

with rate constants greater than $10^6 s^{-1}$. They are thus unlikely to involve moving large portions of the molecule.

We now discuss the relation of our work to that of Klug-Roth *et al.* (1973). Like us, they found that the rate of reduction of native enzyme, the rate of re-oxidation of reduced enzyme and the turnover rate constant were all about equal. Further, they found the extent of steady-state reduction of native enzyme to be 27–30% of the original Cu^{2+}, which is in reasonable agreement with our values of 18–27%. On the other hand, they do not appear to have measured directly the extent of the corresponding reoxidation of reduced enzyme. We suggest that the 'modified form' of the enzyme generated by reaction with H_2O_2 to which they refer and which is crucial to many of their arguments may in fact in some cases at least have been the inactivated species of Bray *et al.* (1974). That this is so is suggested, for example, by close examination of Fig. 4(*b*) of Klug-Roth *et al.* (1973). Calculation from their data indicates that they had exposed 7 μM-enzyme to 350 μM-H_2O_2 for 10 min before recording their trace. At least 50% inactivation would be predicted under these conditions, so their failure to observe a response to O_2^- may not be surprising. Since they were apparently unaware of the inactivation reaction and since their data analysis relied heavily on computer fitting of combined results from experiments of different types, it may not be surprising to find, as noted above, that their enzyme extinction coefficients seem grossly in error. We therefore consider that any conclusion stemming from their computer analysis must be accepted with the greatest caution.

Finally catalysis of O_2^- dismutation by the enzyme has to be compared with that by free Cu^{2+} ions. Though the latter are, of course, quite unphysiological, Rabani *et al.* (1973) have shown that, in acidic media, free copper ions catalyse superoxide dismutation about four times faster than we find the enzyme does in the physiological pH range. Superoxide dismutase is therefore unique among enzymes in that there is a chemical model reaction which is more efficient than the enzymic reaction itself. It is interesting that the enzyme which has apparently evolved (McCord *et al.*, 1971) to carry out so simple a reaction is, in some ways, far from simple, as we have shown.

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