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Reduction and Inactivation of Superoxide Dismutase by Hydrogen Peroxide

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Reactions of H_2O_2 with superoxide dismutase were studied by e.p.r. (electron paramagnetic resonance) spectroscopy and other methods. In agreement with earlier work, the Cu^{2+} of the enzyme is reduced by H_2O_2, although the reaction does not go to completion and its kinetics are not simple. With dilute enzyme the time for half-reduction with 9mM-H_2O_2 is about 150ms. It is suggested that the reaction is a one-electron reduction, involving liberation of O_2^-. On somewhat more prolonged exposure to H_2O_2, the enzyme is inactivated. For enzyme in dilute solution and over a limited range of H_2O_2 concentrations, inactivation is first-order with respect to enzyme and reagent, with $k = 3.1\,M^{-1} \cdot s^{-1}$ at 20–25°C. Inactivation is accompanied by marked changes in the e.p.r. and visible spectra and appears to be associated with destruction of one histidine residue per subunit. It is suggested that this histidine is close to the metal in the native enzyme and essential for its enzymic activity.

The enzyme superoxide dismutase catalyses reaction between two superoxide radicals to yield one molecule each of O_2 and H_2O_2 (McCord & Fridovich, 1969). In the accompanying paper (Fielden *et al.*, 1974) we report detailed studies on the mechanism of action of the enzyme by using pulse radiolysis and e.p.r. (electron paramagnetic resonance) spectroscopy. In the course of our pulse-radiolysis work, we have observed a decrease in activity on leaving the enzyme for some minutes after turnover of superoxide. This loss of activity did not occur if catalase was present, suggesting that H_2O_2 arising from the enzymic reaction might be causing inactivation. The present work describes an investigation of the effects of H_2O_2 on superoxide dismutase. This topic has been discussed in preliminary accounts by Symonyan & Nalbandyan (1972), Rotilio *et al.* (1973) and Fielden *et al.* (1973).

Materials and Methods

Reagents, buffers and visible spectra

H_2O_2 was standardized by titration with $KMnO_4$. Pyrophosphate buffers were prepared from $Na_4P_2O_7$, $10H_2O$ adjusted to the required pH with H_2SO_4. Catalase (crystalline) was obtained from Boehringer Ltd., London W5 2TZ, U.K. Visible absorption spectra were recorded on a Unicam SP.1800 spectrophotometer.

E.p.r. measurements

Spectra were recorded at about 120°K on a Varian E9 spectrometer operating at 9 or 35GHz. Cu^{2+}–EDTA was used as a standard for double integration, with appropriate small corrections for saturation and for dependence of intensity on g value (Aasa & Vänngård, 1970).

Superoxide dismutase

The enzyme was prepared from ox blood in Rome by the method of McCord & Fridovich (1969) and stored as a freeze-dried powder. Solutions were prepared as required, generally in pyrophosphate buffers, and centrifuged before use. Enzyme concentrations were estimated from E_{680}, taking $\varepsilon = 300\,M^{-1} \cdot cm^{-1}$ (McCord & Fridovich, 1969; Fee, 1973*a*). Values obtained in this way agreed well with those found by integrating the e.p.r. signal, assuming $2Cu^{2+}$/mol. Thus, with five samples of enzyme, the concentration determined by e.p.r. averaged $98 \pm 5\%$ (average deviation) of the value determined optically. Minor variations in both e.p.r. and optical spectra suggested the presence of small amounts of impurities. The ratio E_{530}/E_{680} was within the range 0.29–0.36, though residual light-scattering may have been the cause of some of this variability.

Superoxide dismutase assays by pulse radiolysis

The general methods and conditions used for pulse radiolysis are described by Fielden *et al.* (1974). For simple activity measurements, 2mM-sodium pyrophosphate buffer, pH9.0–9.5, containing 85mM-ethanol was bubbled with O_2, and then superoxide dismutase was added to a final concentration of 1–2μM. This solution, at about 25°C, was submitted to a pulse of high-energy electrons, yielding a superoxide ion concentration of around 30μM. Subsequent first-order decay of superoxide was followed optically at 250nm. The rate constant divided by enzyme concentration then gave the second-order rate constant for substrate turnover (see Fielden *et al.*, 1974; Rotilio *et al.*, 1972*a*).

Time-course of reduction of superoxide dismutase by H_2O_2 by using rapid freezing

The rapid-freezing method was as described by Bray *et al.* (1973). As a check for possible inactivation by H_2O_2 under the most drastic conditions used, activity and e.p.r. measurements were carried out in parallel as follows. Portions of the reaction mixture were shot from the jet of the rapid-freezing apparatus into 20ml of catalase solution (10μg/ml) in place of the cold isopentane. These samples were frozen and later assayed by pulse radiolysis for superoxide dismutase activity.

Amino acid analyses

Samples were evaporated to dryness and hydrolysed in 6M-HCl for 24h at 110°C. Samples were analysed by standard chromatographic methods (see Moore & Stein, 1963).

Results and Discussion

Reduction of superoxide dismutase by H_2O_2

We studied the reduction process first, as preliminary indications were that this occurred faster than inactivation. To simplify the kinetics of reduction we used fairly large excesses of H_2O_2 over enzyme and under these conditions fast-reaction techniques were essential. Preliminary stopped-flow measurements on the rate of reduction indicated that the reaction was not a simple second-order one. E.p.r. measurements in conjunction with rapid freezing were in agreement. Using this method we studied the time-course of reduction of about 150μM-enzyme by 2–45mM-H_2O_2. In these experiments inactivation by H_2O_2 caused no problems, amounting to less than 10% even at the longest reaction time (3s) and the highest H_2O_2 concentration (45mM) used. The rate of reduction increased with increasing H_2O_2 concentration. A typical experiment with

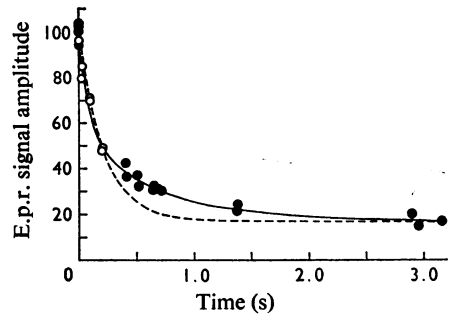

Fig. 1. *Time-course of reduction of superoxide dismutase by H_2O_2*

The rapid-freezing method was used. Relative amplitudes of the e.p.r. spectra are plotted against reaction time. The two symbols refer to different ram speeds and the zero-time points were obtained by replacing H_2O_2 by buffer. Reaction conditions were as follows: superoxide dismutase, 130μM; H_2O_2, 8.9mM, in 10mM-sodium pyrophosphate buffer, pH9.0; temperature 21°C. The dashed curve is a calculated first-order decay with $k = 4.7s^{-1}$, to illustrate the deviation of the experimental results from first-order kinetics.

8.9mM-H_2O_2 is illustrated in Figs. 1 and 2. Fig. 1 shows that the time for half-decay of the Cu^{2+} e.p.r. signal amplitude was about 150ms, but the rate fell markedly compared with that expected for first-order kinetics. Further, the reaction did not go to completion, 17% of the original amplitude (15% of the original integrated intensity) remaining after 3s reaction time. Incomplete reduction was found in all experiments of this type with several samples of enzyme. In a second set of determinations under the conditions of Fig. 1, the residual amplitude at 3s was 15%; another experiment at a higher H_2O_2 concentration (45mM) gave even less complete reduction (24% signal amplitude remaining after 3s). Optical measurements at 680nm have also shown that the Cu^{2+} is incompletely reduced by H_2O_2, to a similar extent (Fielden *et al.*, 1974).

E.p.r. spectra from the experiment of Fig. 1 are reproduced in Fig. 2, illustrating that the residual signal (*c*) is appreciably different from that of native enzyme (*a*). The nature of the unreduced material is uncertain, though it consists in part of impurities present in the starting material (see the Materials and Methods section). Other workers (Fee, 1973*a*) have also noted some inhomogeneity in superoxide dismutase prepared by the method of McCord & Fridovich (1969). It seems significant that an excess of hydrated electrons failed to reduce 13–21% of the enzyme in experiments reported by Fielden *et al.* (1974). Since incomplete reduction of a single species by this reagent seems improbable (but see Faraggi & Pecht, 1971), the remainder must pre-

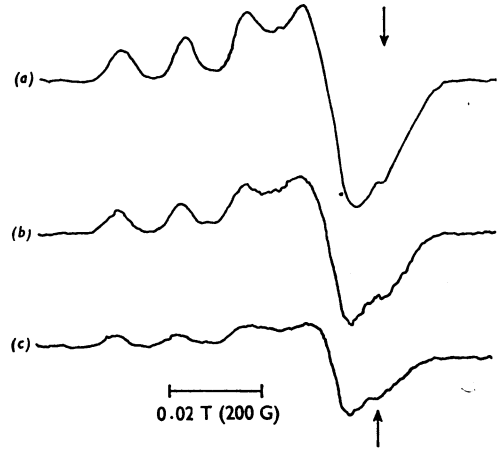

Fig. 2. *E.p.r. spectra (9 GHz) of superoxide dismutase before and during reduction by H_2O_2*

(*a*) Untreated enzyme, (*b*) after a reaction time of 0.4s, (*c*) after 3.0s. The samples correspond to the experiment described in Fig. 1. Spectra were recorded at 122°K with 100mW power and 25×10^{-4}T (25G) modulation. Computer averaging (four scans of 1min duration) and subtraction of appropriate blank signals were used to obtain the spectra in (*b*) and (*c*). Relative gains are: (*a*)×1.0, (*b*)×1.9, (*c*)×2.5. Arrows indicate the diphenylpicrylhydrazyl resonance ($g=2.0036$).

sumably derive from impurities. These might include for example, the peroxide-inactivated enzyme discussed later in this paper. In contrast, all Cu^{2+} in the enzyme was readily reduced by dithionite, as previously observed by Weser *et al.* (1971).

On the other hand, the e.p.r. evidence of Fig. 2 does not exclude the presence of some unreduced native enzyme in the peroxide 'non-reducible' fraction. This could in principle arise from an equilibrium owing to reoxidation by O_2, since reduced superoxide dismutase ultimately reverts to the oxidized form in the presence of air. Unfortunately we have not so far been able to obtain consistent data on this reaction rate. Rapid reoxidation ($t_{\frac{1}{2}}$ about 10s) was found in one experiment in which enzyme was reduced with peroxide, then separated from excess of reagent by anaerobic gel filtration. However, conditions were such that there was probably quite significant peroxide-inactivation in this experiment, which might complicate its interpretation. In contrast, oxygenation of an enzyme solution reduced by the hydrated electron [see Fielden *et al.* (1974) for method] led to reoxidation which was still incomplete after 30min. Clearly, further work is required to determine the precise nature of the 'non-reducible' material.

Mechanism of reduction by H_2O_2

We have not yet attempted a detailed analysis of the kinetics of reduction of superoxide dismutase by H_2O_2. Nevertheless, a significant conclusion may be drawn from the time-course in Fig. 1. Bearing in mind that the catalytic action of the enzyme involves alternate reduction and reoxidation of copper by O_2^- (see Fielden *et al.*, 1974), a plausible scheme for reduction by H_2O_2 may be written:

$$E\text{–}Cu^{2+} + H_2O_2 \rightleftharpoons E\text{–}Cu^{+} + O_2^- + 2H^+ \quad (1)$$

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

It is assumed that the two copper atoms are identical and independent, and that there are no other electron acceptors (see also Symonyan & Nalbandyan, 1972). In the above scheme, the back reaction in eqn. (1) would become faster as reduction proceeded, at the expense of the forward reaction in eqn. (2); this would account qualitatively for the observed slowing down of the overall reaction. The equilibrium in eqn. (2) allows for the possibility referred to above of reoxidation of Cu^+ by O_2. Alternatively, a two-electron reduction might occur, involving copper and a second electron acceptor, as has been suggested by one of us (Rotilio *et al.*, 1973). In this case, there would be no reason to expect other than simple second-order kinetics, contrary to our present observations.

Kinetics of inactivation of superoxide dismutase by H_2O_2

The kinetics governing loss of enzymic activity in the presence of H_2O_2 were then studied in dilute enzyme solutions (around 1μM) by using large excesses of H_2O_2. Results are presented in Fig. 3. Within experimental error, inactivation was first-order with respect to both enzyme and H_2O_2, for concentrations of the reagent between 0.6 and 6.0mM. Within this range, the average observed second-order rate constant was $3.1\,M^{-1}\cdot s^{-1}$. However, deviations were observed at higher and lower peroxide concentrations (rate constants 5.2 and $1.5\,M^{-1}\cdot s^{-1}$ at 0.32 and 15.0mM, respectively), whereas the reaction became somewhat slower at higher enzyme concentrations (cf. Fig. 4). Hence the inactivation must be a complex process. Further experiments showed that the reaction could be stopped but not reversed by addition of catalase. Another noteworthy finding is that all the decay curves in Fig. 3 can be extrapolated back to the 100%-activity point. Thus H_2O_2 has no inhibitory action on the enzyme, as opposed to its inactivating effect.

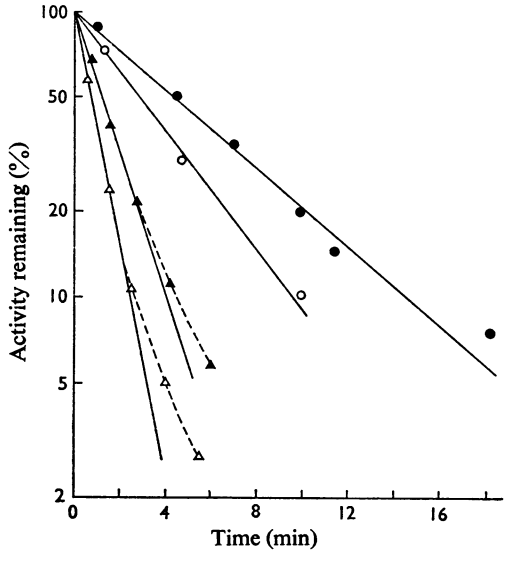

Fig. 3. *Time-course of inactivation of superoxide dismutase by H_2O_2 at various concentrations*

H_2O_2 in 2mM-sodium pyrophosphate buffer (pH9.1, containing 85mM-ethanol) was saturated with O_2; at zero time, superoxide dismutase (approx. 1μM) was added and the solution was rapidly transferred to the cell of the pulse-radiolysis apparatus. Activity measurements were made at suitable intervals, and results are expressed as percentages relative to a control without H_2O_2. The concentrations of H_2O_2 were: ●, 0.76mM; ○, 1.5mM; ▲, 3.0mM; △, 6.0mM. The assay introduced a negligible quantity of H_2O_2.

Properties of the inactivated enzyme

Fig. 4 illustrates some effects of an 11-fold excess of H_2O_2 on more concentrated superoxide dismutase. The E_{680} first fell rapidly, owing to reduction of the enzyme, then gradually increased over a period of some 30min to around 50% of the starting value. The time-course at 490nm was similar but resulted in a final absorbance greater than the initial value. These slow absorbance increases were accompanied by a loss of enzymic activity. After 35min, 34% of the activity remained although absorption changes seemed nearly complete. The spectrum at this stage had no distinctive features other than a slight shoulder in the region of 600nm, the trough in the region of 530nm and the peak at 680nm which are characteristic of the native enzyme, having been abolished. A further addition of peroxide had little effect on the spectrum, and catalase (about 20μg/ml) failed to reverse the changes.

The inactivation process also gave rise to a marked transformation of the e.p.r. spectrum of the enzyme similar to that seen by Symonyan & Nalbandyan

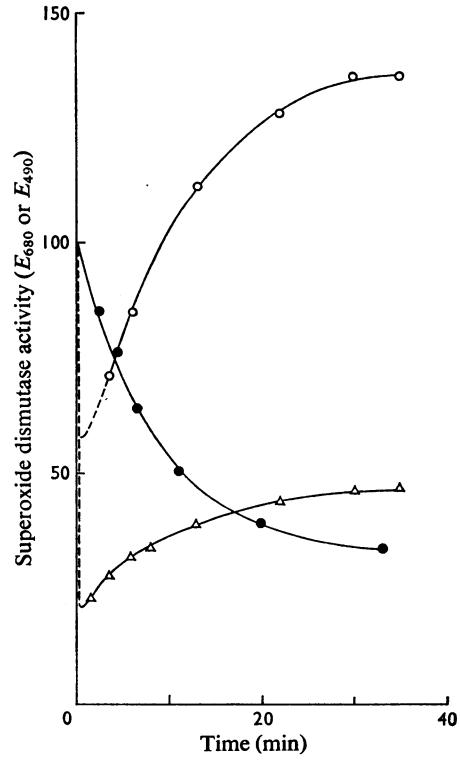

Fig. 4. *Changes in absorbance and in enzyme activity on treating superoxide dismutase with H_2O_2*

The reaction was carried out in a spectrophotometer cell; small portions were removed at intervals and pipetted into a solution of catalase, then assayed by pulse radiolysis (see the Materials and Methods section). Reaction conditions were as follows: superoxide dismutase, 75μM; H_2O_2, 810μM; temperature 20–25°C. ●, Superoxide dismutase activity; ○, E_{490}; △, E_{680}. All data are expressed as percentages of the initial values.

(1972). Fig. 5 illustrates spectra obtained after extended exposure to H_2O_2, when changes in signal form and intensity seemed to be nearing completion. The 35GHz traces show that the product has a more nearly axial type of spectrum than the native enzyme, with a smaller apparent line-width in the $g_\perp$ region (cf. Fielden *et al.*, 1974). However, the product is obviously not a single species, as illustrated in particular by its irregular $g_\parallel$ hyperfine structure at 9GHz. Only 72% of the Cu^{2+} originally present was detectable in the inactivated enzyme at this stage.

Amino acid analyses were carried out on enzyme treated with H_2O_2 under the conditions used for the e.p.r. measurements. Results on native and inactivated enzyme are compared in Table 1. Our values for the native enzyme are in fair agreement with those of

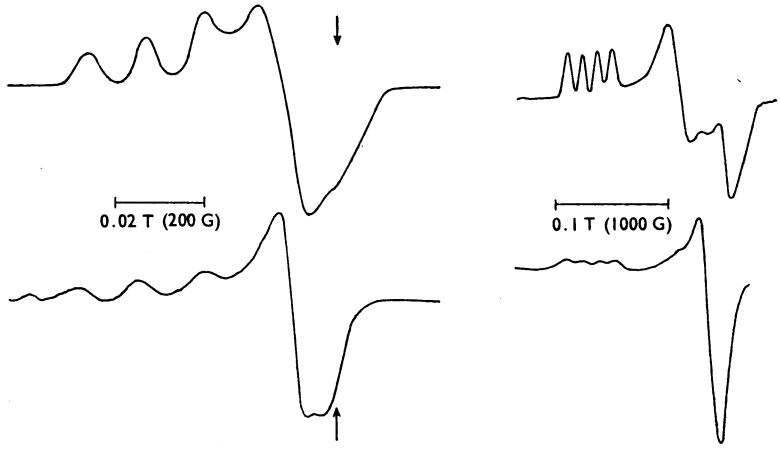

Fig. 5. *E.p.r. spectra of superoxide dismutase before and after inactivation with H_2O_2*

Superoxide dismutase (240μM) was treated with H_2O_2 (4.7mM) in sodium pyrophosphate buffer (10mM, pH9.0) for 120min at 20–25°C. Spectra of the native enzyme are shown at the top and those of the inactivated form at the bottom. Spectra on the left were recorded at 9.1GHz and on the right at 35.3GHz. In all cases the temperature was about 120°K. Other e.p.r. conditions at the two frequencies were, respectively, power 10mW or 8 decibels, modulation 25×10^{-4}T (25G) or 20×10^{-4}T (20G). The arrows indicate the diphenylpicrylhydrazyl resonance. E.p.r. parameters measured from the spectra were, for the native enzyme: g_1 2.257, g_2 2.11, g_3 2.03, A_1(Cu) 0.0132T (132G) (0.0139cm^{-1}); for the inactivated enzyme: g_1 (or $g_\perp$) 2.256, g_m 2.07, A_1(Cu) 0.0139T (139G) (0.0146cm^{-1}).

Fridovich and co-workers (Keele *et al.*, 1971; Forman *et al.*, 1973), although no corrections have been applied for those amino acids expected to be slowly decomposed or slowly liberated during acid hydrolysis. Peroxide inactivation caused few alterations in the amino acid composition. The largest change observed was a decrease of 1.9 in the number of histidine residues. This was accompanied by smaller apparent increases in the serine and proline contents; other changes were not greater than 0.3 residue.

Significance and nature of the inactivation reaction

It is clear that superoxide dismutase is quite readily inactivated by H_2O_2. This effect should be avoided or at least allowed for in all mechanistic studies on the enzyme (cf. Fielden *et al.*, 1974). Thus significant inactivation will occur if dilute enzyme at 25°C is exposed for more than a few minutes to H_2O_2 at concentrations greater than 100μM (whether generated by the enzymic reaction or added to the system). If the peroxide concentration is raised to around 1mM, then the permissible exposure time will fall to below 1min.

The precise nature of the inactivation reaction is not certain. As yet we have not followed in detail the time-course of the changes in the e.p.r. spectrum and amino acid composition, and it is of course possible that these might represent reactions subsequent to the primary inactivation step. On the other hand, the optical changes do parallel the activity loss quite closely. In any case, the course of the nett reaction cannot be simple, since inactivation presumably occurs after Cu^{2+} has been reduced to Cu^{+}, whereas in the products detected both optically and by e.p.r. this must have been reoxidized to Cu^{2+}.

In the overall process it is evident that the environment of the copper is substantially modified. This could possibly be due to conformational changes resulting from a primary reaction at a site remote from the metal, e.g. at a cysteine or cystine residue. However, it is more tempting to relate the change in copper environment directly to the observed fall in histidine content of almost one residue per subunit, and postulate that this histidine is closely associated with the copper in the native enzyme. It seems highly significant that, in several enzyme samples analysed after treatment with H_2O_2, the only residue to decrease appreciably was histidine. Tryptophan was not determined, but this has been shown to be absent from highly purified superoxide dismutase (Fee, 1973*a*). H_2O_2 is capable of oxidizing tryptophan and a number of other amino acid residues in proteins, but not normally histidine (see Hachimori *et al.*, 1964). The generally mild conditions under which inactivation proceeds, coupled with the minimal changes in amino acid composition, even under our most drastic conditions, argue for a high degree of specificity in the reaction. It is conceivable that enzyme-bound Cu^{+} in the presence of H_2O_2 could behave

Table 1. *Amino acid analyses of native and H_2O_2-inactivated superoxide dismutase*

Effect of H_2O_2 inactivation on the amino acid composition of superoxide dismutase. Reaction conditions were as follows: superoxide dismutase, 240μM; H_2O_2, 4.7mM, in 10mM-sodium pyrophosphate buffer, pH9.0; reaction time 120min at 20–25°C. Amino acid analyses were performed as described in the Materials and Methods section. The values for native enzyme are the average of two determinations. Data on the native enzyme, given by Keele *et al.* (1971) and by Forman *et al.* (1973), are presented for comparison. Results from the present experiments are normalized such that Asx+Glx+Ala+Val+Ile+Leu+Phe+Arg = 165 residues (cf. Keele *et al.*, 1971). No corrections have been applied for possible partial decomposition or incomplete liberation of certain amino acids during acid hydrolysis.

	Residues (mol/mol)			
		Native		
	Inactivated	Present work	Keele *et al.* (1971)	Forman *et al.* (1973)
Asx	37.0	36.7	35	—
Thr	24.7	24.7	26	—
Ser	17.2	16.1	20	—
Glx	25.7	26.0	24	—
Pro	15.0	14.3	14	—
Gly	42.7	42.4	50	—
Ala	16.9	16.9	21	23.6
Cys (½)	5.3	5.4	—	5.6
Val	30.4	30.6	28	—
Met	2.2	2.1	0	2.2
Ile	17.1	17.0	17	—
Leu	19.0	18.8	20	20.0
Tyr	2.3	2.3	2	2.6
Phe	9.6	9.5	10	9.6
His	16.4	18.3	16	15.4
Lys	—*	—*	22	—
Arg	9.3	9.5	10	—

* No estimate was possible for lysine, owing to poor resolution from NH_3.

as an analogue of Fenton's reagent and yield hydroxyl radicals. A selective active-site modification might then be expected, possibly even converting histidine into serine, in accordance with our observed increase in serine content.

The proposal that inactivation involves destruction of a histidine residue associated with copper is fully consistent with previous e.p.r. and n.m.r. work relating to the nature of the copper-binding site (Rotilio *et al.*, 1972*b*; Fee, 1973*b*; Stokes *et al.*, 1973). Forman *et al.* (1973) have also shown that photo-oxidation or other chemical modification of histidine in the apoenzyme abolishes recoverable activity. If H_2O_2 is indeed a selective reagent for modifying the active site, further comparison of the properties of the inactivated enzyme with those of the native form should prove valuable in understanding more fully the mechanism of the enzymic reaction.

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