Biochem. J. (1977) **161**, 3–11
Printed in Great Britain

A Pulse-Radiolysis Study of the Catalytic Mechanism of the Iron-Containing Superoxide Dismutase from *Photobacterium leiognathi*

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(*Received* 30 *June* 1976)

The mechanism of the enzymic reaction of an iron-containing superoxide dismutase purified from the marine bacterium *Photobacterium leiognathi* was studied by using pulse radiolysis. Measurements of activity were done with two different preparations of enzyme containing either 1.6 or 1.15 g-atom of iron/mol. In both cases, identical values of the second-order rate constant for reaction between superoxide dismutase and the superoxide ion in the pH range 6.2–9.0 ($k = 5.5 \times 10^8 M^{-1} \cdot s^{-1}$ at pH 8.0) were found. As with the bovine erythrocuprein, there was no evidence for substrate saturation. The effects of reducing agents (H_2O_2, sodium ascorbate or $CO_2^{-\cdot}$ radicals) on the visible and the electron-paramagnetic-resonance spectra of the superoxide dismutase containing 1.6 g-atom of ferric iron/mol indicate that this enzyme contains two different types of iron. Turnover experiments demonstrate that only that fraction of the ferric iron that is reduced by H_2O_2 is involved in the catalysis, being alternately oxidized and reduced by $O_2^{-\cdot}$; both the oxidation and the reduction steps have a rate constant equal to that measured under turnover conditions. These results are interpreted by assuming that the superoxide dismutase isolated from the organism contains 1 g-atom of catalytic iron/mol and a variable amount of non-catalytic iron. This interpretation is discussed in relation to the stoicheiometry reported for iron-containing superoxide dismutases prepared from several other organisms.

McCord & Fridovich (1969) established that the copper-containing protein, erythrocuprein, isolated from bovine erythrocytes is an enzyme catalysing the dismutation of superoxide radicals. This enzyme, and similar Cu- (copper-containing) superoxide dismutases isolated from various eukaryotes, have been extensively studied (see review by Fridovich 1974). Subsequently, superoxide dismutases containing functional manganese (Keele *et al.*, 1970; Vance *et al.*, 1972; Weisiger & Fridovich, 1973; Bridgen *et al.*, 1975; Lavelle & Michelson, 1975; Ravindranath & Fridovich, 1975) and iron (Yost & Fridovich, 1973; Puget & Michelson, 1974; Lumsden & Hall, 1974; Asada *et al.*, 1975; Misra & Keele, 1975; Yamakura, 1976) have been purified and their properties investigated. The most convenient method of generating relatively large amounts of substrate, $O_2^{-\cdot}$, is by ionizing radiation, and pulse radiolysis provides an ideal method for the study of the catalytic behaviour of these metalloenzymes. Such studies have been restricted largely to erythrocuprein (Rotilio *et al.*, 1972; Klug *et al.*, 1972; Bannister *et al.*, 1973; Klug-Roth *et al.*, 1973; Fielden *et al.*, 1974) and only one report has appeared on the pulse radiolysis of manganese-containing superoxide dismutase isolated from *Escherichia coli* (Pick *et al.*, 1974).

In the present paper, pulse radiolysis has been used to investigate the catalytic properties and mode of action of a bacterial Fe-(iron-containing) superoxide dismutase.

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Materials and Methods

General

Optical spectra were recorded with a Cary 15 spectrophotometer and e.p.r. (electron-paramagnetic-resonance) spectra with a Varian E9 spectrometer. Catalase was from Boehringer Laboratories, London W5 2TZ, U.K. Triple-distilled water was used for all experiments; other chemicals were of the highest purity available.

Pulse-radiolysis experiments

The pulse-radiolysis system and the general method of enzyme assay have been described previously (Fielden *et al.*, 1974). Unless stated otherwise the standard medium contained 2mM-sodium pyrophosphate, 100μM-EDTA and 0.1M-sodium formate. For experiments involving analysis at 350nm a filter was used, between the light-source and the irradiation cell, to exclude light of wavelength less than 300nm. All experiments were performed at room temperature (about 25°C).

Bacterial Fe-superoxide dismutase

The Fe-superoxide dismutase was purified from the marine bacterium *Photobacterium leiognathi* by the method of Puget & Michelson (1974). The enzyme (mol.wt. 40660), composed of two identical subunits, yielded a single band in polyacrylamide-gel electrophoresis. Unless stated otherwise the preparation used in the present experiments contained 1.6g-atom of iron/mol, and for this preparation protein concentrations were measured by using either the A_{280} or the A_{350}, by using a measured $A_{280}^{1\%} = 17.1$ and a calculated $\varepsilon_{350} = 2500\,\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ (Puget & Michelson, 1974). Storage of this sample at 3°C as a precipitate in saturated $(NH_4)_2SO_4$ solution for 1 year resulted in no loss of activity.

However, enzyme preparations with a lower iron content (cf. Puget & Michelson, 1974) have been isolated from bacteria grown in nutrient media of different iron concentrations. Iron-rich medium was obtained by adding $FeSO_4$ (50μM) to the medium previously described (Puget & Michelson, 1974). For low iron content the medium was treated with alumina (10g/litre, Alcoa A 305, Bacteriological grade) at 110°C for 20min, cooled, filtered and re-sterilized (110°C; 15min). Iron content was determined by using *o*-phenanthroline (Massey, 1957) to be 4.8μM. Bacteria were grown at 28°C in the presence of O_2.

From 12 litres of medium the 'high'-iron system yielded 109g wet wt. of bacteria, and the 'low'-iron system yielded 75g of bacteria. In the former case the iron content of the isolated enzyme was 1.15g-atom of iron/mol (average of four determinations) and in the latter case 1.12g-atom of iron/mol (average of four determinations). The results of a few experiments performed with enzyme containing 1.15g-atom of iron/mol are reported.

Concentrated enzyme solutions were dialysed against buffer (2mM-sodium pyrophosphate/100μM-EDTA, pH8) and centrifuged (15000*g*, 30min) before use.

Results

Catalytic properties of Fe-superoxide dismutase

The decay of $O_2^{-\cdot}$ in the presence of catalytic quantities of enzyme is illustrated in Fig. 1. The decay was studied as a function of pH (Table 1) and found to be always first-order. The rate of decay was proportional to the enzyme concentration, and second-order rate constants were calculated from the observed rates. The correction applied for the spontaneous decay of $O_2^{-\cdot}$ in buffer is significant only at pH values less than 7. A double-reciprocal plot of initial velocity against substrate concentration (Fig. 2) is a straight line passing through the origin.

Decay of $O_2^{-\cdot}$ in the presence of enzyme containing 1.15g-atom of iron/mol was measured at three different pH values (Table 1).

Action of reducing agents

Previous e.p.r. and spectrophotometric data have established that the iron in the native enzyme is in a high-spin ferric form (Puget & Michelson, 1974). The absorption band of the enzyme has a shoulder at 350nm, attributable in part to the ferric iron. Thus it may be expected that reducing agents should bleach (decrease) the A_{350} of the enzyme.

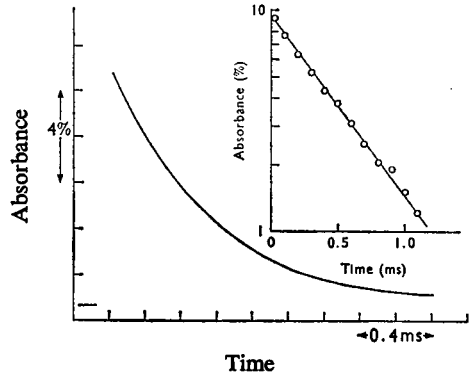

Fig. 1. *Decay of $O_2^{-\cdot}$ in the presence of Fe-superoxide dismutase*

The standard medium at pH8 contained ethanol (0.1M) in place of formate. The enzyme concentration was 3.35μM and the $O_2^{-\cdot}$ concentration was 33μM. The path length was 0.7cm and the decay of $O_2^{-\cdot}$ was followed at 250nm. Inset is a semi-logarithmic plot of *A* against time.

Table 1. *Effect of pH and of enzyme concentration on the second-order catalytic rate constant*

$O_2^{-\cdot}$ concentrations were approx. 30 μM.

pH	Enzyme concn. (μM)	$10^{-8} \times k$ ($M^{-1} \cdot s^{-1}$)
6.2	3.35	6.1
7.0	3.35	5.4
7.0	0.33*	6.4
8.0	0.20	5.0
8.0	3.35	5.4
8.0	0.33*	5.6
8.5	3.35	4.7
8.8	3.35	4.2
9.0	1.34	3.8
9.0	3.35	4.2
9.0	0.33*	4.2
9.5	3.35	2.2
10.1	3.35	1.3

* The enzyme used in these assays contained 1.15 g-atom of iron/mol.

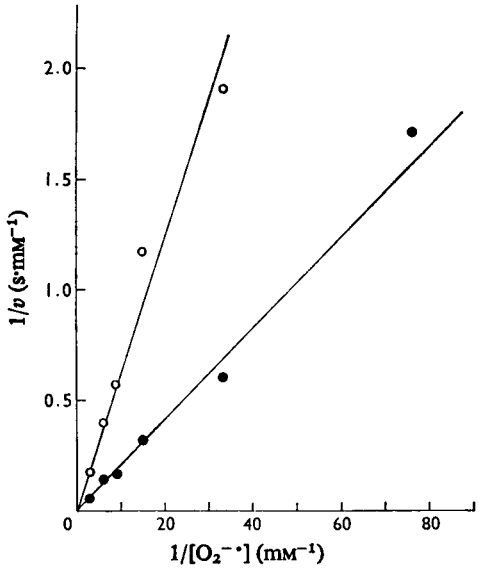

Fig. 2. *Double-reciprocal plot of initial velocity against initial $O_2^{-\cdot}$ concentration*

Measurements were made in the standard medium (pH 9.5) by using a cell of path length 0.7 cm and the decay of $O_2^{-\cdot}$ was followed at 250 nm. ○, Enzyme concentration of 0.17 μM; ●, enzyme concentration of 0.5 μM.

At pH 7.9 (2 mM-pyrophosphate/100 μM-EDTA), sodium ascorbate (2.5 mM) caused a rapid (<2 min) bleaching (two spectrophotometric determinations gave 64% and 66% decreases) of the A_{350} of the ferric enzyme (87.5 μM). The final A_{350} remained constant for more than 20 min after reduction and full activity was retained. Under the same conditions the e.p.r. spectrum completely disappeared (Fig. 3).

Radiolysis of deoxygenated sodium formate solution, saturated with N_2O, generates the formate radical anion, $CO_2^{-\cdot}$ (Neta *et al.*, 1969), as the only radical species. $CO_2^{-\cdot}$ is a powerful reducing agent. Deoxygenation and N_2O saturation were achieved by gently bubbling N_2O through the enzyme solution for 15 min; this process led to no inactivation. A train of pulses caused a maximal net bleaching of 66% of the A_{350} of the ferric enzyme (Fig. 4). Assay of the enzyme after reduction by $CO_2^{-\cdot}$ showed that full activity was retained.

Reduction and inactivation by H_2O_2

Since H_2O_2 is a product of the catalytic reaction, the effect of this reagent on the oxidation state and on the activity of the enzyme was investigated. As with bovine erythrocuprein (Bray *et al.*, 1974), H_2O_2 was found to act as both a reducing and an inactivating agent. The changes produced in the visible absorption spectrum are shown in Fig. 5. Reduction of the iron in the enzyme by H_2O_2 led to about 50% bleaching of the A_{350} within 2 min (four spectrophotometric determinations gave values ranging from 48 to 54%); at longer times a recovery in the A_{350} occurred and further addition of H_2O_2 at this stage resulted in no further change in the A_{350} (see Fig. 5). In another experiment using higher concentrations (140 μM-Fe-superoxide dismutase, 3.13 mM-H_2O_2), this recovery led to a final absorbance greater than the initial value (cf. Fig. 5). E.p.r. experiments indicate that a residual signal at about $g = 4.3$ is present after treatment with H_2O_2. At longer times and higher peroxide concentrations (when considerable inactivation has occurred), this signal remains virtually unchanged and there is apparently no new signal in this region (see Fig. 3).

The reduction of the enzyme has been studied also by observation of the effects of H_2O_2 generated during turnover. This post-steady-state bleaching of the A_{350} is illustrated in Fig. 6 and is absent in the presence of catalase. Experiments under similar conditions support a value of $1.75 \times 10^2 M^{-1} \cdot s^{-1}$ as an approximate second-order rate constant for the reduction process. However, this reaction mechanism may not be simple and the second-order rate constant quoted may apply only under the stated conditions (cf. bovine erythrocyte enzyme; Bray *et al.*, 1974).

The rate of inactivation by H_2O_2 was investigated under conditions similar to those prevailing in most of the experiments involving turnover studies (Fig. 7). The average second-order rate constant for peroxide inactivation derived from this and related experiments is $1.15 M^{-1} \cdot s^{-1}$. This value is subject to the qualifications expressed for the rate constant for peroxide-

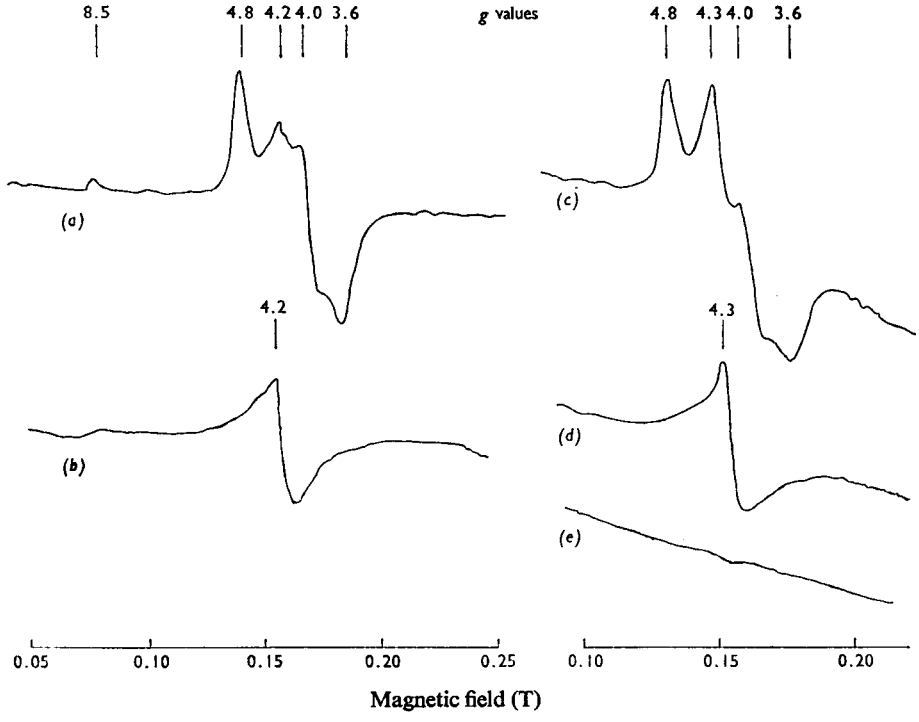

Fig. 3. *E.p.r. of Fe-superoxide dismutase*

All samples were in buffer (2mM-sodium pyrophosphate/100μM-EDTA) at pH8. Spectra (*a*) and (*b*) were obtained by using freshly prepared enzyme, whereas spectra (*c*), (*d*) and (*e*) were obtained by using the same enzyme preparation after storage for 5 months in saturated $(NH_4)_2SO_4$ solution. Spectrum (*a*) refers to native enzyme (370μM) and spectrum (*b*) to enzyme (370μM) treated with H_2O_2 (18.5mM; 20min at about 25°C). Spectrum (*c*) refers to native enzyme (87.5μM) and spectrum (*d*) to enzyme (87.5μM) treated with H_2O_2 (2mM; 36min at about 25°C). Spectrum (*e*) refers to enzyme (87.5μM) treated with sodium ascorbate (2.5mM) at about 25°C until the extent of bleaching of the A_{350} of the enzyme was constant (see the Results section). All spectra were recorded at 77K with a modulation frequency of 100kHz. For spectra (*a*) and (*b*) the other conditions were: microwave frequency 9.148GHz; modulation amplitude 10^{-3}T (10G); microwave power 5mW; gain 2500. For spectra (*c*), (*d*) and (*e*) the other conditions were: microwave frequency 9.151GHz; modulation amplitude 1.25×10^{-3}T (12.5G); microwave power 50mW; gain 2000. The numbers above the spectra are *g* values.

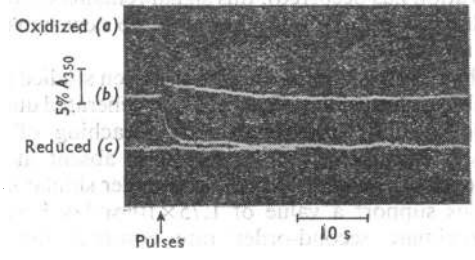

Fig. 4. *Changes in A_{350} of Fe-superoxide dismutase on reduction by $CO_2^{-\cdot}$*

The standard medium (pH8.2), saturated with N_2O, contained 3.68μM-enzyme. The light-path was 7.5cm and the pulse repetition rate was 50 pulses/s. Each pulse introduced 1.1μM-$CO_2^{-\cdot}$. (*a*) Effect on the A_{350} of the native enzyme of 24 successive pulses. (*b*) Further bleaching of the A_{350} caused by a train of 124 pulses applied approx. 1min after the first train. (*c*) A third train of pulses (124), approx. 2min after the first train, caused no further bleaching.

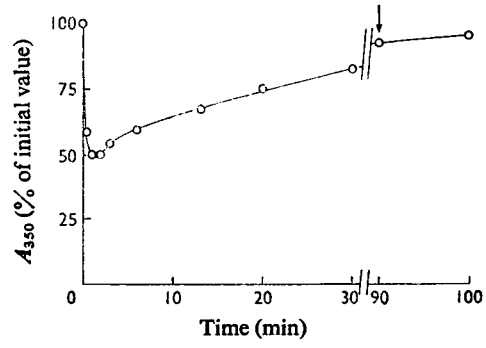

Fig. 5. *Effect of H_2O_2 on the A_{350} of the enzyme*

The buffer (2mM-sodium pyrophosphate/100μM-EDTA, pH7.4) contained 31.9μM-enzyme and H_2O_2 was added at zero time to a final concentration of 588μM. The light-path was 1cm. The arrow corresponds to further addition of H_2O_2 to give a total peroxide concentration of 1.18mM.

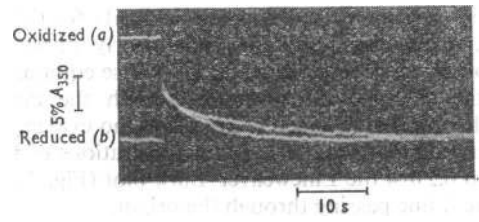

Fig. 6. *Post-steady-state bleaching of A_{350} of Fe-superoxide dismutase*

The standard medium (pH 8.2) was saturated with O_2 and the enzyme was added to a final concentration of 7.9 μM. The light-path was 7.5 cm. The pulse repetition rate was 400 pulses/s, and each pulse yielded 21 μM-$O_2^{-\cdot}$. Trace (*a*) shows the effect of 32 such pulses on the A_{350} of the native enzyme and trace (*b*) shows the effect of a similar train of pulses on the A_{350} of the enzyme after reduction with the H_2O_2 (approx. 400 μM) generated during turnover. The second train of pulses was introduced approx. 50 s after the first train.

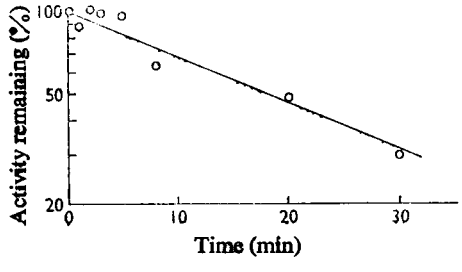

Fig. 7. *Inactivation of enzyme by H_2O_2*

The buffer (2 mM-sodium pyrophosphate/100 μM-EDTA pH 8.0) contained 8.15 μM-enzyme, and H_2O_2 was added at zero time to a final concentration of 450 μM. During the course of the reaction samples were removed, diluted into the standard medium (pH 8.0) containing catalase (15 units/ml) and assayed by the pulse-radiolysis technique. In the absence of Fe-superoxide dismutase, the catalase had negligible effect on the decay rate of $O_2^{-\cdot}$.

induced reduction (see above and Bray *et al.*, 1974).

Oxidation states of iron during and after catalysis

The enzyme was effectively maintained under turnover conditions if the solution was irradiated (to produce $O_2^{-\cdot}$) by a train of closely spaced electron pulses. This, as shown in Fig. 8, caused the A_{350} of the oxidized native enzyme solution to decrease by an average of 30% (seven determinations gave decreases ranging from 27 to 32%) to a steady value. With the reduced enzyme, obtained either by treatment with sodium ascorbate or by exposure to H_2O_2 (added to native enzyme solution or generated during catalysis) under non-inactivating conditions, successive pulsing caused a net increase in the A_{350} of the solution (Fig. 8). The average increase, expressed as a percentage of the A_{350} of the native enzyme solution, was 20% (seven determinations gave increases ranging from 18 to 24%).

The observed steady increase in A_{350} (Fig. 8) during turnover is not associated with enzymic processes, but is an artifact associated with the silica irradiation cell. Such an effect was observed both when the buffer-filled cell was irradiated (Fig. 8*a*) and when the empty cell was irradiated. It was reproducible and the corrections were easily made. The effect is not caused by a build-up of any residual A_{350} attributable to $O_2^{-\cdot}$; indeed, parallel experiments at 250 nm show that $O_2^{-\cdot}$ disappears completely between pulses.

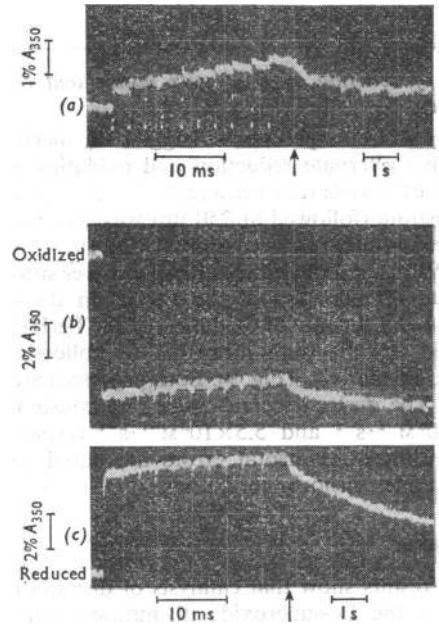

Fig. 8. *Changes in the A_{350} of Fe-superoxide dismutase during turnover*

The standard medium (pH 8.5) was saturated with O_2 and then enzyme was added to a final concentration of 6.7 μM. The light-path was 7.5 cm. The pulse repetition rate was 400 pulses/s and the yield of $O_2^{-\cdot}$ was 32 μM per pulse; 11 successive pulses were introduced in each case. The traces refer to: (*a*) buffer without enzyme; (*b*) native enzyme; (*c*) enzyme reduced by exposure (1 min) to H_2O_2 (approx. 500 μM) generated during turnover. The significance of the changes in absorption seen in (*a*) are discussed in the Results section. The vertical arrows indicate changes in time-scale.

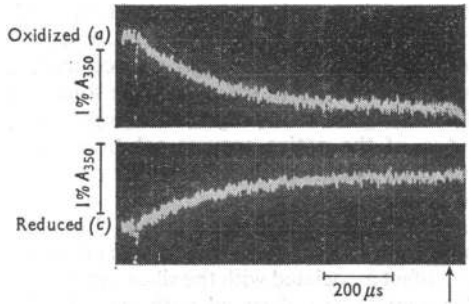

Fig. 9. *Rates of reaction of $O_2^{-\cdot}$ with native and reduced enzyme*

The standard medium (pH8.0) contained 7.65 μM-enzyme. The wavelength was 350nm and the light-path 7.5cm. Trace (*a*) shows the partial reduction of the native enzyme by 0.7 μM-$O_2^{-\cdot}$, and (*b*) shows the partial oxidation of the reduced enzyme (exposure to 460 μM-H_2O_2 for 1 min) by a similar amount of $O_2^{-\cdot}$. The vertical arrow indicates a change in time-scale, the second part of each trace being followed for 10ms.

Rates of reaction of $O_2^{-\cdot}$ with native and reduced enzymes

The above experiments suggest a mechanism involving alternate reduction and oxidation of the iron; the turnover rate measured by the disappearance of substrate (followed at 250nm) was characterized by a second-order rate constant of 5.2×10^8 M$^{-1}\cdot$s^{-1} at pH8. With a tenfold excess of enzyme over substrate, the rate of reduction of the Fe(III) in the native enzyme and the rate of oxidation of the Fe(II) in the reduced enzyme were measured by following the change of the A_{350} (Fig. 9). Both processes are first-order. The derived second-order rate constants are 5.2×10^8 M$^{-1}\cdot$s^{-1} and 5.5×10^8 M$^{-1}\cdot$s^{-1} respectively, and within experimental errors are equal to that found under turnover conditions.

Discussion

The results show that catalysis of dismutation of $O_2^{-\cdot}$ by the Fe-superoxide dismutase from *leiognathi* is first order with respect to substrate concentration, and there is no evidence for a biphasic process, as found for the manganese-containing superoxide dismutase isolated from *E. coli* (Pick *et al.*, 1974). Although the enzyme is stable between pH6 and 10, the value of the rate constant decreases as pH increases (Table 1). A similar pH-dependence has been reported for the Fe-superoxide dismutase from *E. coli* by using an indirect activity assay (Forman & Fridovich, 1973). At all pH values, the rate constants are lower than that for the bovine Cu-superoxide dismutase (Fielden *et al.*, 1974), where there was found to be no pH-dependence. The nature of the small variation of rate constant with pH for the Fe-superoxide dismutase does not clearly imply the involvement of any particular acid–base equilibrium in the catalytic mechanism. As with the bovine erythrocuprein (Fielden *et al.*, 1974), no evidence of saturation was obtained; for concentrations of $O_2^{-\cdot}$ up to 0.2mM the Lineweaver–Burk plot (Fig. 2) is a straight line passing through the origin.

Assay of the enzyme sample containing a lower iron content (1.15g-atom/mol) shows that its activity is nearly identical with that of the sample with higher iron content (Table 1). Indirect activity assay by the procedure of Puget & Michelson (1974) showed no difference between the specific activities of samples of enzyme containing 1.15, 1.25, 1.25, 1.34 and 1.53g-atom of iron/mol. These findings are in disagreement with those of Yamakura (1976) for the Fe-superoxide dismutase isolated from *Pseudomonas ovalis* (see below).

It must be noted that for the assays of the 'low'-iron enzyme it was assumed that $A_{280}^{1\%}=17.1$. The similarity of the two sets of rate constants suggests that this is the case, implying that the iron species present have no appreciable A_{280}.

The state of the iron in the enzyme (as measured by A_{350}) under various conditions is illustrated in Fig. 10. The reducing agent sodium ascorbate caused the A_{350} to decrease by 65% and simultaneous disappearance of the characteristic e.p.r. spectrum (Fig. 3). $CO_2^{-\cdot}$ caused a 66% bleaching of the A_{350} (Fig. 4). The remaining absorbance (approx. 35%) can be attributed to the residual absorption of aromatic amino acid residues at this wavelength. By comparison with the spectrum of a typical non-metalloprotein, such as bovine serum albumin, the expected A_{350} of such residues can be predicted by considering the $\varepsilon_{280}/\varepsilon_{350}$ ratio in the reference protein and assuming that such a ratio holds for the Fe-superoxide dismutase. Such a prediction agrees well with the residual absorbance obtained in the above experiments.

H_2O_2 causes a similar, but significantly smaller (only 50%) bleaching of the A_{350}. The extent of the bleaching as measured after turnover (e.g. Fig. 6) agrees well with this value. As with the bovine Cu-superoxide dismutase (Bray *et al.*, 1974) this reagent also inactivates the enzyme; the rates of inactivation of the two enzymes are similar for the respective conditions used.

The difference between the extents of bleaching by sodium ascorbate or $CO_2^{-\cdot}$ and by H_2O_2 (amounting to approx. 20% of the bleachable absorption) is not fully understood. E.p.r. data suggest that the signal originally present at $g=4.3$ is unaffected by H_2O_2, although it cannot be certain that the signal remaining after treatment with excess of peroxide is indeed that which was originally present. Double integration (considering only the region $2.5<g<5.5$) of the spectra obtained with samples before and after

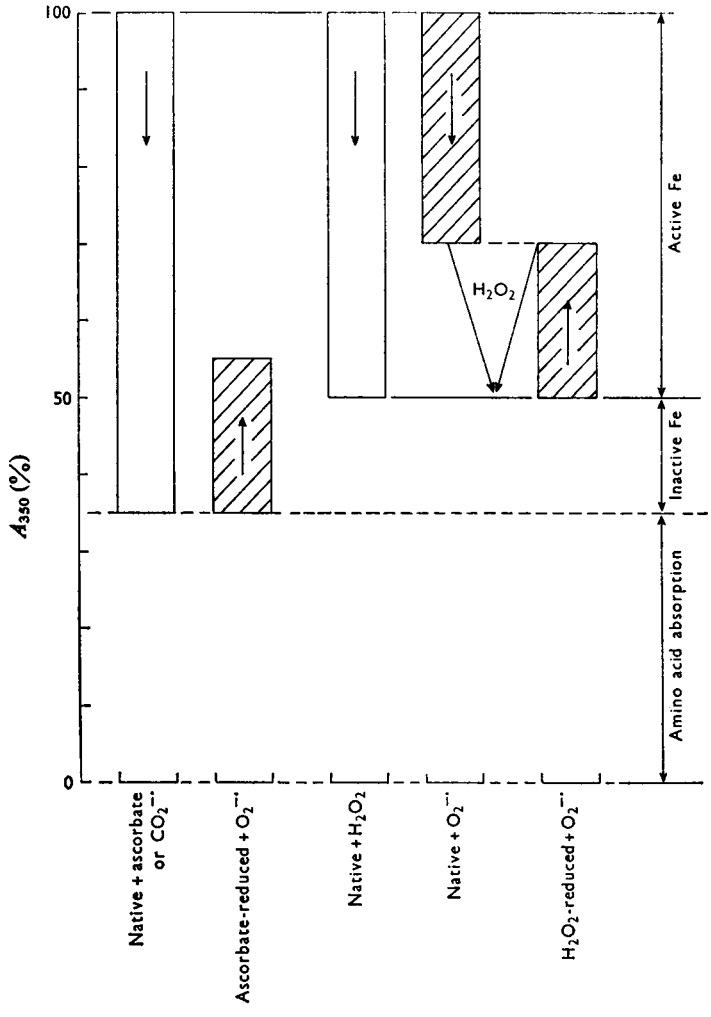

Fig. 10. *Data from optical experiments: effects of substrate and of reducing agents on* A_{350}

storage indicates that in both cases the remaining signal amounts to about 30% of that originally present; this method of comparison is only approximate and it is clear that the $g = 4.3$ signal is sharper in the spectrum of the stored sample (Figs. 3*a* and 3*c*). Asada *et al.* (1975) report a similar increase in a $g = 4.3$ signal on storage of Fe-superoxide dismutase isolated from *Plectonema boryanum*. This was attributed to denatured enzyme, although in our case storage of the sample resulted in no loss of specific activity.

A possible interpretation of the results is that the signal at $g = 4.3$ is due to non-specifically bound iron and it is the absorption of this iron which is responsible for the different extents of bleaching observed with different reagents. For instance, ascorbate and CO_2^- are able to reduce the non-specifically bound iron, whereas H_2O_2 is not capable of so doing (see Fig. 10). The presence of such iron 'impurities' in biological materials, characterized by a $g = 4.3$ e.p.r. signal, has been reported elsewhere (see Beinert & Palmer, 1965). In view of our observation of no change in activity on storage, we must conclude that the change in the $g = 4.3$ signal is associated with only the non-specifically bound iron, which appears to be catalytically inactive.

Inactivation by H_2O_2 is accompanied by an increase in A_{350} (Figs. 5 and 7), but it seems improbable that this is due to re-oxidation of the active iron to some inactive ferric form, since no new e.p.r.

signals were seen (cf. bovine Cu-superoxide dismutase; Bray *et al.*, 1974). Hachimori *et al.* (1964) have shown that H_2O_2 reacts with aromatic amino acids, either in free solution or as residues in proteins (e.g. lysozyme) to yield products absorbing in the 300–350nm region. Such a process may be occurring here. However, the rate of inactivation is slow compared with the rate of reduction by peroxide and any products of inactivation absorbing at 350nm did not interfere with kinetic observations at this wavelength.

The results of experiments performed under turnover conditions suggest catalytic involvement of only that iron which has an absorbance at 350nm and is bleachable by H_2O_2. This functional iron centre is alternately oxidized and reduced by substrate (Figs. 6 and 8).

$$\text{Fe(III)–enzyme} + O_2^{\cdot-} \rightarrow \text{Fe(II)–enzyme} + O_2 \quad (1)$$

$$\text{Fe(II)–enzyme} + O_2^{\cdot-} + 2H^+ \rightarrow \text{Fe(III)–enzyme} + H_2O_2 \quad (2)$$

As with the bovine erythrocyte enzyme, the rate of oxidation or reduction of the metal centre (Fig. 9) is the same as the rate of disappearance of substrate. Thus catalysis is a single-step electron-transfer process.

The decrease (approx. 30% of the total absorbance) in A_{350} observed when the native enzyme is subjected to turnover concentrations of $O_2^{\cdot-}$ is greater than the corresponding increase (approx. 20%) seen when the reduced enzyme is treated similarly, despite the equality of the rate constants for the two processes. The latter change is identical whether ascorbate or H_2O_2 is used as reducing agent, indicating that the different effects observed for the two reducing agents (by using optical or e.p.r. spectrometry) concern an iron species not involved in catalysis. The consistent difference between the percentage change in absorbance occurring in going from native enzyme to enzyme at turnover and that in going from reduced enzyme to enzyme at turnover is reminiscent of a similar, but smaller, effect observed for some preparations of bovine erythrocuprein (Fielden *et al.*, 1974). The effect for the Fe-superoxide dismutase is larger, since only approx. 65% of the A_{350} is attributable to the Fe(III), whereas the A_{680} in bovine erythrocuprein is wholly attributable to Cu(II) centres.

It is to be noted that at high enzyme and low substrate concentrations (Fig. 9) the total absorbance changes observed at 350nm are different depending on whether native or reduced enzyme is being studied. Under these conditions, turnover conditions are not attained (since only a small fraction of the total enzyme reacts with substrate), although the relative absorbance changes for the two cases are again in the ratio 3:2. By using the known $O_2^{\cdot-}$ concentration it is possible to estimate an extinction coefficient of about 1700 litre·mol^{-1}·cm^{-1} at 350nm for the species changing from the native state and about 1200 litre·mol^{-1}·cm^{-1} for the species changing from the reduced state. In several of the assays carried out at 250nm a small permanent net bleaching was seen after the disappearance of substrate; no residual absorption was seen on subsequent irradiation of the same sample.

It may be that the so-called 'inactive' iron in the protein is able to participate in electron transfer with the substrate, although by a non-catalytic mechanism. Alternatively, the similarity of the results to those obtained with bovine erythrocuprein suggest that a more general explanation may be applicable. Further studies are necessary to see whether the effects are due to some conformational difference (around the active iron) between the native enzyme and the ferric enzyme generated by the reaction of substrate with ferrous enzyme [such an effect leading to different extinction coefficients at 350nm for the Fe(III) forms] or whether the non-catalytic iron is involved. Rapid-freeze e.p.r. experiments to study both the exact time-course of peroxide reduction and inactivation, and to investigate the changes in e.p.r. spectra on subjecting the enzyme to turnover concentrations of $O_2^{\cdot-}$, will help to clarify the mechanism more exactly.

Conclusions

The simplest interpretation of the data obtained is that the isolated dismutase contains 1g-atom of catalytic iron/mol and a variable amount of non-catalytic iron. The catalytic centre has an e.p.r. spectrum characteristic of high-spin ferric iron in a nearly rhombically symmetric environment (see Puget & Michelson, 1974). Comparison of published data (Yost & Fridovich, 1973; Asada *et al.*, 1975; Misra & Keele, 1975; Yamakura, 1976) shows that Fe-superoxide dismutases that have been isolated by a method involving exposure to low pH (approx. 5.5) contain 1 iron atom per molecule, whereas those isolated by procedures involving exposure to conditions of pH no lower than about 7 contain up to two iron atoms per molecule. In particular, the Fe-superoxide dismutase from *Plectonema boryanum* was found to contain two iron atoms per molecule by Asada *et al.* (1975), but only one iron atom per molecule by Misra & Keele (1975). The specific activity found in the former case was only 1.3 times that found in the latter. The results of Yamakura (1976) suggest that the native enzyme from *Pseudomonas ovalis* is truly a dismutase with two iron atoms per molecule. In contrast, our results indicate that, for the *P. leiognathi* enzyme as isolated, the second iron atom is catalytically inactive. In the truly native state the enzyme may or may not contain two catalytically active iron centres per molecule.

It is hoped that a further comparative study of various isolations of Fe-superoxide dismutase from *P. leiognathi* with different iron contents will reveal the exact nature of both iron atoms and particularly whether both were originally catalytically active.

We thank Dr. R. C. Bray and Dr. D. J. Lowe of the University of Sussex for their help with e.p.r. measurements, and Dr. R. A. Fox for much valuable discussion. The work was supported by grants to the Division of Physics, Institute of Cancer Research, from the Medical Research Council and the Cancer Research Campaign, and by grants to the Service de Biochimie-Physique, Institut de Biologie Physico-Chimique, from the Centre National de la Recherche Scientifique (E.R. no. 103) the Délègation à la Recherche Scientifique et Technique (Contrat no. 75 70 196) and the Fondation pour la Recherche Médicale Française.

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