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The Interaction of Bovine Erythrocyte Superoxide Dismutase with Hydrogen Peroxide: Inactivation of the Enzyme†

Ellen K. Hodgson and Irwin Fridovich*

ABSTRACT: Bovine erythrocyte superoxide dismutase was slowly and irreversibly inactivated by hydrogen peroxide. The rate of this inactivation was directly dependent upon the concentrations of both H_2O_2 and of enzyme, and its second-order rate constant at pH 10.0 and 25° was 6.7 M^{-1} sec^{-1}. Inactivation was preceded by a bleaching due to rapid reduction of Cu^{2+} on the enzyme, and following this there was a gradual reappearance of a new absorption in the visible region, which was coincident with the loss of catalytic activity. Inactivation of the enzyme was pH-dependent and indicated an essential ionization whose pK_a was approximately 10.2. Replacement of H_2O by D_2O raised this pK_a but did not diminish the catalytic activity of superoxide dismutase, measured at pH 10.0. Several compounds, including xanthine, urate, formate, and azide, protected the enzyme against inactivation by H_2O_2. Alcohols and benzoate, which scavenge hydroxyl radical, did not protect. Compounds with special affinity for singlet oxygen were similarly ineffective. The data were interpreted in terms of the reduction of the enzyme-bound Cu^{2+} to Cu^+, by H_2O_2, followed by a Fenton's type reaction of the Cu^+ with additional H_2O_2. This would generate Cu^{2+}–OH· or its ionized equivalent, Cu^{2+}–$O\cdot^-$, which could then oxidatively attack an adjacent histidine and thus inactivate the enzyme. Compounds which protected the enzyme could have done so by reacting with the bound oxidant, in competition with the adjacent histidine.

Superoxide dismutases catalyze the reaction $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. By thus scavenging O_2^- they serve to protect respiring cells against its deleterious reactivities.

The superoxide dismutase of bovine erythrocytes is characteristic of the corresponding enzymes found in the cytosols of eukaryotes and it is the most thoroughly studied of these enzymes (Fridovich, 1974, 1975). Its molecular weight is 32000 and it is made up of two identical subunits, each of which contains one Cu^{2+} and one Zn^{2+}. X-ray diffraction analysis has shown (Richardson et al., 1975) that the Cu^{2+} and Zn^{2+} are in close proximity, as was predicted by Fee and Gaber (1972). They are, in fact, joined by a common ligand, which is the imidazole ring of histidine-61. In addi-

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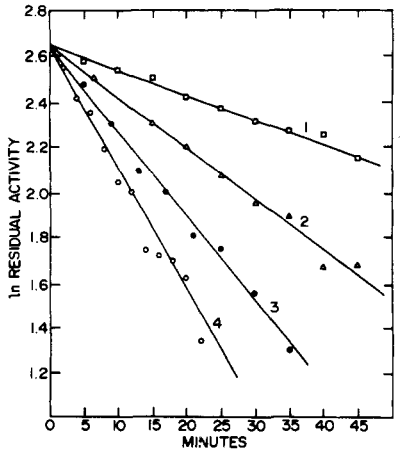


FIGURE 1: Inactivation of the enzyme as a function of the concentration of H_2O_2. Bovine erythrocyte superoxide dismutase, at 4.1×10^{-7} M in 0.05 M sodium pyrophosphate at pH 10.0 and 25°, was exposed to H_2O_2 and, at intervals, aliquots were removed and assayed for residual enzymatic activity. The initial concentrations of H_2O_2 were: line 1 = 4.2×10^{-5} M, line 2 = 8.5×10^{-5} M, line 3 = 12.8×10^{-5} M, and line 4 = 17×10^{-5} M. Plotted on the ordinate is the logarithm of the concentration of active enzyme remaining at the indicated times after addition of H_2O_2.

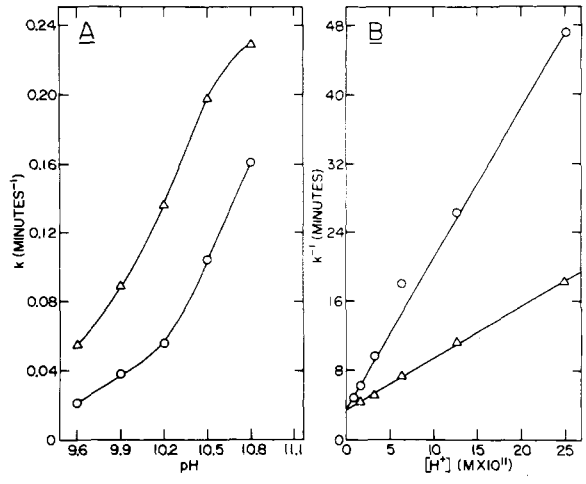


FIGURE 2: The inactivation of superoxide dismutase by H_2O_2 as a Function of pH, in H_2O and in D_2O. In A the rate of inactivation of superoxide dismutase, initially at 3.8×10^{-7} M by 1.28×10^{-4} M H_2O_2, was assayed, as a function of pH, as described in the legend of Figure 1. The measurements were then repeated in 99.7% D_2O. The pseudo-first-order rate constants determined from the slopes of lines such as those presented in Figure 1 are plotted here as a function of pH. In B the reciprocal of the rate constants is plotted as a function of the hydrogen ion concentration.

tion to the bridging group, the Cu^{2+} is ligated to the imidazole rings of three other histidine residues, while the Zn^{2+} is similarly bonded to two other histidines and to one aspartyl residue (Richardson et al., 1975). The copper alternates between the cupric and cuprous states, during the catalytic cycle.

The reversal of the superoxide dismutase reaction has been demonstrated. Thus, with tetranitromethane as a scavenger of O_2^-, the enzyme was seen to produce O_2^- from $H_2O_2 + O_2$ (Hodgson and Fridovich, 1973). This reversal implied the following reactions, the second of which was certainly rate limiting:

$$\text{Enz-Cu}^{2+} + H_2O_2 \rightleftarrows \text{Enz-Cu}^{+} + 2H^+ + O_2^-$$

and

$$\text{Enz-Cu}^{+} + O_2 \rightleftarrows \text{Enz-Cu}^{2+} + O_2^-$$

The reduction of superoxide dismutase by H_2O_2 has indeed been reported (Symonyan and Nalbandyan, 1972; Rotilio et al., 1972c; Klug et al., 1973; Fielden et al., 1973; Bray et al., 1974). It is a rapid process and is followed by a slower irreversible inactivation of the enzyme (Symonyan and Nalbandyan, 1972; Klug et al., 1973; Fielden et al., 1973; Bray et al., 1974; Beauchamp and Fridovich, 1973). One histidine residue per subunit is reportedly destroyed during this inactivation by H_2O_2 (Bray et al., 1974) but the mechanism of this reaction has not yet been explored.

We now report studies of the inactivation of bovine erythrocyte superoxide dismutase by H_2O_2. The ability of a variety of compounds to protect against this inactivation is also described. The following paper (Hodgson and Fridovich, 1975) describes the chemiluminescence which accompanies this inactivation and the peroxidative activity of superoxide dismutase. A mechanism is proposed to account for the data.

Materials and Methods

Superoxide dismutase was prepared from bovine erythrocytes and was assayed as previously described (McCord and Fridovich, 1969) but with the modification that the assays were performed at pH 10.0. Xanthine oxidase was prepared from cream by a procedure which avoids proteolysis (Waud et al., 1975). Hydrogen peroxide of high purity and at a concentration of at least 98% was kindly provided by Dr. Peter Smith, Department of Chemistry, Duke University. D_2O (99.7%) was purchased from the General Dynamics Corporation or from the Bio-Rad Laboratories. The pD of buffers prepared in D_2O was determined with the glass electrode by applying a correction of +0.4 pH unit, as suggested by Lumry et al. (1951). Rates of inactivation were determined by adding superoxide dismutase and H_2O_2, in that order, to buffered reaction mixtures at 25°. At intervals thereafter, aliquots were withdrawn for assay of residual superoxide dismutase activity. The inactivation reaction was stopped by the large dilution (at least 300-fold) inherent in the activity assay. Spectrophotometric assays of reaction rates were performed on a Gilford Model 2000. The absorption spectrum of the peroxide-inactivated enzyme was recorded on an Aminco DW-2; while the spectrum of the azide-treated superoxide dismutase was taken with a Cary Model 15.

Results

Kinetics. The rate of inactivation was a second-order process depending upon the concentrations of both H_2O_2 and of the enzyme. Figure 1 presents the inactivation of 4×10^{-7} M enzyme by H_2O_2, whose concentration was varied from 0.42 to 1.7×10^{-4} M, all at pH 10.0 and at 25°. The slopes of the lines in Figure 1 are directly proportional to the concentration of H_2O_2. Similarly, when H_2O_2 was held constant at 8×10^{-5} M, while the enzyme was varied from 1.9 to 16.9×10^{-7} M, the rate of activity loss varied directly with the concentration of enzyme. The second-order rate constant for this inactivation at pH 10.0 and at 25° was approximately 6.7 M^{-1} sec^{-1}.

Effects of pH. Raising the pH increased the rate of inactivation. Figure 2A presents the pseudo-first-order rate constants for the inactivation of 3.8×10^{-7} M enzyme by 1.28×10^{-4} M H_2O_2 in carbonate buffers whose pH was varied

Table I: The Effects of Various Compounds on the Inactivation of Superoxide Dismutase by Hydrogen Peroxide.[a]

Compound	Concn (M)	Protection (%)
Benzoate[b]	0.025	0
tert-Butyl alcohol[b]	0.50	0
Ethanol[b]	30%	0
Formate[b]	0.05	85
Urate[b]	0.0025	95
Xanthine[b]	0.01	100
Histidine[b]	0.50	70
Triethylamine[b]	1%	23
Azide[c]	0.01	96
D_2O[d]	100%	50
Diphenylisobenzofuran[e]	1×10^{-5}	0
Bilirubin[e]	5×10^{-5}	0
Ferrocytochrome *c*[e]	5×10^{-5}	0

[a] The ability of these compounds to prevent the inactivation of superoxide dismutase by peroxide in 0.05 M carbonate buffer containing 1×10^{-4} M EDTA, at pH 10.0 and 25°, was determined as described in Materials and Methods. The concentrations of enzyme and peroxide used is indicated for each experiment. [b] Enzyme concentration, 3.4×10^{-7} M; H_2O_2 concentration, 1.8×10^{-4} M. [c] Enzyme concentration, 1.6×10^{-6} M; H_2O_2 concentration, 8.7×10^{-5} M. [d] Enzyme concentration, 3.8×10^{-7} M; H_2O_2 concentration, 1.28×10^{-4} M. [e] Enzyme concentration, 9.1×10^{-7} M; H_2O_2 concentration, 1.1×10^{-4} M.

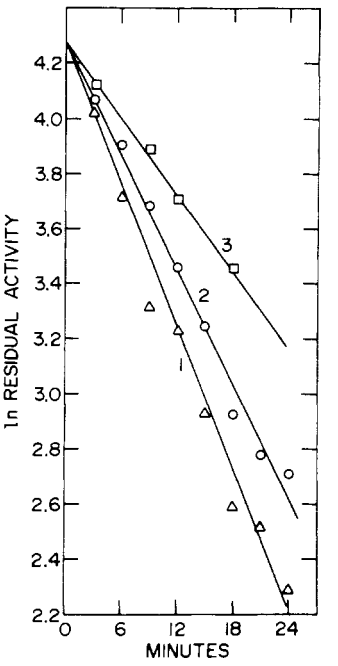


FIGURE 3: The effect of oxygen on the inactivation of superoxide dismutase by H_2O_2. Enzyme at 5.3×10^{-7} M in 0.05 M sodium carbonate–1×10^{-4} M EDTA at pH 10.2 and at 25°, was equilibrated with air (line 2), nitrogen (line 1), or with 100% oxygen (line 3) prior to the addition of 7×10^{-5} M H_2O_2. Samples of the reaction mixture were assayed for residual activity at intervals after the addition of H_2O_2, as described in the legend of Figure 1.

from 9.6 to 10.8. Under these conditions the half-life of the enzyme was 27 min at pH 9.6 and 3 min at pH 10.8. If this effect of pH is due to the ionization of some critical species, it should be possible to calculate an apparent pK_a from a plot of the reciprocal of the rate constant vs. the hydrogen ion concentration (Dixon and Webb, 1964). Such a plot is shown in Figure 2B and from these data, the apparent pK_a involved here is determined to be about 10.2.

Protection. If the inactivation of the enzyme by H_2O_2 is associated with destruction of a single histidine residue per subunit, as has been reported (Bray et al., 1974), then it seemed likely that the attack on that residue was an oxidative one. Since H_2O_2 itself does not attack histidine, it is likely that interaction of H_2O_2 with the active center of the enzyme must generate a potent oxidant. In that case exogenous compounds capable of reacting with that oxidant might compete with the endogenous electron donor and thus protect the enzyme. Table I summarizes the effects of a variety of compounds. Formate, urate, xanthine, histidine, and azide all protected the enzyme against inactivation by H_2O_2; while ethanol, *tert*-butyl alcohol, benzoate, diphenylisobenzofuran, bilirubin, and ferrocytochrome *c* were without effect. Triethylamine had a weak protective effect. In control experiments it was shown that these compounds did not noticeably react directly with H_2O_2. Since benzoate and the alcohols are effective scavengers of OH· (Dorfman and Adams, 1973) we conclude that *free* OH· was not the reactive oxidant responsible for inactivation of the enzyme. Diphenylisobenzofuran and bilirubin are effective scavengers of singlet oxygen (Merkel et al., 1972; Wilson, 1966; Bonnett and Stewart, 1972) and their failure to protect thus excludes this excited form of oxygen from further consideration. That singlet oxygen was not responsible for the inactivation was also supported by the data shown in Figure 2A. Replacement of H_2O by D_2O has been reported to increase the lifetime of singlet oxygen, so that its effectiveness as an oxidant is greater in D_2O than in H_2O (Nilsson and Kearns, 1973). If singlet were responsible for the inactivation, the rates of inactivation in D_2O would be expected to be greater than those in H_2O at the same pH. As shown in Figure 2A, however, replacement of H_2O by D_2O actually had a protective effect on the inactivation process. Since deuteration is known to raise the pK_a of acids (La Mer and Chittum, 1936), this protective effect of D_2O could be explained by assuming that the pK_a for the critical ionization involved in the inactivation process had been increased. From the data shown in Figure 2B, the apparent pK_a for this species in D_2O is calculated to be about 10.7.

The effect of replacement of H_2O by D_2O, upon the catalytic activity of the superoxide dismutase, was also investigated. The activity of the enzyme was as great in D_2O, at pH 10.0, as it was in H_2O at this pH. This was assessed by observing the ability of superoxide dismutase to inhibit the O_2^--dependent reduction of cytochrome *c* by xanthine oxidase acting upon xanthine, in the presence of oxygen (McCord and Fridovich, 1969). The situation is very complex in that D_2O could conceivably affect the O_2^--generating system, i.e., the xanthine oxidase reaction; the O_2^--detecting system, i.e., the reaction of O_2^- with cytochrome *c*; or the O_2^--scavenging enzyme, i.e., the superoxide dismutase. The first effect was obviated by adjusting the concentration of xanthine oxidase, such that the uninhibited rate of reduction of cytochrome *c* was the same in the D_2O as it was in the H_2O system. Thus the rate of production of O_2^- was then the same in both solvents. The ability of superoxide dismutase to inhibit the reduction of cytochrome *c*, equally well in the D_2O as in the H_2O systems, means that the effect of this solvent change on the reaction of O_2^- with cytochrome *c* was the same as on its reaction with superoxide dismutase.

Oxygen. The effect of oxygen upon the inactivation was also investigated. If the reduced form of the enzyme is an

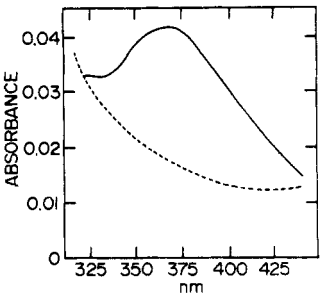


FIGURE 4: The effect of azide on the near-ultraviolet absorbance by superoxide dismutase. The absorption spectrum of the enzyme at 3.8×10^{-6} *M* in 0.05 *M* sodium carbonate–1 $\times 10^{-4}$ *M* EDTA at pH 10.2 and at 25° was recorded in the absence (- - -) and in the presence (—) of 0.01 *M* sodium azide. Cuvettes with a path length of 10 cm were used to obtain these spectra.

obligatory intermediate in the inactivation reaction, then oxygen, by slowly reoxidizing the cuprous enzyme, should afford some protection. As shown in Figure 3 oxygen did have a protective effect and the rate of inactivation in the absence of oxygen was almost twice the rate obtained in 100% O_2.

Azide. At a concentration of 0.01 *M*, azide almost completely protected the enzyme against inactivation by H_2O_2. Azide does complex with the metals at the active site of superoxide dismutase (Fee and Gaber, 1972). Figure 4 illustrates the absorption band at 373 nm which appeared when the enzyme was exposed to 0.01 *M* azide, under the conditions used in these studies. Such absorption is characteristic of the cupric–azide system (Hatfield and Wyman, 1969). Azide at 0.01 *M* did not at all inhibit the catalytic activity of superoxide dismutase and it did not interfere with the rapid reduction of this enzyme by H_2O_2. Its ability to completely protect against the inactivation by H_2O_2 probably relates to its activity as an electron donor. Thus azide reacts with OH· at a rate of 1×10^{10} M^{-1} sec^{-1} (Dorfman and Adams, 1973).

Spectral Changes during Inactivation. The action of 1 m*M* H_2O_2 upon superoxide dismutase was clearly biphasic. As shown in Figure 5 there was a rapid bleaching of absorption in the visible and near-ultraviolet, which was followed by the slow appearance of a new absorption spectrum. The spectrum of the inactivated enzyme shows that the absorption due to the Cu^{2+} is markedly different from that of the native enzyme, indicating that the integrity of the active site has in some way been disrupted during the inactivation process. The absorbance at 440 nm reached half of its maximum value in approximately 4 min. Under these conditions the enzyme was also 50% inactivated in this time interval. The spectral changes shown in Figure 5 and the inactivation of the enzyme were thus coincident. It has been reported that H_2O_2 does not react with the copper of superoxide dismutase in the presence of cyanide (Rotilio et al., 1972b). Figure 6 demonstrates that this is not quite the case. Thus the absorption spectrum of the enzyme-cyanide complex was gradually bleached by 1 m*M* H_2O_2. It was certainly true that cyanide prevented the rapid reduction of the enzyme-bound Cu^{2+} by H_2O_2.

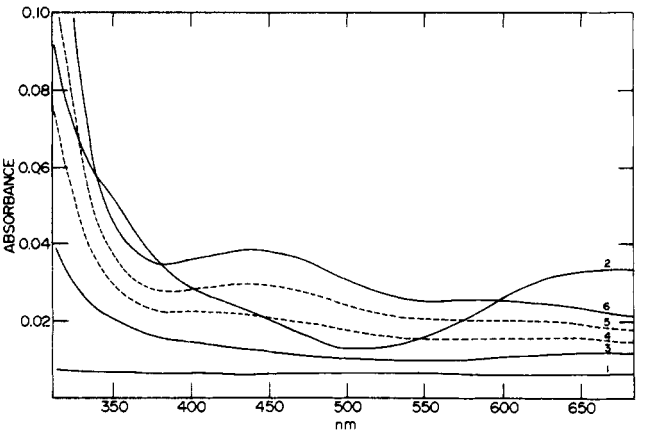


FIGURE 5: The effects of H_2O_2 upon the absorption spectrum of bovine erythrocyte superoxide dismutase. Enzyme at 9.4×10^{-5} *M* in 0.02 *M* sodium pyrophosphate at pH 9.0 and 25° was treated with 1.0×10^{-3} *M* H_2O_2 and optical spectra were recorded at intervals. Line 1 = base line with buffer in both reference and sample cuvettes; line 2 = enzyme prior to the addition of H_2O_2; line 3 = enzyme within 1 min after addition of H_2O_2; line 4 = enzyme at 4 min after exposure to H_2O_2; line 5 = enzyme at 10 min after exposure to H_2O_2; line 6 = enzyme at 30 or 60 min after addition of H_2O_2.

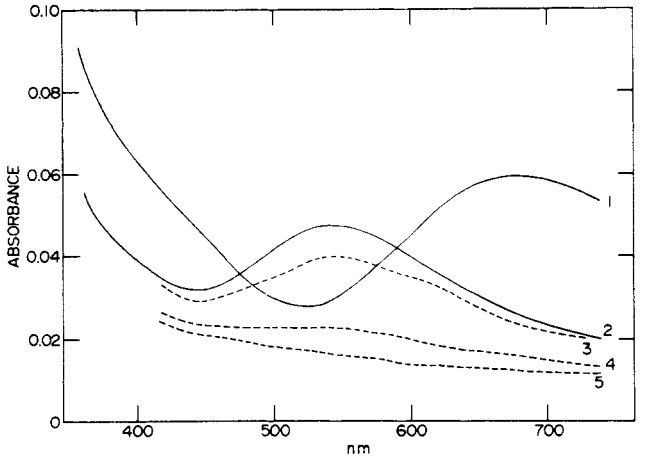


FIGURE 6: The effects of H_2O_2 upon the absorption spectrum of the enzyme in the presence of cyanide. Enzyme at 1.67×10^{-4} *M* in 0.05 *M* potassium phosphate–1 $\times 10^{-4}$ *M* EDTA at pH 7.8 and 25° was treated with 33 m*M* cyanide and then with 1.0 m*M* H_2O_2. Line 1 is the absorption spectrum of enzyme alone while line 2 presents the spectrum after addition of the cyanide. Lines 3, 4, and 5 record the absorption spectra of the enzyme–cyanide complex as soon as possible after addition of H_2O_2, line 3; approximately 5 min later, line 4; and, either 7.5 or 12.5 min later, line 5.

Discussion

H_2O_2 caused a rapid and reversible reduction of the enzyme-bound Cu^{2+} which was associated with a bleaching of its absorption in the visible and near-ultraviolet. There followed a slower process during which a new visible absorption spectrum was generated and the enzyme was irreversibly inactivated. These changes are accommodated by the following reactions:

$$\text{Enz-Cu}^{2+} + H_2O_2 \rightleftharpoons \text{Enz-Cu}^{+} + O_2^{-} + 2H^{+} \quad \text{(a)}$$

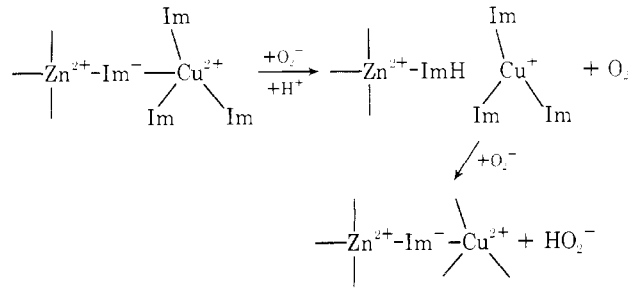
$$\text{Enz-Cu}^{+} + H_2O_2 \rightleftharpoons \text{Enz-Cu}^{2+}\text{-OH}\cdot + OH^{-} \quad \text{(b)}$$

$$\text{Enz-Cu}^{2+}\text{-OH}\cdot + \text{ImH} \rightarrow \text{Enz-Cu}^{2+} + \text{Im}\cdot + H_2O \quad \text{(c)}$$

Reaction b is analogous to the Fenton reaction and it generates a bound hydroxyl radical or its ionized equivalent, i.e., $\text{Enz-Cu}^{2+}\text{-O}\cdot^{-}$. This bound oxidant then attacks an adjacent imidazole (ImH) as in reaction c. We assume that the oxidant generated in reaction b remains bound to the metal because it did not react with alcohols or benzoate, which are known to react with free hydroxyl radical (Dorfman and

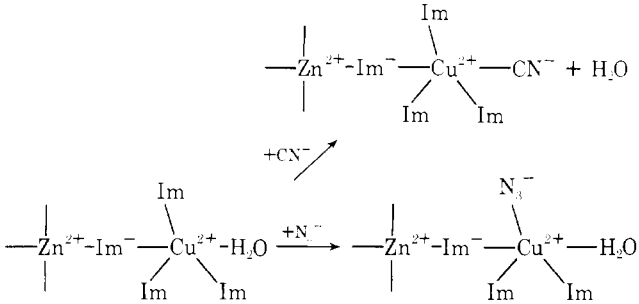
Adams, 1973) and in order to account for the specificity of its attack upon histidine. Exogenous electron donors, such as formate, azide, and urate, which could gain access to the active site, could compete with the endogenous donor (ImH) in reaction c and could thus prevent inactivation of the enzyme. Oxygen protects by competing with H_2O_2 for reaction with the reduced enzyme, as in (b).

The activity of superoxide dismutase at pH 10.0, as assayed in terms of its ability to inhibit the superoxide mediated reduction of cytochrome *c* by the xanthine oxidase system, was not affected by replacing H_2O by D_2O. Minimally this means that the effects of D_2O on the reaction of O_2^- with superoxide dismutase and with cytochrome *c* were identical. It could mean that D_2O has no effect on the rate of either reaction. In that case we could conclude that proton transfer is not rate limiting in the action of superoxide dismutase. This would be in accord with the lack of a pH effect on the catalytic rate constant, in the range pH 5.0–10.0 (Klug et al., 1972; Rotilio et al., 1972a). Since the overall dismutation reaction does require two protons, i.e., $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, and since the enzyme-catalyzed dismutation proceeds at a rate close to the diffusion limit, this lack of a deuterium isotope effect and of a pH effect requires some comment. It may be that the product of the enzymatic dismutation is HO_2^- rather than H_2O_2 so that only one proton need be of concern during a single turn of the catalytic cycle. Furthermore the bridging imidazole may function as a proton carrier, to facilitate protonation of that O_2^- which interacts with the cuprous enzyme. This may be represented as follows:



Here, reduction of the Cu^{2+} by the first O_2^- is accompanied by release of the bridging imidazole, which becomes protonated. When the reduced enzyme then collides with the next O_2^-, the proton on the bridging ligand and the electron on copper are both transferred to it, generating HO_2^- and restoring the enzyme to its original condition.

The contrasting effects of azide and cyanide deserve comment. Thus, both bind to copper on the enzyme, but the cyanide inhibits the enzyme and interferes with its reduction by H_2O_2, whereas azide does not. We tentatively suggest that azide occupies a position normally taken by one of the imidazole ligands to the copper whereas cyanide ligates to that position which is normally available for H_2O, O_2^-, or H_2O_2. Thus:



In this situation the binding of cyanide blocks the position available for interaction with O_2^- or H_2O_2 and thus inhibits; whereas the binding of azide merely substitutes a new nitrogenous ligand for one of the imidazoles which normally constitutes a stable aspect of the ligand field of the copper. Azide binding would therefore not interfere with activity.

Irreversible inactivation of an essential enzyme by one of its products could pose a serious problem. There is, however, no measurable inactivation of superoxide dismutase in vivo. Thus the level of superoxide dismutase in mature human erythrocytes does not diminish during the lifespan of these cells (J. M. McCord, personal communication). This is vivo stability of superoxide dismutase and its irreversible inactivation by H_2O_2, as studied in vitro, are readily reconciled. Thus the level of H_2O_2 in vivo must at all times be several orders of magnitude lower than the 10^{-3}–10^{-5} *M* range used in these studies. In addition, the pH inside cells is far below the alkaline range in which the inactivation occurs rapidly. Furthermore, compounds which can protect the enzyme against this inactivation must be plentiful within cells. Finally since the inactivation is dependent upon a reaction between the reduced enzyme and H_2O_2, we must expect that O_2^- would itself prevent the inactivation by competing with H_2O_2.

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The Interaction of Bovine Erythrocyte Superoxide Dismutase with Hydrogen Peroxide: Chemiluminescence and Peroxidation†

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ABSTRACT: Reaction of bovine erythrocyte superoxide dismutase with H_2O_2 was accompanied by a luminescence whose intensity was a function of the concentration of H_2O_2 and whose duration was coincident with the inactivation of the enzyme by this reagent. Oxygen, which protected against inactivation, also diminished the luminescence. Several other compounds which prevented the inactivation by H_2O_2 also modified the luminescence. Thus urate, formate, and triethylamine inhibited luminescence whereas imidazole and xanthine augmented it. These seemingly contrary effects can be explained by assuming that the compounds which protected the enzyme were peroxidized in competition with the sensitive group on the enzyme. The luminescence arises because that group on the enzyme was oxidized to a product in an electronically excited state, which could return to the ground state by emitting light. Imidazole and xanthine gave electronically excited products whose quantum efficiency was greater than that of the group on the enzyme, whereas urate, formate, and triethylamine gave products with much lower luminescent efficiencies. This superoxide dismutase could catalyze the peroxidation of a wide range of compounds, including ferrocytochrome *c*, luminol, diphenylisobenzofuran, dianisidine, and linoleic acid. In control experiments, boiled enzyme was inactive. This peroxidative activity can lead to unexpected effects when superoxide dismutase is added to H_2O_2-producing systems, as a probe for the involvement of O_2^-. Several examples from the literature are cited to illustrate the misinterpretations which this previously unrecognized peroxidative activity can generate.

The preceding paper (Hodgson and Fridovich, 1975) describes the inactivation of the copper- and zinc-containing superoxide dismutase by H_2O_2. A mechanism was proposed in which H_2O_2 first reduces the Cu^{2+} and then reacts with the Cu^+, so generated, to give a potent oxidant, which remains bound to the metal. This bound oxidant, in turn, attacks an adjacent histidine residue and so destroys the integrity of the catalytic site. In the course of these studies we observed a chemiluminescence during the reaction of H_2O_2 with the enzyme and noted additionally that superoxide dismutase can act as a peroxidase. Since superoxide dismutase is often used as a test for O_2^- in oxidative and in chemiluminescent reactions and since H_2O_2 is often a product of such reactions, this peroxidative action of superoxide dismutase can, if not appreciated, lead to misinterpretation of the observations. The chemiluminescence and the peroxidations which accompany the interaction of superoxide dismutase with H_2O_2 were therefore studied both to gain understanding of their mechanisms and to expose the dangers of uncritically applying superoxide dismutase as a test for O_2^- in peroxide-generating systems.

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Materials and Methods

The manganese-containing superoxide dismutase was prepared from *Escherichia coli* as previously described (Keele et al., 1970). Luminescent intensity was measured with the photometer described by Mitchell and Hastings (1971) which was calibrated with the stable standard light source described by Hastings and Weber (1963). Some of the measurements were made with a Nuclear Chicago Mark I liquid scintillation counter with the coincidence circuit inactivated. Rates of change of absorbance were recorded with a Gilford Model 2000. The absorption spectrum of cytochrome *c* was recorded with an Aminco DW-2 whereas the spectrum of linoleic acids was taken with a Cary Model 15. The bleaching of diphenylisobenzofuran was followed at 410 nm (Merkel et al., 1972). The peroxidation of dianisidine was followed at 460 nm (Fridovich, 1963). All other materials and procedures were exactly as described in the preceding paper (Hodgson and Fridovich, 1975).

Results

Chemiluminescence. Admixture of superoxide dismutase