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Effect of Hydrogen Peroxide on the Iron-Containing Superoxide Dismutase of *Escherichia coli*†

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ABSTRACT: The iron-containing superoxide dismutase from *Escherichia coli* is inactivated by H_2O_2 to a limit of ~90%. When corrected for the H_2O_2-resistant portion, this inactivation was first order with respect to residual activity and exhibited a pseudo-first-order rate constant of 0.066 min^{-1} at 25 °C in 0.24 mM H_2O_2 at pH 7.8. The superoxide dismutase activity remaining after treatment with H_2O_2 differed from the activity of the native enzyme with respect to heat stability, inhibition by azide, and inactivation by light in the presence of rose bengal and by *N*-bromosuccinimide. The native and the H_2O_2-modified enzymes were indistinguishable by electrophoresis on polyacrylamide gels. Inactivation of the enzyme by H_2O_2 was accompanied by loss of tryptophan and some loss of iron, but there was no detectable loss of histidine or of other amino acids. H_2O_2 treatment caused changes in the optical spectrum of the enzyme. Inactivation of the enzyme by H_2O_2 depends upon the iron at the active site. Thus, the apoenzyme and the manganese-substituted enzyme were unaffected by H_2O_2. We conclude that reaction of H_2O_2 with the iron at the active site generates a potent oxidant capable of attacking tryptophan residues. A mechanism is proposed.

Superoxide dismutases containing copper and zinc (McCord & Fridovich, 1969), manganese (Keele et al., 1970), and iron (Yost & Fridovich, 1973) have been isolated and characterized. The manganese and the iron enzymes show a great deal of amino acid sequence (Steinman, 1978; Harris et al., 1980) and structural (Stallings et al., 1984) homology. H_2O_2 inactivates the Cu,Zn and the Fe, but not the Mn, superoxide dismutases SODs[1] (Steinman, 1982). In the case of the Cu,ZnSOD, bleaching of the Cu(II) preceded inactivation, and the rate of inactivation increased with pH in a manner defining a pK_a of ~10.2 (Hodgson & Fridovich, 1975). This effect of pH was subsequently explained in terms of HO_2^- being the active species, rather than H_2O_2 (Blech & Borders, 1983). Inactivation of Cu,ZnSOD by H_2O_2 is associated with the loss of one histidine residue per subunit (Bray et al., 1974).

Inactivation of the FeSOD from *Pseudomonas ovalis* has been correlated with losses of tryptophan, histidine, and cysteine residues (Yamakura, 1984). We have previously

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[1] Abbreviations: FeSOD, iron-containing superoxide dismutase; Cu,ZnSOD, copper- and zinc-containing superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; SDS, sodium dodecyl sulfate; NBS, *N*-bromosuccinimide; TSY, trypticase–soy yeast extract; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Da, dalton(s).

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noted that the FeSOD of *Escherichia coli* is inactivated by H_2O_2 to a limit of 90% and had supposed that the 10% of peroxide-resistant activity might reflect a heterogeneity of the electrophoretically homogeneous preparation (Clare et al., 1984). This unusual situation led us to reexplore the inactivation of the *E. coli* FeSOD by H_2O_2. We now present results indicating that the H_2O_2-resistant activity reflects residual activity of the H_2O_2 modified FeSOD and that H_2O_2 treatment causes losses of iron and tryptophan, but not of histidine. In addition, we present data suggesting that the H_2O_2-resistant activity is substantially different from the native activity with respect to thermostability, sensitivity to sodium azide, and photoinactivation in the presence of rose bengal. We also present evidence that iron is essential for the inactivation by H_2O_2 and propose a mechanism for this inactivation.

Materials and Methods

Escherichia coli (ATCC 29682) was grown aerobically to late log phase in TSY medium at 37 °C and was harvested with a Sharples continuous-flow centrifuge. Cell paste either was used immediately or was stored at −70 °C until needed. Cell paste was slurried into 5 volumes of 50 mM potassium phosphate buffer containing 0.1 mM EDTA at pH 7.8, and cytolysis was achieved by 6-fold passage through a Gaulin 18 M8TA laboratory homogenizer operated at 450 kg/cm^2. FeSOD was isolated from this cell homogenate as previously described (Yost & Fridovich, 1973) with the modifications that 30 mM $MnCl_2$ was used, in place of streptomycin, to precipitate DNA (Britton et al., 1978) and column chromatography over hydroxylapatite C was added as a last fractionation procedure. The final product exhibited a single protien band after electrophoresis in the presence of SDS plus mercaptoethanol, as described by Laemmli (1970). Electrophoresis in the native state (Davis, 1964) demonstrated two bands, one major and one minor, and both were active as demonstrated by staining for SOD activity (Beauchamp & Fridovich, 1971). We have previously noted the existence of electromorphs of the *E. coli* FeSOD (Clare et al., 1984).

Protein concentrations were measured as described by Murphy and Kies (1960) or by measuring A_{280nm} and using $A^{1\%}_{280nm,1cm} = 25.4$ which was based upon dry weight measurements.[2] Treatment of FeSOD with H_2O_2 altered A_{280nm} but did not alter $A_{215nm} - A_{225nm}$. Hence, the concentration of H_2O_2-treated FeSOD was based upon measurements at 215 and 225 nm (Murphy & Kies, 1960), after dialysis to remove H_2O_2. Superoxide dismutase activity was estimated from its ability to compete with ferricytochrome *c* for an enzymic flux of O_2^- (McCord & Fridovich, 1969). Protein bands on polyacrylamide gels were stained with Coomassie Blue R-250 followed by destaining in methanol/acetic acid/water (3:1:9 v/v). Hydrogen peroxide solutions were standardized by using $\epsilon_{240nm} = 43.6\ M^{-1}\ cm^{-1}$ (Hildebrandt & Roots, 1975).

Absorption spectra were recorded with a Shimadzu Model UV-260 recording spectrophotometer. Difference spectra were recorded on a Cary 14 with an attached computer from On-Line Instrument System, Inc.

Metal analyses were performed on a Perkin-Elmer 3030 atomic absorption spectrophotometer equipped with AC-Zeeman effect background correction and with an HGA-600 programmable graphite furnace. The furnace was fitted with a L'vov platform within a pyrolytically coated graphite tube. Samples of 20 μL were introduced with a Perkin-Elmer AS-60 autosampler. Data reported are the average of five measurements ± the standard deviation. Samples were dialyzed exhaustively against changes of 10 g/L Chelex-100, suspended with stirring, in 50 mM potassium phosphate and 0.1 mM EDTA at 4 °C and at pH 7.8. Samples and standards were diluted into 0.2% HNO_3 (AR grade). The dialysate was used as a blank sample. $Mg(NO_3)_2$ was added to all samples to 50 μg/20 μL, as a matrix modifier.

Samples intended for amino acid analysis were dialyzed for 24 h at 25 °C against 1000 volumes of 0.2 M Na_2CO_3, 10 mM EDTA, and 1.0 mM dithiothreitol, at pH 11.2, followed by exhaustive dialysis against glass-distilled water containing 10 g/L Chelex-100. The dialyzed apoprotein was lyophilized and hydrolyzed at 110 °C in 6.0 N HCL and 0.1% phenol, when general amino acid analysis was to be performed, or in 4.0 N methanesulfonic acid, when tryptophan was to be measured (Simpson et al., 1976). In the latter case, tryptamine was not added to the samples prior to hydrolysis.

Inactivation of FeSOD at 0.25 mg/mL in 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.8, was achieved by exposure to 1 mM H_2O_2 at 25 °C in a shaking water bath for 2 h. H_2O_2 was then separated from the enzyme by gel exclusion chromatography over PD-10. The protein-containing effluent peak was dialyzed at 4 °C against several changes of the phosphate/EDTA buffer and was then concentrated to 5 mg/mL by ultrafiltration over an Amicon PM-10 membrane. The modified enzyme so obtained typically exhibited ~10% of the initial specific activity. Exposure to severalfold higher concentrations of H_2O_2 of allowing the exposure to H_2O_2 to proceed beyond 2 h did not further lower the activity of the modified enzyme.

Results

Inactivation of FeSOD by H_2O_2. When native *E. coli* FeSOD was exposed to 0.24 mM H_2O_2 at pH 7.8 and at 26.1 °C, it suffered a progressive loss of activity, to a limit of 90% inactivation, as shown in Figure 1A. This limit was not exceeded even when the concentration of H_2O_2 was raised to 50 mM (data not shown). When corrected for H_2O_2-resistant activity, the inactivation of FeSOD by H_2O_2 was seen to be first order dependent of the residual activity, as shown in Figure 1B. The pseudo-first-order rate constant, calculated from the data in Figure 1B, is 0.066 min^{-1}, and the half-life equals 10.5 min.

Effects of N_3^- on Native and on H_2O_2-Modified FeSOD. If the 10% activity remaining after H_2O_2 treatment were due to residual native enzyme, then it should exhibit the properties of native enzyme. In contrast, if the residual activity reflected H_2O_2-modified FeSOD, then it might well differ from the native enzyme. Since N_3^- inhibits all SODs, but to different degrees (Misra & Fridovich, 1978), we chose to begin our examination of this expectation with N_3^-. Control experiments indicated that N_3^-, up to 25 mM, did not significantly interfere with the xanthine oxidase/cytochrome *c* reaction, used for assaying SOD activity. The data in Figure 2 (line 1) demonstrate that the native FeSOD was much more susceptible to inhibition by N_3^- than was the H_2O_2-treated enzyme (line 2).

Thermal Stability of Native and H_2O_2-Modified FeSODs. Changes in thermal stability often accompany even subtle changes in enzyme structure. The native and the H_2O_2-inactivated enzymes were incubated at 70 °C, and changes in activity as a function of time were measured. The H_2O_2-treated enzyme had been exhaustively dialyzed, to remove H_2O_2, as described under Materials and Methods. As shown in Figure 3, thermal inactivation was a first-order process and was slower ($t_{1/2}$ = 24 min) for the native than for the H_2O_2-modified ($t_{1/2}$ = 12 min) enzyme. Native or H_2O_2-

[2] Dr. Yas Nozaki, unpublished results.

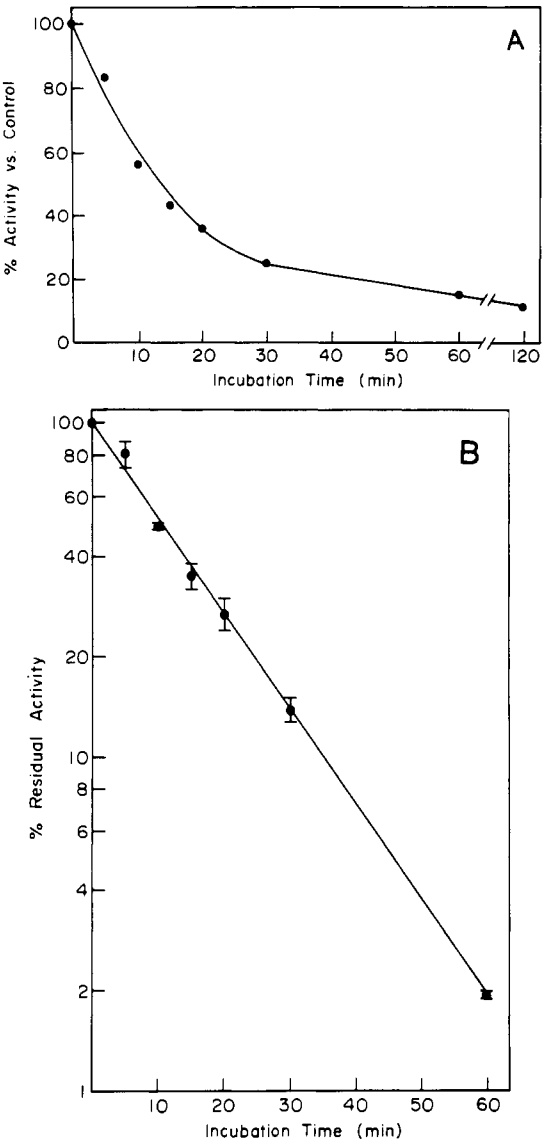

FIGURE 1: Inactivation of native FeSOD by H_2O_2. Reaction mixtures contained 0.25 mg/mL FeSOD, 0.24 mM H_2O_2, 0.1 mM EDTA, and 50 mM potassium phosphate at pH 7.8 and at 26 ± 1 °C. At intervals, aliquots were withdrawn, quenched with 100 units of cold catalase, and then assayed for residual SOD activity. (A) Residual activity is graphed as a function of time. (B) The log of residual activity, corrected for 12.9% H_2O_2-resistant activity, is graphed as a function of time.

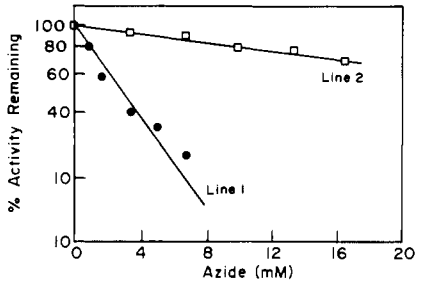

FIGURE 2: Inhibition of native and of H_2O_2-modified FeSODs by azide. Native FeSOD and FeSOD modified by H_2O_2 to 11% residual activity and then dialyzed were assayed as described under Materials and Methods in the presence of a range of concentrations of NaN_3. Line 1 = native FeSOD; line 2 = H_2O_2-modified FeSOD.

treated FeSOD, after inactivation for 12 or 24 min at 70 °C (one or two half-lives, respectively), was analyzed by native gel electrophoresis, as described under Materials and Methods. Thermal inactivation of both proteins at both time points

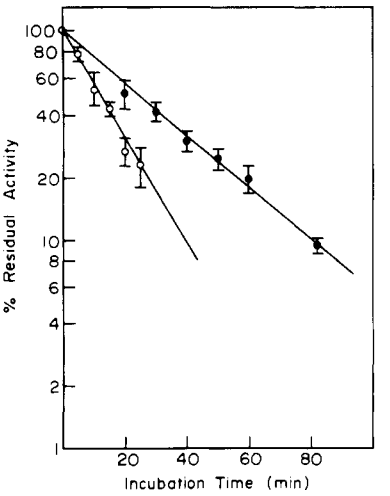

FIGURE 3: Thermal inactivation of native and of H_2O_2-modified FeSODs. Reaction mixtures contained either native FeSOD at 0.54 mg/mL (●) or H_2O_2-modified FeSOD at 5.4 mg/mL (○) in 50 mM potassium phosphate and 0.1 mM EDTA at pH 7.8 and at 70 ± 2 °C. At intervals, aliquots were removed, chilled in an ice bath, and then assayed for residual SOD activity.

resulted in no detectable change in the electrophoretic pattern of the SOD-active bands, suggesting that the remaining SOD activity was due to enzyme that was unaltered during heat treatment. It is conceivable that during the thermal treatment a scrambling of active and inactive subunits might occur, resulting in a "hybrid" enzyme. Such a hybridization might be expected to produce a sublte changes in the electrophoretic mobility of these chimeric proteins. On the basis of the aforementioned data, this possibility is not likely.

Photosensitized Inactivation. FeSOD or H_2O_2-modified FeSOD was exposed to the beam of a 300-W slide projector lamp. The glass reaction vessel was water jacketed, and the temperature was held at 25 ± 1 °C. Rose bengal was used as the photosensitizer. This dye had no effect on enzymic activity in the absence of light. At intervals, 100-μL aliquots of the reaction mixture were removed and stored in the dark on ice, until assayed for residual activity. As shown in Figure 4, photosensitized inactivation was first order dependent of residual activity, and native FeSOD was more rapidly inactivated than was the H_2O_2-modified enzyme.

Inactivation by N-Bromosuccinimide. Native FeSOD at 0.09 mg/mL and H_2O_2-modified FeSOD at 1.02 mg/mL were exposed to *N*-bromosuccinimide for 30 min in 50 mM potassium phosphate and 0.1 mM EDTA, at pH 7.8 and at 25 °C, and residual activity was then measured after appropriate dilution. Control experiments demonstrated that the amount of N-bromosuccinimide which carried over into the assay for SOD activity had no effect on that assay. Under these conditions, native enzyme lost 50% of its activity when exposed to 4.0 mol of *N*-bromosuccinimide/mol of protein, while for the H_2O_2-modified enzyme the corresponding number was 5.3. Moreover, the native enzyme was completely inactivated by 13 mol of NBS/mol of enzyme, whereas the H_2O_2-modified enzyme retained 5% of its initial activity even when exposed to 20 mol of NBS/mol of enzyme. The tendency of NBS to cause oxidiation of tryptophan residues (Spande & Witkop, 1967) and the relative resistance of the H_2O_2-modified enzyme toward this reagent suggested that H_2O_2 modification might have involved modification of tryptophan residues which were then unavailable for reaction with *N*-bromosuccinimide.

Effect of H_2O_2 Treatment on the Optical Spectrum of FeSOD. Modification of FeSOD by H_2O_2 was accompanied by changes in both the UV and the visible regions of its ab-

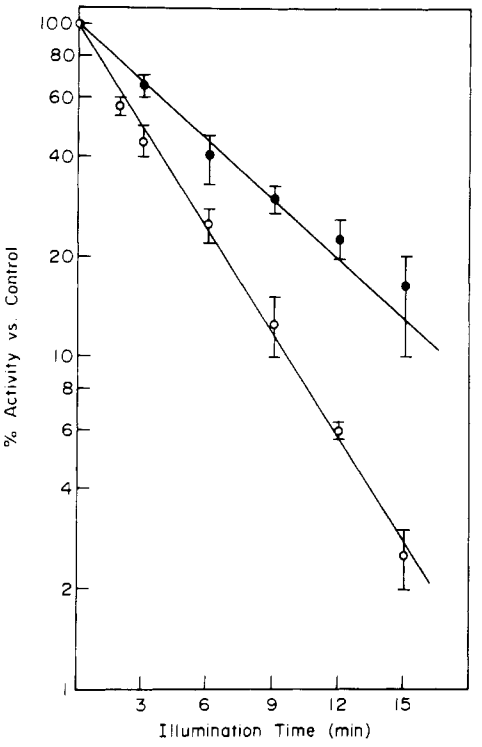

FIGURE 4: Photosensitized inactivation of native and of H_2O_2-modified FeSODs. Reaction mixtures contained native FeSOD at 0.097 mg/mL (○) or H_2O_2-modified FeSOD at 0.92 mg/mL (●) in 90 μM rose bengal, 50 mM potassium phosphate, and 0.1 mM EDTA at pH 7.8 and at 25 ± 1 °C in a water-jacketed glass reaction vessel illuminated by a 300-W slide projector at a distance of 12 cm. At intervals, samples were removed and assayed for residual SOD activity. A dark control showed no loss of activity in 15 min (data not shown).

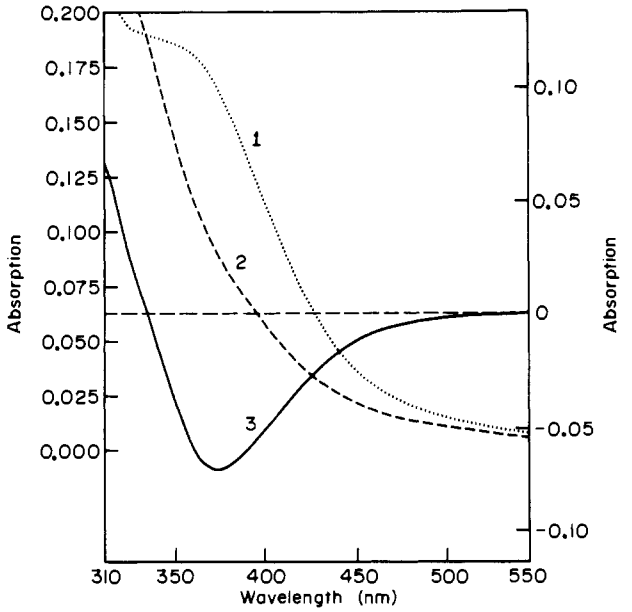

FIGURE 5: Optical spectra of native and of H_2O_2-modified FeSODs. Cuvettes contained the proteins at 6.2 mg/mL in 5.0 mM potassium phosphate and 0.1 mM EDTA at pH 7.8 and at 25 °C. Line 1 = native FeSOD; line 2 = H_2O_2-modified FeSOD; line 3 = difference spectrum (native minus modified).

sorption spectrum. Figure 5 illustrates the changes seen in the 310–500-nm range. The bleaching which accompanied H_2O_2 inactivation was maximal at 375 nm, as shown by the difference spectrum (line 3). H_2O_2 modification of FeSOD was also accompanied by a decrease in absorbance at 290 nm whose time course paralleled the loss of activity (data not shown). This also suggested that H_2O_2 inactivation might

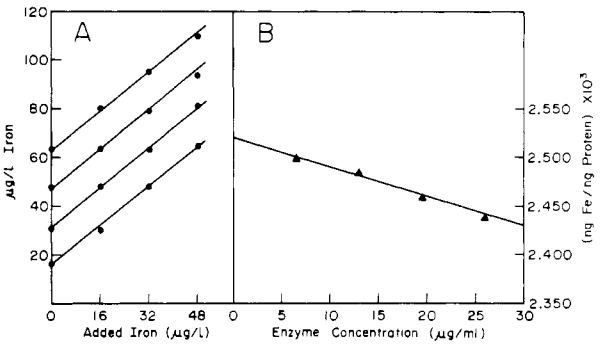

FIGURE 6: Determination of the iron content of FeSOD. Solutions of FeSOD, containing 6.42, 12.84, 19.27, and 25.69 μg of enzyme/mL plus 0, 40, 80, or 120 μg of added Fe/mL, were prepared in 0.2% HNO_3. Twenty-microliter aliquots of these solutions were assayed for Fe content by atomic absorption spectrophotometry. (A) The data are plotted as iron measured as a function of iron added. From bottom to top, lin 1 = 25.69 μg of FeSOD/mL, line 2 = 19.27 μg of FeSOD/mL, line 3 = 12.84 μg of FeSOD/mL, and line 4 = 6.42 μg of FeSOD/mL. (B) The ordinate intercepts from (A) are here graphed as a function of enzyme concentration. The ordinate intercept of this secondary plot represents the iron content of the enzyme corrected for protein matrix effects.

entail attack on tryptophan residues.

Content of Iron and of Manganese. We previously proposed that the 10% of H_2O_2-resistant activity of FeSOD might have been due to the presence of a MnSOD which copurified with the FeSOD and also comigrated with it during electrophoresis on polyacrylamide gels (Clare et al., 1984). We have now examined the Fe and Mn contents of FeSOD by using Zeeman effect atomic absorption spectroscopy. The native enzyme, at 540 μg/mL, was dialyzed for 24 h at 4 °C against several changes of 1000 volumes of 25 mM Tris-HCl buffer at pH 7.8, in which 10 g/L Chelex-100 was suspended by continuous stirring. The fully active dialyzed enzyme was diluted to 6.4, 12.8, 19.2, and 25.7 μg/mL with 0.2% HNO_3 containing known amounts of added iron (0–50 μg/L), and the iron content of each sample was then determined by atomic absorption as described under Materials and Methods.

For each concentration of FeSOD, iron content was then graphed as a function of iron added (Figure 6A). The points for each concentration of enzyme fell on a straight line whose ordinate intercept represented the amount of iron endogenous to that quantity of FeSOD. The endogenous iron content per unit FeSOD was then graphed as a function of FeSOD concentration. These results are shown in Figure 6B.

These measurements, taken together with a molecular weight of 45 800, indicated 2.05 ± 0.10 atoms of Fe per molecule of FeSOD, while comparable measurements of Mn contents indicated only 0.03 atom of Mn per molecule of FeSOD. The Mn content of FeSOD is thus too low to account for the 10% of H_2O_2-resistant activity of FeSOD given that the specific activities of MnSOD and FeSOD are comparable. The iron content of FeSOD did fall during exposure to H_2O_2. Thus, as shown in Figure 7, during exposure to 0.25 mM H_2O_2 the iron content fell by 50% while the activity declined by 90%. Since iron loss from an active site must be accompanied by complete activity loss, it appears that the H_2O_2-resistant activity of FeSOD is actually 20%, when expressed per iron-retaining active site.

Reversible Resolution and Metal Replacement. The role of the iron of FeSOD in its inactivation by H_2O_2 could be explored by removal of that metal and by its replacement with manganese. ApoFeSOD was prepared as described by Yamakura (1976). It contained less than 0.02 atom of Fe per molecule of enzyme and exhibited no significant dismutase

Table I: Reversible Resolution of FeSOD and Susceptibility to H_2O_2 Inactivation

sample[a]	sp act. (units/mg)	iron content[b] (mol/45 800 Da)	Mn content[b] (mol/45 800 Da)	% residual act. after H_2O_2 treatment: 1.0 mM H_2O_2, 30 min	% residual act. after H_2O_2 treatment: 2.0 mM H_2O_2, 120 min
native FeSOD	5000	2.05 ± 0.10	0.03	10.5	10.0
apoFeSOD	<100	<0.03	0.03	ND	ND
Fe-reconstituted apoFeSOD[c]	5010	1.8 ± 0.20	0.05		
Mn-reconstituted apoFeSOD	<200	<0.03	1.6 ± 0.2	ND	ND
H_2O_2-treated apoFeSOD,[d] iron reconstituted	4900	ND[e]	ND	10.0	10.0
H_2O_2-treated[d] Mn-reconstituted apoFeSOD, reconstituted with iron	3900	ND	ND	ND	ND

[a] Iron-free (apoFeSOD) SOD was prepared by the alkaline method described by Yamakura and Suzuki (1976). $Fe(NH_4)_2(SO_4)_2$ was used to reconstitute the apoenzyme with iron. $MnCl_2$ was used to reconstitute the apoenzyme with manganese. Enzyme manipulations were performed under anaerobic conditions in the presence of excess reducing agent. [b] Determined by Zeeman graphite atomic absorption; protein concentration was determined by UV absorption using $A^{1\%}_{280,1cm} = 25.4$. In the case of H_2O_2-treated FeSOD, the method of Murphy and Kies (1960) was used. [c] Eight-five percent of the original activity was recovered. [d] Enzymes were treated with 1.0 mM H_2O_2 for 60 min, followed by reconstitution with iron. [e] ND, not determined.

Table II: Amino Acid Composition of FeSOD and H_2O_2-Modified FeSOD[d]

	hydrolysis method: 6 N HCl[a] control	6 N HCl[a] H_2O_2 modified[c]	6 N HCl[a] difference	4 N methanesulfonic acid[b] control	4 N methanesulfonic acid[b] H_2O_2 modified[c]	4 N methanesulfonic acid[b] difference
aspartic acid	48.1 ± 0.6	47.8 ± 0.3	−0.3	40.6	39.3	−1.3
threonine	31.0 ± 0.4	32.4 ± 0.2	1.4	27.1	26.3	−0.8
serine	22.3 ± 0.1	25.6 ± 2.0	3.3	18.7	18.1	−0.6
glutamic acid	36.0 ± 0.7	35.6 ± 0.4	−0.4	33.1	34.2	1.1
proline	19.8 ± 0.2	19.8 ± 0.2	0	20.1	23.1	3.0
glycine	32.3 ± 0.4	34.3 ± 2.0	2.0	27.9	29.6	1.7
alanine	64.2 ± 0.9	62.5 ± 2.0	−1.7	55.5	56.7	1.2
valine	20.4 ± 0.4	19.9 ± 0.4	−1.0	20.9	20.7	−0.2
methionine	0	0	0	0	0	0
isoleucine	19.2 ± 0.3	19.7 ± 0.5	0.5	12.9	12.9	0
leucine	35.8 ± 0.5	36.2 ± 0.5	0.4	37.2	37.2	0
tyrosine	20.6 ± 0.8	19.6 ± 0.3	−0.4	19.6	19.5	−0.1
phenylalanine	25.9 ± 0.7	24.8 ± 0.6	−1.1	24.1	24.1	0
histidine	14.8 ± 0.2	15.2 ± 0.2	0.4	14.9	15.6	0.7
lysine	22.9 ± 0.1	23.0 ± 0.1	0.1	21.2	20.4	−0.8
arginine	8.5 ± 1.5	7.3 ± 0.5	−1.2	5.4	6.1	0.7
tryptophan[e]	ND[g]	ND		21.8	12.9	−8.9
cysteine[f]	1.60	1.20	−0.4	ND	ND	

[a] 24-h hydrolysis; average of four determinations. [b] 24-h hydrolysis; two determinations. [c] FeSOD was modified to 10% residual activity by incubation in 0.24 mM H_2O_2 for 120 min. [d] Values are expressed as residues per 45 800 daltons. [e] Values are corrected for destruction based on a lysozyme standard; recovery was estimated to be 74%. [f] Determined by using 5,5′-dithiobis(2-nitrobenzoic acid). [g] ND, not determined.

activity. Treatment of the apoenzyme with ferrous ammonium sulfate, as described by Yamakura (1976), followed by removal of unbonded Fe by repeated dialysis against Chelex-100 in phosphate/EDTA, restored 1.8 ± 0.2 atoms of Fe per molecule of enzyme and an activity of 5000 units/mg of protein. When apoFeSOD was reconstituted with $MnCl_2$, it bound 1.6 ± 0.2 atoms of manganese per molecule of enzyme and then exhibited an activity of less than 200 units/mg of protein.

Neither apoFeSOD nor Mn-substituted FeSOD was inactivated when exposed to 1.0 mM H_2O_2, whereas Fe-reconstituted FeSOD was as susceptible as was the original native enzyme. Since both apoFeSOD and Mn-substituted FeSOD were inactive, it was not possible to assess the effects of H_2O_2 by directly assaying their activity. This problem was circumvented, in the case of the apoenzyme, by reconstituting with iron, and in the case of the Mn-substituted enzyme by removing the metal and then reconstituted with iron by the procedure of Yamakura (1976). These results, which are presented in Table I, demonstrate that iron at the active site is essential for expression of the sensitivity toward H_2O_2 and suggest that interaction of H_2O_2 with the active-site iron generates the attacking oxidant. Manganese evidently cannot substitute for iron in this interaction with H_2O_2.

Amino Acid Analyses. Both native and H_2O_2-inactivated FeSOD were hydrolyzed and analyzed for amino acid contents, as described under Materials and Methods. Cysteine contents were measured colorimetrically with 5,5′-dithiobis(2-nitro-

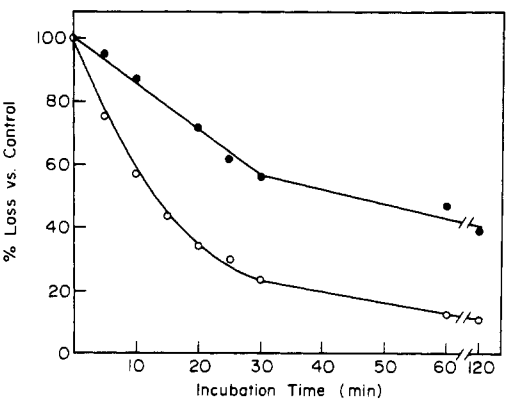

FIGURE 7: Loss of iron during H_2O_2 modification of FeSOD. Reaction mixtures contained 0.25 mg/mL FeSOD, 0.25 mM H_2O_2, 50 mM potassium phosphate, and 0.1 mM EDTA at pH 7.8 and at 25 °C. At intervals, 100-μL samples were removed, freed of H_2O_2 by addition of 100 units (2.5 μg) of catalase, and then exhaustively dialyzed against the phosphate/EDTA buffer, prior to analysis for protein content, for SOD activity (○), and for iron content (●). Control measurements, in the absence of FeSOD, demonstrated that the catalase contributed no more than 3% to the iron content. The protein content was corrected for the known amount of catalase.

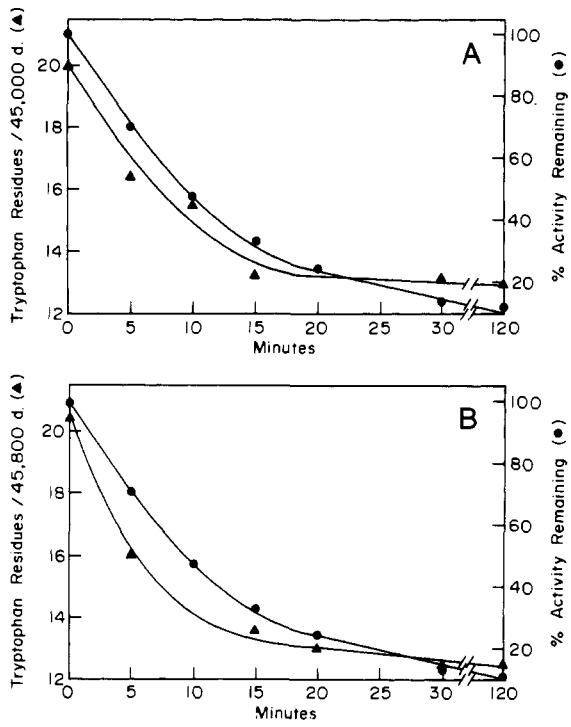

FIGURE 8: (A) Loss of tryptophan and of activity during H_2O_2 modification of FeSOD: amino acid analysis. Reaction mixtures contained 0.25 mg/mL FeSOD, 0.24 mM H_2O_2, 50 mM potassium phosphate, and 0.1 mM EDTA at pH 7.8 and at 25 °C. At intervals, 1.0-mL aliquots were withdrawn and were treated with 100 units (2.5 μg) of catalase prior to measurement of SOD activity (●) and of tryptophan content (▲). Since the catalase:FeSOD ratio was 1:100 (w/w), the contribution of catalase to the tryptophan content was negligible. (B) Loss of tryptophan and of activity during H_2O_2 modification of FeSOD: second-derivative spectrophotometry. Reaction mixtures contained 0.25 mg/mL FeSOD, 0.24 mM H_2O_2, 50 mM potassium phosphate, and 0.1 mM EDTA at pH 7.8 and at 25 °C. At intervals, 1.0-mL aliquots were removed, treated with 100 units (2.5 μg) of catalase, and then analyzed for tyrosine SOD activity (●) and tryptophan by second-derivative spectrophotometry. Tryptophan content (▲) was then calculated on the basis of 20 residues of tyrosine per 45 800 Da, which had been determined by amino acid analysis.

benzoic acid) in the presence of 0.1% sodium dodecyl sulfate (Yamakura, 1976). The results of these measurements, shown in Table II, indicate that H_2O_2 treatment was associated with substantial losses only of tryptophan (4.5 residues/subunit). When FeSOD was exposed to 0.24 mM H_2O_2, the rate of loss of tryptophan was found to parallel the loss of activity, as shown in Figure 8. There was no loss of histidine during this incubation with H_2O_2 (data not shown).

The effect of H_2O_2 treatment on the tryptophan content of FeSOD was also examined by second-derivative spectroscopy (Servillo et al., 1982). This method allows measurement of the tyrosine:tryptophan ratio of the samples dissolved in 6.0 M guanidinium chloride. Since the results of amino acid analyses had already indicated that tyrosine content was unaffected by exposure of FeSOD to H_2O_2 (Table II), we could use the method of Servillo et al. (1982) to follow the loss of tryptophan during exposure to H_2O_2. The results (Figure 8) were in excellent agreement with the data obtained by amino acid analysis of hydrolysates and again indicated concomitant losses of tryptophan and of activity.

DISCUSSION

H_2O_2 inactivates Cu,ZnSOD (Fielden et al., 1973) and FeSOD, but not MnSOD (Asada et al., 1975). In the case of Cu,ZnSOD, bleaching of the active-site Cu(II) precedes inactivation, and a mechanism proposing reduction of Cu(II) to Cu(I) by H_2O_2, followed by a Fenton-like reaction between Cu(I) and H_2O_2, has been proposed (Hodgson & Fridovich, 1975). The strong oxidant generated at the copper by the latter reaction, presumably Cu^{II}–O, then oxidizes an adjacent histidine residue, accounting for the observed loss of one histidine per subunit during inactivation (Bray et al., 1974). A similar mechanism may now be proposed for the inactivation of FeSOD by H_2O_2. X-ray crystallography has shown that MnSOD and FeSOD, which exhibit a great deal of amino acid sequence homology, are structural homologues (Stallings et al., 1984). Moreover, the ligated manganese in the MnSOD from *Thermus thermophilus* is adjacent to three tryptophan residues, which are strongly conserved in related SODs (Stallings et al., 1985). It is thus not surprising that we found a loss of tryptophan during H_2O_2 inactivation of the FeSOD of *E. coli*.

It is however, surprising that we observed no loss of histidine, since three of the four ligands to the Mn in MnSOD, and presumably to the Fe in FeSOD, are provided by histidine residues (Stallings et al., 1984, 1985) and since H_2O_2 inactivation of Cu,ZnSOD does result in oxidation of a liganding histidine residue (Blech & Borders, 1983). It may be that, in the case of FeSOD, primary electron removal from a liganding imidazole is followed, almost immediately, by electron conduction to that imidazole from adjacent tryptophan residues. This could lead to net oxidation of tryptophan, not of histidine. Yamakura (1984) has noted loss of tryptophan, histidine, and cysteine during H_2O_2 inactivation of the FeSOD from *P. ovalis*. Possibly electron conduction is less efficient in the FeSOD from *P. ovalis* than it seems to be in the corresponding enzyme from *E. coli*.

Inactivation of the *E. coli* FeSOD is entirely dependent upon the iron since the apoprotein and the Mn-substituted enzyme are refractory to this reagent. This result is in accord with the proposal that the attacking oxidant is generated from reactions of the iron with H_2O_2. Inactivation by H_2O_2 approaches a limit of 10% residual activity, which appears to reflect activity of the modified FeSOD, rather than inadequate treatment with H_2O_2, or an impurity of active MnSOD which happens to comigrate on gels and to copurify with the FeSOD. Thus, the FeSOD contains 2.0 atoms of Fe per molecule, but not more than 0.03 atom of manganese. Moreover, the residual activity of the H_2O_2-modified enzyme differs from that of the native enzyme with respect to heat stability, azide sensitivity, and susceptibility toward inactivation by *N*-bromosuccinimide or by light plus rose bengal. These changes presumably reflect modification of the active-site environment, due to oxidation of the tryptophan residues which contribute to the hydrophobic milieu which surrounds the liganded metal (Stallings et al., 1984, 1985).

Inactivation of FeSOD by H_2O_2 was accompanied by loss of half of the iron. This does not reflect decreased affinity of the stably modified enzyme for iron, since this modified enzyme may be extensively dialyzed against EDTA without inactivation. We may suppose that an intermediate form of the enzyme, which appears transiently during H_2O_2 inactivation, exhibits diminished affinity for iron. This hypothetical intermediate may well be one in which oxidation of a liganding imidazole has not yet been followed by electron conduction from tryptophan to that imidazole. Were it is possible to have prevented this loss of turn, during H_2O_2 inactivation of FeSOD, we would have observed 20% residual activity. This substantial activity of the modified enzyme probably reflects the limited essentially of those tryptophan residues which lie outside of

the ligand field of the iron and which contribute to the hydrophobicity of that second layer of residues.

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Registry No. SOD, 9054-89-1; Fe, 7439-89-6; H_2O_2, 7722-84-1; L-Trp, 73-22-3.

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