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Reactions of H_2O_2 with the Iron-Containing Superoxide Dismutase from *Escherichia coli*

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Near-stoichiometric concentrations of H_2O_2 partially bleached the 350-nm absorption band of the iron-containing superoxide dismutase from *Escherichia coli*. Concomitantly, the high-spin Fe(III) EPR signals decreased in intensity. Both the absorption and EPR spectral changes were largely reversible. Thus, peroxide reduces the Fe(III) ions in superoxide dismutase, which then slowly reoxidize. The pH dependence of this reaction implicates HO_2^- as the actual reductant. Magnetic circular dichroism and fluorescence spectroscopy indicate that tryptophan oxidation occurs at higher H_2O_2 concentrations. The tryptophan content of the native superoxide dismutase was determined to be 12.4 ± 0.5; about 2.5 tryptophans are modified by a 20-fold excess of peroxide over iron. Negligible tryptophan oxidation was observed by magnetic circular dichroism spectroscopy when $[H_2O_2] \simeq [Fe]$. Furthermore, the reaction with stoichiometric peroxide produced an increase in the tryptophan fluorescence intensity at 340 nm, suggesting that the fluorescence from the native superoxide dismutase is partially quenched via energy transfer to the 350-nm chromophore. Azide significantly decreased the rates of Fe(III) reduction and tryptophan oxidation; azide also protected the dismutase against inactivation by high concentrations of peroxide. Fe(III) reduction by peroxide is sufficiently rapid to be involved in protein modification and inactivation. Collectively, the data suggest that these processes result from the reaction of $HO_2^-(H_2O_2)$ with the reduced enzyme.

Several aspects of the mechanism of the iron-containing superoxide dismutase (FeSOD) from *E. coli* are now well-defined.[1] Cyclic inner-sphere oxidation–reduction steps appear to be involved, as shown in eq 1 and 2. Proton uptake and reduction are

$$\text{Fe(III)} + O_2^- \rightleftharpoons \text{Fe(III)}\text{–}O_2^- \rightarrow \text{Fe(II)} + O_2 \qquad (1)$$

$$\text{Fe(II)} + O_2^- \overset{H^+}{\rightleftharpoons} \text{Fe(III)}\text{–}O_2H^- \overset{H^+}{\rightleftharpoons} \text{Fe(III)} + H_2O_2 \qquad (2)$$

concurrent, and the rate-limiting step in catalysis involves proton transfer, possibly in a general-acid-assisted breakdown of an Fe(III)–peroxo intermediate[1,2] Extensive kinetics studied on the $Fe^{3+/2+}$–EDTA-catalyzed superoxide dismutation reaction have provided an essential framework for interpreting the enzyme-catalyzed reaction.[2] General-acid-catalyzed breakdown of an Fe(III)–peroxo intermediate is important in the Fe–EDTA system as well.[2] In fact, a stable peroxo complex can be observed under some conditions; its structure is believed to contain either a cyclic Fe(III)–peroxo moiety[1] with side-bound O_2^{2-} or a monodentate HO_2^- ligand.[3,4] Reactions of H_2O_2 with superoxide dismutases have been previously investigated.[5–16] Both the eukaryotic Cu,ZnSOD and FeSOD (from various sources) are inactivated or inhibited by H_2O_2, whereas the MnSOD is generally resistant. No peroxo complex, analogous to that formed by $Fe^{3+/2+}$–EDTA, has been definitely detected in FeSOD reactions with H_2O_2. Some aspects of the reaction(s) of H_2O_2 with FeSOD remain obscure. Irreversible inactivation is often, but not invariably, observed. H_2O_2 has been reported to reduce Fe(III) to Fe(II) in FeSODs from *Anacystis nidulans*[13] and *Photobacterium leiognathi*[12] but did not reduce the *Propionibacterium shermanii* FeSOD.[17] This latter enzyme was apparently inhibited by H_2O_2 reversibly despite the fact that H_2O_2 permanently alters its Fe(III) EPR spectrum. Recent spectroscopic and analytical work indicates that H_2O_2 oxidizes 1.5–1.8 tryptophan residues per dimer ($M_r \sim 38\,000$) of the *Pseudomonas ovalis* FeSOD.[11] There are several tryptophans in this enzyme, and the 2.9-Å X-ray structure showed that one tryptophan is near each of the two iron atoms in the dimer.[18] The structure of the *E. coli* enzyme is very similar.[19] Tryptophan oxidation by H_2O_2 is well precedented, particularly in basic conditions.[20,21]

H_2O_2 inactivates the yeast or bovine Cu,ZnSOD in alkaline conditions by modifying one histidine residue per subunit.[5–9] The inactivation mechanism is believed to involve HO_2^- coordination to Cu(II) to form a reactive complex that subsequently oxidizes a histidine ligand. At pH 7.4 the combination of superoxide and peroxide also inactivates Cu,ZnSOD.[22] In principle, similar reactions are not implausible for the FeSOD or MnSOD because the metal ion ligands are probably three histidines and an aspartic acid.[18,19,23–25] Since O_2^- and HO_2^- have been shown to rapidly reduce SOD–Cu(II), the reactive complex likely contains Cu(I) or is produced by reaction of Cu(I) with a second equivalent of H_2O_2.[5,10] Several compounds protect Cu,ZnSOD against H_2O_2 inactivation, notably CN^- and N_3^-, which may block HO_2^- coordination to Cu(II).[5,7–10] Azide slightly decreased the rate of inactivation of *Chromatium vinosum* FeSOD, but other compounds that protected Cu,ZnSOD were ineffective.[13]

Superoxide dismutase inactivation by H_2O_2 is an extremely interesting case of product inhibition. The question of whether H_2O_2 is an active-site-specific reagent deserves careful scrutiny.

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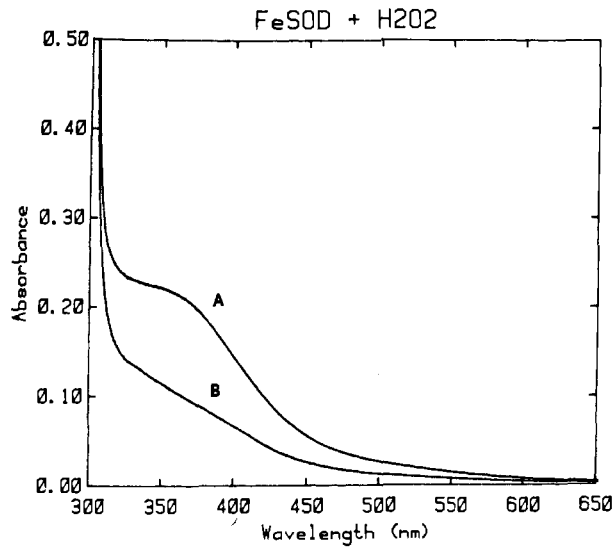

Figure 1. Absorption spectra in the absence and presence of H_2O_2: (A) spectrum of 6.5×10^{-5} M FeSOD in 5 mM potassium phosphate buffer, pH 7.4; (B) maximum bleaching produced by 1.5×10^{-4} M H_2O_2.

Previous results on the reactivity of H_2O_2 with FeSOD have been obtained under conditions where peroxide was present in large excess ($[H_2O_2] \geq 20[FeSOD]$); we were interested in studying the reactivity at near-stoichiometric $[H_2O_2]/[FeSOD]$ ratios because such conditions may correspond more closely to the physiological situation. Investigating the effects of exogenous Fe(III) ligands (i.e. N_3^-) on the peroxide reactivity is also of interest. In this paper we describe extensive spectroscopic studies on the *E. coli* FeSOD reactions with H_2O_2, which indicate that H_2O_2 reduces and, especially at higher concentrations, modifies the *E. coli* FeSOD. Azide decreases the rates of Fe(III) reduction, tryptophan modification, and inactivation. Finally, possible mechanisms for these reactions are evaluated.

Experimental Procedures

E. coli B was purchased from Grain Products, Inc. (Muscatine, IA). FeSOD was purified by the procedure described by Slykhouse and Fee,[26] with some minor modifications, using a hydroxylapatite (Calbiochem fast flow) column as the final step. The enzyme was homogeneous as judged by SDS gel electrophoresis (12.5%, Coomasie Blue staining) and displayed spectroscopic properties identical with those previously reported. Protein was estimated by the Bradford assay.[27] FeSOD concentrations were routinely measured via the extinction coefficients $\epsilon_{280} = 1.01 \times 10^5$ M^{-1} cm^{-1} and $\epsilon_{350} = 1850$ M^{-1} cm^{-1};[26] the latter value is based on the iron concentration and served as the basis for quantitating iron. The cytochrome *c* reduction assay was employed to measure SOD activity.[28] FeSOD samples were shell-frozen in liquid N_2 and stored at −70 °C until needed. H_2O_2 solutions were freshly prepared in distilled, deionized water for all experiments.

All absorption spectra were obtained on a Cary 219 spectrophotometer, interfaced to an Apple IIe computer. EPR spectra were measured with a Varian E-9. A Spex Fluorolog 112 photon-counting spectrofluorometer with a Hamamatsu R928 photomultiplier tube was employed for emission spectroscopy. The PM tube was cooled with a PRA, Inc., thermoelectric cooler. Excitation and emission slits were set to afford spectral bandwidths of 5.0 and 2.0 nm, respectively. Data were collected with 0.5-nm monochromator steps and a 0.5 s/step acquisition (counting) time. Fluorescence intensities are reported as S/R, where S is the number of fluorescent photons detected (at 90°) and R is a reference signal generated by using a beam-splitter in the excitation path together with a Rhodamine 6-G quantum counter, and thus are corrected for any fluctuations in lamp intensity. The data have also been corrected for wavelength variations in photomultiplier sensitivity and optical throughput. CD and MCD spectra were recorded with a modified JASCO J-40A equipped with a Morvue photoelastic modulator, a PAR lock-in amplifier, and an Alpha Scientific electromagnet. Standard data acquisition parameters were 1-nm resolution, 25 nm min^{-1} scanning rate, and ~1.0-T magnetic field. CD/MCD data were collected and manipulated with a Bascom-Turner digital recorder. 10-Camphorsulfonic acid (recrystallized from glacial acetic acid, mp 195 °C) and *N*-acetyltryptophanamide served as CD and MCD calibration standards, respectively.

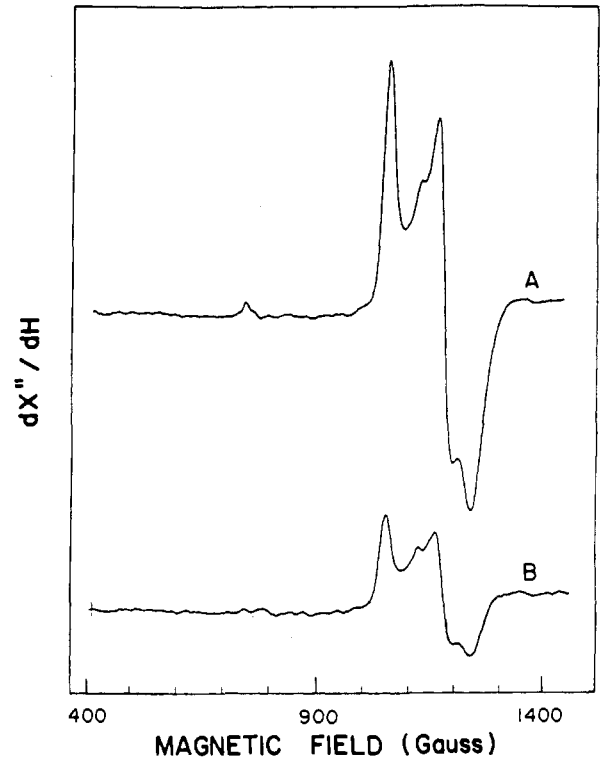

Figure 2. 77 K EPR spectra in the absence and presence of H_2O_2: (A) spectrum of 4.25×10^{-4} M FeSOD; (B) spectrum obtained following reaction with 9×10^{-4} M H_2O_2 for ~10 min at room temperature. Conditions for both spectra: frequency, 9.38 GHz; modulation amplitude, 10 G; power, 10 mW.

Peroxide inactivation of FeSOD was evaluated by using the cytochrome *c* reduction assay. Volumes of H_2O_2 solutions sufficient to produce a 10–100-fold stoichiometric excess (relative to Fe) were added to FeSOD solutions in 50 mM phosphate buffer, pH 7.4 or 7.8. Aliquots were withdrawn at various times, the peroxide reaction was quenched by dilution into the assay buffer, and then the samples were assayed immediately. Assays were initiated by xanthine addition. Anions were allowed to react with FeSOD for approximately 10 min prior to the addition of H_2O_2.

Results

Stoichiometric H_2O_2 partially bleaches the 350-nm absorption band of oxidized FeSOD (Figure 1). Since this band is not present in the apoprotein or the dithionite-reduced enzyme, it is reasonably assigned as an Fe(III) charge-transfer transition. H_2O_2 also decreases the high-spin Fe(III) EPR signals characteristically displayed by FeSOD (Figure 2). No new features, suggestive of H_2O_2 binding, are evident. The data are entirely consistent with H_2O_2 acting as a reductant because (1) high-spin ($S = 2$) Fe(II) is virtually EPR-nondetectable at 77 K and (2) an LMCT transition would shift to higher energy upon metal reduction (an MLCT transition would red-shift, in contrast to the results in Figure 1). On the basis of the difference between the absorption spectra of the native, oxidized FeSOD and the apoprotein (or dithionite reduced FeSOD), the bleaching in Figure 1 corresponds to ~70% of the Fe(III) sites being reduced. Inspecting Figure 2, we see that the EPR signal centered near $g \sim 4.1$ is reduced to approximately 30% of its original intensity (peak-to-trough) by stoichiometric H_2O_2.

FeSOD absorption and EPR spectra also display similar time-dependent behavior. Maximum bleaching occurs after approximately 15 min at pH 7.4; subsequently the Fe(III) EPR signals and the 350-nm absorption band increase slowly. Maximum recovery was generally observed 2-4 hours after exposure to near-stoichiometric ((0.75–2.0)[Fe]) H_2O_2 under our conditions.

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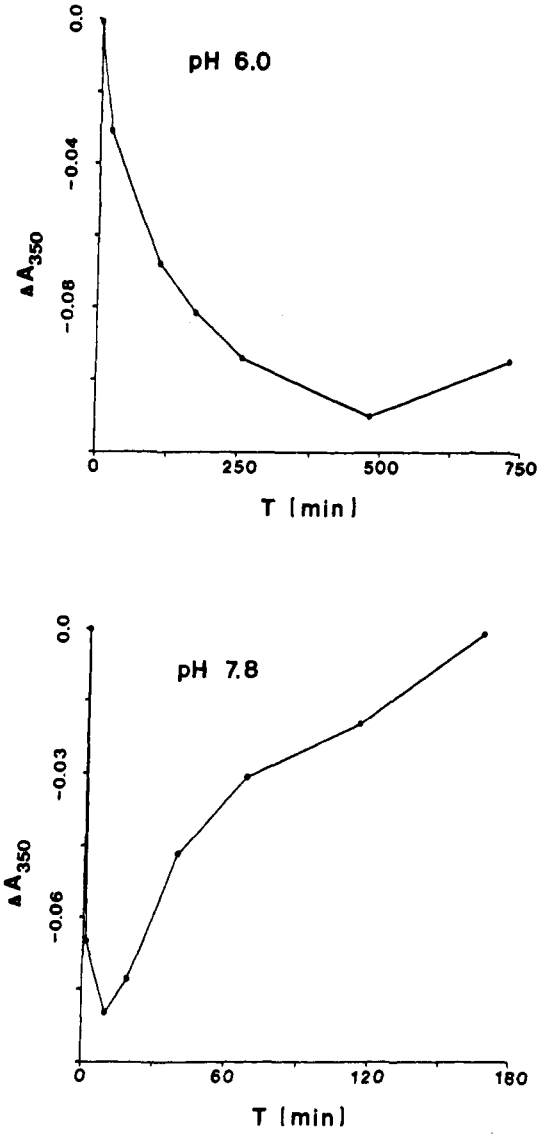

Figure 3. Peroxide-induced absorbance changes at 350 nm at two pH values: top, 1.0×10^{-5} M FeSOD plus 1.5×10^{-5} M H_2O_2 in 50 mM potassium phosphate buffer, pH 6.0; bottom, concentrations as above, pH 7.8.

Typical absorbance vs. time data are shown in Figure 3. It is clear that both components of the FeSOD–H_2O_2 reaction are considerably faster at pH 7.8 compared to those at pH 6.0. Quantitatively the reaction monitored at 350 nm is about 50 times faster at higher pH, which correlates very well with the increase in $[HO_2^-]$, assuming $pK_a = 11.6$.[29] Spectral changes induced by stoichiometric peroxide were not consistently reversible. Further, irreversible effects were more pronounced at higher $[H_2O_2]/[Fe]$ ratios. Neither extensive dialysis nor treatment with O_2^- or $Fe(CN)_6^{3-}$ significantly improves the recovery of the oxidized enzyme's spectroscopic properties in this case, suggesting that iron may be lost from the reduced enzyme (vide infra).

Azide is known to coordinate to Fe(III) in FeSOD, producing $N_3^- \rightarrow$ Fe(III) LMCT transitions at 320 and 440 nm (Figure 4).[1,26,30] These bands are also bleached by H_2O_2, but the reaction is considerably slower than the reaction of the native enzyme with H_2O_2. Peroxide also reduces the intensity of the FeSOD–N_3^- EPR spectrum at a lower rate than observed for the native FeSOD (data not shown).

A UV–visible difference absorption spectrum of FeSOD plus excess H_2O_2 ($[H_2O_2] \simeq 100[Fe]$) is shown in Figure 5. In addition to the decrease at 350 nm, minima are observed at 280 and <240 nm, while maxima are evident at 250 and 310 nm. Our spectra are similar to those reported for the tryptophan oxidation product generated by reaction with H_2O_2/aqueous dioxane[20] and to spectra displayed by *Pseudomonas ovalis* FeSOD following H_2O_2 addition.[11]

MCD spectroscopy provides unambiguous evidence for tryptophan modification by H_2O_2. Tryptophan is the only amino acid found in proteins that displays an intense, positive MCD band at ~290 nm, associated with the ^1L_b transition of the indole ring.[31-33] MCD transitions arise from external magnetic field

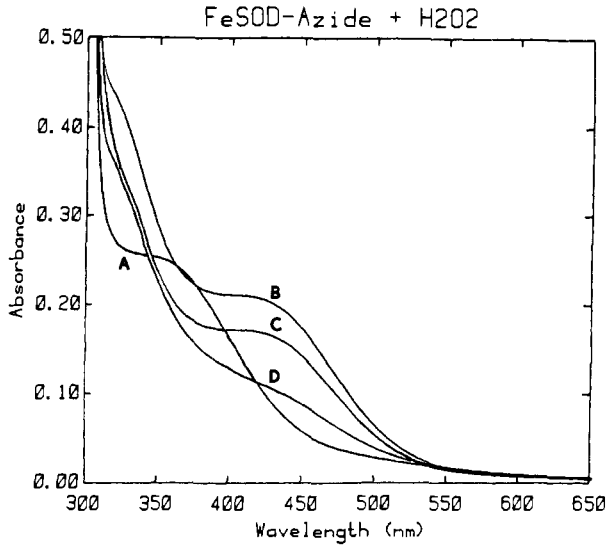

Figure 4. Absorption spectral changes accompanying H_2O_2 addition to the FeSOD–azide complex: (A) native 7.0×10^{-5} M FeSOD in 5.0 mM potassium phosphate buffer, pH 7.4; (B) azide complex; (C and D) azide complex following reaction with 3×10^{-4} M H_2O_2 for 15 min and 8 h, respectively.

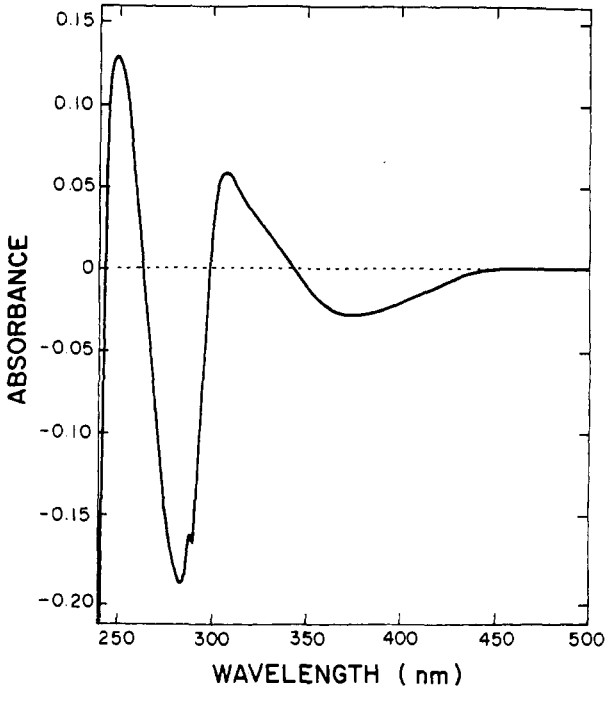

Figure 5. UV difference absorption spectrum produced by adding excess H_2O_2 to FeSOD. This spectrum was obtained ~2 min following the addition of H_2O_2 to the enzyme in 50 mM potassium phosphate buffer, pH 7.8. Final concentrations were 2.5×10^{-5} M for FeSOD and 5×10^{-3} M for H_2O_2.

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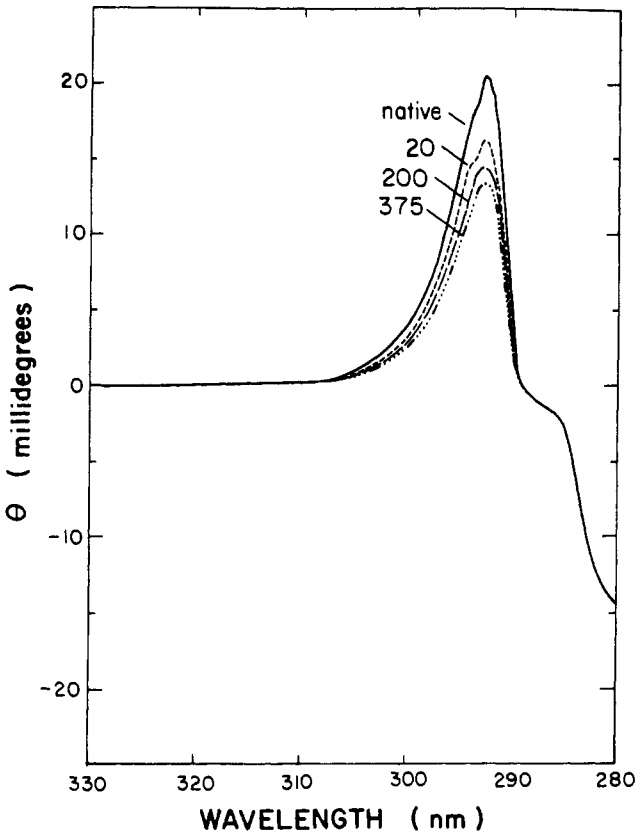

Figure 6. MCD spectral changes accompanying H_2O_2 addition to FeSOD: (—) native FeSOD; (---) FeSOD plus a 20-fold excess of H_2O_2; (– –) FeSOD plus a 200-fold excess of H_2O_2; (-·-·-) FeSOD plus a 375-fold excess of H_2O_2. The enzyme concentration is 2.0×10^{-5} M in 50 mM potassium phosphate buffer, pH 7.8. In each case the spectrum shown was obtained after the reaction was complete.

Table I. Tryptophan Content of FeSOD from MCD

sample	$[H_2O_2]/[Fe]$	no. of tryptophans[a]
native FeSOD	0	12.4
FeSOD + H_2O_2[b]	20	9.9 (7 min)
	195	9.0 (3 min)
	375	8.2 (3 min)
FeSOD-N_3^- + H_2O_2[c]	20	11.3 (8 min)
		10.5 (20 min)
		9.9 (45 min)
	170	9.4 (4 min)

[a] The stated uncertainty in the MCD method[31-33] corresponds to ±0.5 residue for FeSOD. [b] Spectral changes were complete by the elapsed time from the H_2O_2 addition indicated within parentheses. [c] Spectra were recorded after the addition of H_2O_2 at the times indicated. No further changes were observed for times longer than the last one listed for each ratio.

effects on the energies and degeneracies of molecular electronic states and thus are relatively insensitive to environmental and conformational effects. Consequently the molar magnetic differential extinction coefficient at ~290 nm, $\Delta\epsilon_M = (\epsilon_L - \epsilon_R)(H)^{-1}$, for a given protein is a linear function of the tryptophan content. This property has been extensively exploited to quantitate tryptophan residues in proteins.[31-33] MCD spectroscopy is also much easier to use for kinetics experiments compared to other methods for tryptophan determination. Effects of H_2O_2 on the MCD spectrum of FeSOD are shown in Figure 6. Near-stoichiometric H_2O_2 only slightly (≤5%) decreased $\Delta\epsilon_M^{295}$. Under conditions where the 350-nm absorption band is substantially bleached there is little change in $\Delta\epsilon_M^{295}$, indicating the MCD spectral changes observed at 295 nm at higher $[H_2O_2]$ are not associated with

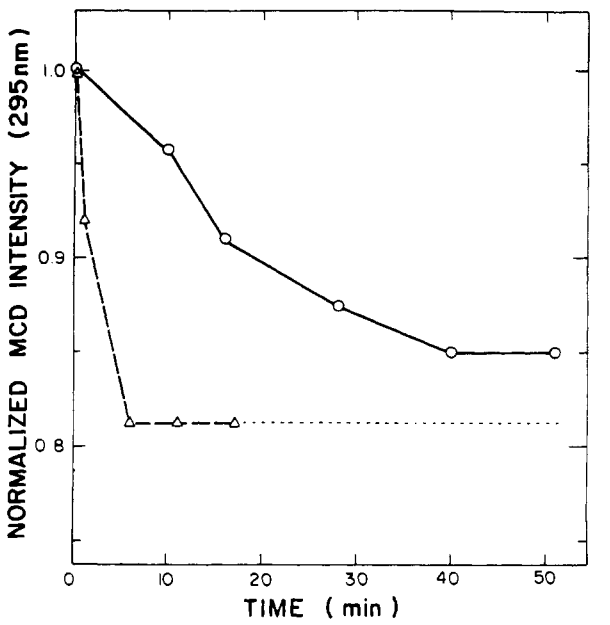

Figure 7. Time dependence of the tryptophan 295-nm MCD transition: (△) native FeSOD; (○) FeSOD–azide complex, $[N_3^-] \simeq 50[Fe(III)]$. $[H_2O_2] = 20[Fe(III)]$ in 50 mM potassium phosphate buffer, pH 7.8.

iron-localized electronic transitions. Both the magnitude and rate of the decrease in $\Delta\epsilon_M^{295}$ are proportioinal to $[H_2O_2]$. Quantitative MCD data are reported in Table I. The number of tryptophans in native *E. coli* FeSOD found by MCD spectroscopy is in remarkably good agreement with the number (11.9 ± 0.2) determined by amino acid analysis for the *P. ovalis* enzyme.[11,34] Clearly, then, tryptophans in FeSOD are modified by H_2O_2, especially when $[H_2O_2] \geq 10[Fe]$. A decrease in $\Delta\epsilon_M^{295}$ is consistent with oxidation of the indole ring to a product similar to 2-oxindole. Similar effects were observed following H_2O_2 addition to FeSOD-N_3^-, but the reaction rate was considerably slower (Figure 7). Assuming a rate law for tryptophan modification of the form $k[Fe(III)][H_2O_2]$, second-order rate constants are 0.59 and 0.087 M^{-1} s^{-1} for native and azide-bound FeSOD, respectively. Difference MCD spectra (oxidized-H_2O_2 treated) are consistent with tryptophan modification (cf. ref 35).

Fluorescence data are also consistent with Fe(III) reduction and tryptophan modification by H_2O_2. To minimize self-quenching, much lower concentrations of FeSOD were used in the fluorescence experiments, which required higher peroxide concentrations to produce reaction rates comparable to those observed by absorbance, EPR, and MCD spectroscopy. When peroxide was present in 1-10-fold excess over iron, tryptophan emission (λ_{max} = 340 nm) was significantly enhanced relative to native FeSOD fluorescence (Figure 8). This result suggests that tryptophan fluorescence from native FeSOD is partially quenched via energy transfer to the 350-nm chromophore. Given the proximity of a tryptophan residue to the Fe(III) site,[18,19,23,25] energy-transfer quenching is certainly plausible. At higher H_2O_2 concentrations the fluorescence intensity at 340 nm decreased markedly in a time-dependent manner (Figure 8). Oxidation by *N*-bromosuccinimide has been shown to abolish the characteristic fluorescence of tryptophan.[36,37] Considerable tryptophan emission

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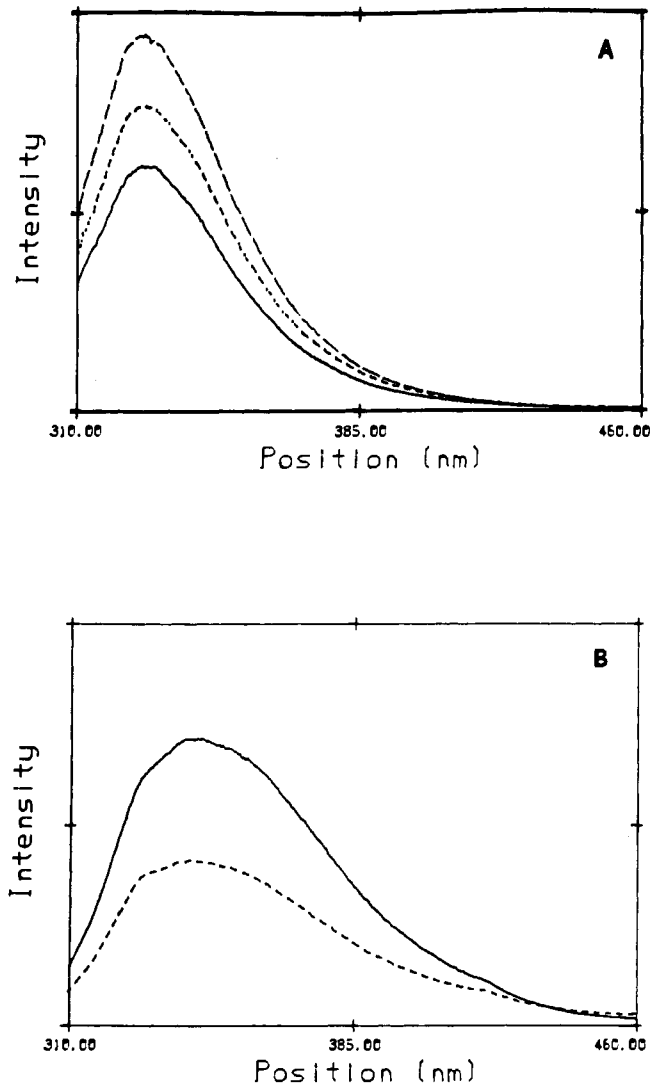

Figure 8. Fluorescence spectra of FeSOD in the absence and presence of H_2O_2. (A) Spectrum of 3.0×10^{-6} M FeSOD in 50 mM Tris–sulfate buffer, pH 7.4 (—); spectrum obtained immediately after H_2O_2 addition to give $[H_2O_2] = 5 \times 10^{-5}$ M (- - -); spectrum of previous sample after an additional 20 min at room temperature (– –). (B) Spectrum of 1×10^{-6} M FeSOD in 50 mM Hepes–sulfate buffer, pH 7.4 (—); spectrum obtained following the reaction of 1.2×10^{-4} M H_2O_2 with FeSOD for 45 min at room temperature (- - -). Excitation wavelength was 295 nm for all spectra.

could be detected following prolonged exposure to high levels of H_2O_2, indicating that some of the tryptophan residues in FeSOD are unreactive toward aqueous peroxide.

We have confirmed that excess H_2O_2 inactivates *E. coli* FeSOD, as observed previously for several iron-containing superoxide dismutases.[11-16] Again assuming that the rate law is first order in FeSOD and peroxide, we find $k = 0.36 \pm 0.09$ M^{-1} s^{-1} (average of three measurements), which is about one-third of the inactivation rate constant reported for *P. ovalis*.[11] Parenthetically, we note that the rate law for inactivation of Cu,ZnSOD by HO_2^- has been shown to be second order.[8,9,38] Finally, azide protects FeSOD from inactivation by H_2O_2. Under conditions where peroxide decreases the activity of native FeSOD by ~35% in 30 min, no decrease in activity is observed if 20 mM NaN_3 is present.[39] Recently, azide has been shown to protect *Bacteroides* FeSODs from inactivation by peroxide as well.[40] In contrast, other anionic or neutral compounds that scavenge radicals apparently afford little protection against the peroxide inactivation of *E. coli* and other FeSODs.[13,40,41]

Discussion

Hydrogen peroxide clearly reacts with *E. coli* FeSOD in two different ways. H_2O_2 reduces Fe(III) to Fe(II), which is then slowly reoxidized, probably by O_2. In addition, tryptophan residues are modified, especially at higher peroxide concentrations. Although the products of the tryptophan reaction with H_2O_2 have not been identified, the MCD data are consistent with oxidation to an oxindole or dione moiety.[32] Minor perturbations of the FeSOD UV absorbance and MCD spectra were observed for near-stoichiometric H_2O_2 concentrations. Further, the overall reaction is largely reversible when $[H_2O_2] \leq [Fe]$. Thus at relatively low $[H_2O_2]$, Fe(III) reduction is the predominant reaction and the extent of tryptophan modification is small and variable. Evidence consistent with Fe(III) reduction by H_2O_2 has also been presented for FeSOD isolated from other sources.[11,12] The correlation between the reduction rates at pH 6.0 vs. pH 7.8 and the HO_2^- concentration suggests that HO_2^- is the actual reductant.

Fe(III)–O_2H^- complexes are likely intermediates in superoxide dismutation catalyzed by FeSOD and Fe(III)–EDTA.[1,2] Apparently, the inner-sphere electron-transfer reaction in such a complex to give an Fe(II) species is slow. Formation of an Fe(III)–O_2H^- complex, followed by Fe(III) reduction to Fe(II), is a plausible mechanism for the reaction examined here. If the reduction step was rate-limiting, some perturbation of the Fe(III) EPR spectrum that reflects complex formation might be anticipated. The Fe(III)–EDTA–peroxide complex displays a moderately intense absorption band at 520 nm ($\epsilon = 530$ M^{-1} cm^{-1}).[42,43] Cyclic, bidentate[1] and monodentate structures[3,4] for the chromophore have been proposed. Whichever structure is correct for the Fe(III)–EDTA complex, the structure of the FeSOD–peroxide complex cannot be the same, since a similar complex between FeSOD and H_2O_2 would have been detected under our experimental conditions (Figure 1) but was not observed. It is possible that ligand substitution is rate-limiting (i.e., HO_2^- binds slowly) or that outer-sphere electron transfer occurs. However, the mechanistic work on FeSOD, as well as extensive studies of the reactions of Fe(II, III) complexes with peroxide and superoxide,[3,44] can be best understood in terms of inner-sphere redox reactions. Therefore, we tentatively suggest that inner-sphere redox steps are also involved in the FeSOD reaction with peroxide. The lack of an EPR signal (at 77 K) attributable to an Fe(III)–O_2H^- complex may reflect temperature or freezing effects on the equilibria.

Azide inhibits FeSOD reduction by HO_2^- at high and near-stoichiometric $[H_2O_2]/[Fe]$ ratios. As there are no large conformational differences between native and azide-bound FeSOD at 3.1-Å resolution,[18,19] the lower reactivity of the latter form is probably intrinsic to the Fe(III)–N_3^- complex. This result is not unexpected if an inner-sphere mechanism for the peroxide reduction is operative. Azide must also reduce the reoxidation rate; otherwise, no bleaching at 350 nm would be apparent (or its magnitude would be reduced). Only a semiquantitative comparison between the reduction rates of FeSOD, with or without added azide, was feasible: $k \simeq 1 \times 10^{-2}$ s^{-1} and $k \simeq 5 \times 10^{-3}$ s^{-1} for native and azide-FeSOD, respectively (pseudo-first-order conditions, $[H_2O_2] \simeq 100[Fe]$). The fact that Fe(III) reduction, reoxidation, and tryptophan modification all contribute to the absorbance changes at 350 nm prevented a more rigorous comparison.

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(39) Conditions: [FeSOD] = 4.7×10^{-6} M; $[H_2O_2] = 8.1 \times 10^{-4}$ M in 50 mM KPO_4 buffer, pH 7.8. The decrease in activity was linear over the first 30 min. Note that negligible tryptophan oxidation would be expected for the FeSOD–N_3^- complex during this time.
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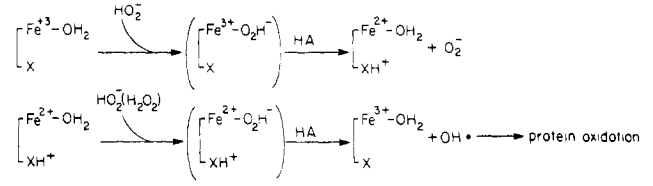
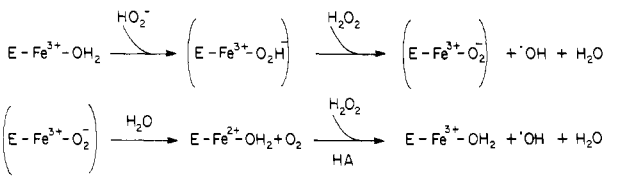
Scheme I



Based on the mechanism proposed by Bull and Fee (reference 1). HA is a general acid. The complexes in parentheses have not been observed spectroscopically. Either HO_2^- or H_2O_2 binding to the reduced enzyme is consistent with the data

Scheme II



Based on the proposal by Lawrence and Sawyer (reference 43) for the mechanism of the H_2O_2 inactivation of Cu,ZnSOD. The complexes in parentheses have not been observed spectroscopically.

At higher peroxide concentrations, additional chemistry occurs, leading to irreversible protein modification and inactivation. The inferred second-order rate constant for Fe(III) reduction ($\simeq$1.3 M^{-1} s^{-1}) is similar to the inactivation rate constant ($\simeq$1.0 M^{-1} s^{-1}) reported for the *P. ovalis* FeSOD.[11] The relative magnitudes of the rate constants, k(reduction) > k(tryptophan oxidation) > k(inactivation), permit the former reactions to be involved in the irreversible inactivation of *E. coli* FeSOD by peroxide. Furthermore azide decreases all three rates by comparable magnitudes. It is possible that the greater attenuation of the tryptophan modification and inactivation rates by azide reflects a decrease in the reaction rate of reduced FeSOD with a second equivalent of peroxide (vide infra). Yamakura demonstrated that 50-min exposures to a 100-fold excess of H_2O_2 ($\simeq$50[Fe]) modified 1.5–1.8 tryptophan residues/*P. ovalis* FeSOD dimer.[11] Histidine and cysteine ($\sim$1 residue/dimer) were also modified under these conditions. However, inactivation does not correlate with histidine loss in *E. coli* FeSOD.[41] Approximately 2.5 tryptophans/dimer of the *E. coli* FeSOD are modified by 20-fold excess (over iron) of H_2O_2 (Table I). A tryptophan residue is located near each iron site in both FeSODs, and Yamakura suggested that these residues are closely related to catalytic activity.[11] The suggestion that tryptophans near the iron site are modified is supported by our observation that azide decreases the rate of tryptophan modification. Since azide is an Fe(III) ligand, its protective effect implicates the metal-site vicinity as the locale for inactivation. Azide has been proposed to protect the Cu,ZnSOD via coordination to Cu(II).[5] Azide may react with peroxide instead of histidine or compete with peroxide for a Cu(II) coordination position. The former suggestion was based in part on the erroneous impression that azide did not inhibit Cu,ZnSOD and that it bound differently from cyanide, which also decreased the rate of the peroxide reaction.[5] More recent investigations of the Cu,ZnSOD coordination chemistry are consistent with similar structures for the Cu(II)–N_3^-/CN^- complexes.[45] We believe that the simplest interpretation of these, and our, results is that anions such as N_3^- and HO_2^- compete for the same metal ion binding site. Solvent ^1H NMR relaxation measurements suggest that the metal site in FeSOD cannot simultaneously accommodate more than one anionic ligand.[46]

Schemes I and II summarize possible mechanisms for the reduction and modification of FeSOD. Scheme I is based on the dismutation mechanism proposed by Bull and Fee[1] and on previous proposals for peroxide reactions with the Cu,ZnSOD.[5,10,47] Scheme II is essentially the mechanism first advanced by Lawrence and Sawyer.[48] The mechanism in Scheme I is more consistent with our data. Note that 2 equiv of H_2O_2 is required to reduce the metal according to Scheme II. This is difficult to reconcile with the observation that low concentrations of peroxide reduce the absorption and EPR features associated with Fe(III) by more than expected on the basis of a 2:1 stoichiometry. Further, highly reactive $^{\bullet}OH$ is also a necessary product of Fe(III) reduction according to Scheme II. If so, then even relatively low H_2O_2 concentrations should modify and presumably inactivate the protein, in contrast with experiment. On the other hand, Scheme I readily accommodates the results obtained to date on FeSOD reactions with H_2O_2. FeSOD takes up one H^+ upon reduction[1] that we assume is involved in the breakdown of the Fe(II)–peroxide complex. When $[H_2O_2]/[Fe] \simeq 1$, Fe(III) is reduced and then slowly reoxidized in a largely reversible process. Protein modification most readily occurs in excess H_2O_2, suggesting that reaction with a second equivalent of H_2O_2 is required to generate the inactivating reagent, namely $^{\bullet}OH$. Additional tryptophan modification, apparent when H_2O_2 is present in large excess, may be produced by further turnover or by direct reactions between peroxide and tryptophan. Evidence for radical formation, produced by $^{\bullet}OH$ abstraction, in the breakdown of the Fe(III)–EDTA peroxy complex, has recently been presented.[3] In addition to the evidence from the pH dependence, HO_2^- is implicated as the reductant because it is a much better one-electron reductant than H_2O_2.[49] Reducing conditions facilitate iron removal from various FeSODs including that from *E coli*.[50] Hence loss of iron from the peroxide-reduced protein may also contribute to the irreversibility of the absorbance and EPR changes and to inactivation,[41] particularly at relatively low H_2O_2 concentrations where reduction occurs to a much greater extent than tryptophan modification. Conceivably, iron is lost more readily following tryptophan modification, but this question warrants further study.

Regardless of the mechanism of H_2O_2 inactivation, superoxide dismutases (at least the FeSOD and Cu,ZnSOD) are peculiar enzymes in that the reaction product can irreversibly modify and inactivate the enzyme that produced it at physiological pH. The results reported here indicate that, as long as local peroxide concentrations do not build up, the peroxide–FeSOD reaction is slow and nearly reversible and that little inactivation would occur. The local pH, the rate of peroxide formation, and the in vivo distribution of catalase, peroxidases, or any other peroxide scavenging system, and the superoxide dismutase may therefore be critical for protection against oxygen toxicity.[51-53] SOD could also be protected against inactivation by small molecules (presumably anionic or neutral) with access to the active site that could intercept $^{\bullet}OH$.[5,22]

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