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A Simple Proposal That Can Explain the Inactivity of Metal-Substituted Superoxide Dismutases

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Abstract: We propose that the apparent catalytic inactivity of Mn- and Fe-substituted superoxide dismutases (SODs) reflects $E°$s that are either lower (Fe-sub-(Mn)SOD) or higher (Mn-sub-(Fe)SOD) than those of native Fe- or Mn-SODs. In support, we show that the $E°$ of Fe-sub-(Mn)SOD (Fe substituted into Mn-SOD protein) is −240 mV vs NHE, almost 0.5 V lower than our $E°$ of 220 mV for Fe-SOD. The $E°$ of Fe-sub-(Mn)SOD is lower than that of $O_2/O_2^{\bullet-}$ and therefore is sufficient to explain Fe-sub-(Mn)SOD's inactivity. Indeed, Fe-sub-(Mn)SOD is shown to be unable to oxidize $O_2^{\bullet-}$. Alternate causes of inactivity are ruled out by our demonstration that Fe-sub-(Mn)SOD retains the ability to reduce $O_2^{\bullet-}$. Thus, the active site remains active with respect to substrate binding and proton and electron transfer. Finally, we show that Fe-sub-(Mn)SOD's inactivity with respect to $O_2^{\bullet-}$ oxidation cannot be solely due to competitive inhibition by OH^-. Thus, our proposal provides a simple chemical basis for the observed catalytic inactivity of metal-exchanged Mn- or Fe-SODs and suggests that these strongly homologous enzymes may provide important insights into mechanisms of redox midpoint potential tuning in proteins.

Fe- and Mn-containing superoxide dismutases (SODs[1]) constitute a family of closely related enzymes that catalyze the two-step disproportionation of $O_2^{\bullet-}$:

$$O_2^{\bullet-} + M^{3+}\text{-SOD} \rightarrow O_2 + M^{2+}\text{-SOD} \quad (1a)$$

$$O_2^{\bullet-} + M^{2+}\text{-SOD} + 2\,H^+ \rightarrow H_2O_2 + M^{3+}\text{-SOD} \quad (1b)$$

where M signifies the active site Fe or Mn ion.[2,3] The mechanism and kinetic parameters of Fe-SOD and Mn-SOD are similar except that the latter is subject to reversible inhibition by a side reaction.[4] Fe- and Mn-SODs share high amino acid sequence and structural homology, and the active sites are virtually identical.[5] The Fe of Fe-SODs is coordinated in a trigonal bipyramid by three His, an Asp^-, and in most cases a solvent molecule.[6−8] Mn-SODs employ the same ligands[9−12]

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(1) Abbreviations: DCIP, dichloroindophenol; DMSO, dimethyl sulfoxide; $E°$, reduction midpoint potential, $E°'$, reduction midpoint potential at pH 7; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; LMCT, ligand-to-metal charge transfer; NHE, normal hydrogen electrode; SOD, superoxide dismutase, only Fe- and Mn-SODs are discussed in this paper.

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in the same geometry with only very slight differences in ligand side chain rotations or distances to the metal ion.[5] Thus the Fe- and Mn-SODs appear to be variants of the same enzyme. Nonetheless, with the exceptions of a few so-called cambialistic SODs,[13−15] when Mn- or Fe-SOD protein, (Mn)SOD or (Fe)-SOD, is prepared with the other's metal ion in the active site, the resulting Fe-sub-(Mn)SOD (Fe substituted into Mn-SOD protein) or Mn-sub-(Fe)SOD is unable to catalyze disproportionation of $O_2^{\bullet-}$ in the standard assay.[16−19]

Relatively little information is available to explain why many metal-exchanged SODs appear to be catalytically inactive. Possible reasons include distortion of the active site by the nonnative metal ion, inability to bind substrate, and inability to supply the required protons. The non-native metal ion has been reported to bind in the active site similarly to the native metal ion based on EPR and NMR studies.[20,21] Oxidized Fe^{3+}-sub-(Mn)SODs also retain the ability to coordinate substrate analogues[20−22] and thus presumably the substrate itself. However, Yamakura et al. have observed that the p*K* of 8.5−9 of the oxidized active site,[23] which is ascribed to coordination of OH^- to Fe^{3+} in Fe^{3+}-SOD,[24] is depressed to near 7 in Fe^{3+}-sub-(Mn)SOD from *S. marcescens*[20] and lower in Fe^{3+}-sub-(Mn)SOD from *E. coli*.[25] We note that since OH^- acts as a competitive inhibitor, the higher affinity for OH^- could render Fe^{3+}-sub-(Mn)SOD more susceptible to inhibition by OH^- than native Fe^{3+}-SOD and thus account for its lower activity which increases at decreasing pHs. Alternately, or in addition, metal-substituted SODs might be unable to oxidize or reduce substrate.

We propose that Fe-sub-(Mn)SODs and Mn-sub-(Fe)SODs appear inactive, at least in part, because the *E*° of the substituent metal ion is either too low or too high, respectively, to mediate both half-reactions effectively.[26] Specifically, because the *E*°s of the 3+/2+ couple of high-spin Mn compounds are typically significantly higher than the *E*°s of analogous Fe complexes,[27] we note that Mn-specific SOD proteins must depress the *E*° of Mn^{3+}/Mn^{2+} considerably more than Fe-specific proteins depress that of Fe^{3+}/Fe^{2+}, to achieve the optimal *E*°′ of ≈0.36 V (vs NHE) and the *E*°s of 0.2−0.4 V observed in most Fe- and Mn-SODs.[28] Therefore, we conjecture that, when Fe is bound in the (Mn)SOD protein, its *E*° is depressed to a value well below 0.2−0.4 V and, similarly, the *E*° of Mn bound in (Fe)-SOD is insufficiently depressed, to a value well above 0.2−0.4 V. This simple, chemically rational hypothesis is both informative and testable and is supported by the experiments described below.

Materials and Methods

Mn-SOD and Fe-SOD were purified from *E. coli* [29,30] and had activities of 4500−6000 and 6000−7000 units/mg of protein, respectively. The activities of native and metal-exchanged SODs were measured at pH 7.8 in the "standard" indirect assay of McCord and Fridovich.[16] Fe-sub-(Mn)SOD was prepared from Mn-SOD with a yield close to 100%, as was Mn-sub-(Fe)SOD, building on published examples.[17,18] Briefly, Fe-sub-(Mn)SOD was prepared by partially unfolding Mn-SOD protein in 3.5 M guanidinium HCl and 10 mM EDTA at pH 3.1, dialyzing against EDTA in the presence of 2.5 M guanidinium HCl at pH 8.0, reconstituting with Fe^{2+} at pH 8.0 under N_2, and removing extraneous Fe by dialysis against 1 mM EDTA and 1 mM ascorbate under N_2.[21] Mn-sub-(Fe)SOD was prepared by removing Fe at pH 11 and 37 °C and reconstituting with Mn^{2+} by dialysis at pH 8.5.

Oxidation of Fe^{2+}-SOD and Fe^{2+}-sub-(Mn)SOD by $O_2^{\bullet-}$ was conducted at 25 °C in a medium of 100 mM phosphate, 100 mM KBr, and 0.5 mM glucose supplemented with 500 units of catalase (Sigma No. C-3155) and 50 units of glucose oxidase (Sigma No. G-6891) to eliminate any effects of H_2O_2 and O_2 formed upon spontaneous disproportionation of $O_2^{\bullet-}$. A pH of 7.8 was used, except where stated otherwise. The complete reaction medium including approximately 0.1 mM SOD dimers was kept anaerobic and SOD was reduced by titration with methylviologen or dithionite prior to oxidation by $O_2^{\bullet-}$. One to four times stoichiometric[31] aliquots of $O_2^{\bullet-}$ were injected as a stock solution in dry DMSO. Initial additions were approximately one stoichiometric equivalent, but the addition volumes were increased as the system approached the steady state. Injection of DMSO alone produced no effect. Reduction of Fe^{3+}-SOD and Fe^{3+}-sub-(Mn)SOD by $O_2^{\bullet-}$ was conducted similarly after degassing but not reducing the reaction mixture. Oxidation of Fe^{2+}-SOD or Fe^{2+}-sub-(Mn)SOD by O_2 was achieved by injection of 60 mL of O_2 at atmospheric pressure directly into the medium, which was prepared without glucose oxidase or glucose for these experiments, and reduced as above. Reduction by H_2O_2 was performed by injecting 1.5 stoichiometric equivalents of H_2O_2 in aqueous solution into the medium, initiating a drop in absorbance in Fe^{3+}-SOD when catalase was absent.

Potentiometric titrations were performed at 25 °C in an optical cell analogous to the one described by Stankovich.[32] A combination Ag|AgCl and Pt electrode was inserted in one port, a syringe containing titrant was mounted in a second, and the third port was connected to a vacuum line and maintained a low flow of N_2 gas treated to remove residual O_2. The reaction mixture comprised 100 mM phosphate buffer at pH 7.4 (Fe-SOD) or pH 7.8 (Fe-sub-(Mn)SOD), 100 mM KBr, 0.1−0.2

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mM SOD dimers, and 25 μM *p*-benzoquinone, 10 μM DCIP, or 200 μM benzylviologen.[33] Before the titration and after each addition of titrant, the approach to equilibrium was monitored optically using the absorbance of Fe^{3+}-SOD (or Fe^{3+}-sub-(Mn)-SOD) between 350 and 370 nm and the optical signature(s) of the mediator and titrant, and electronically using the potential measured by the electrode. At equilibrium, the optical spectrum was recorded and in many instances a sample was withdrawn for EPR spectroscopy. The percent oxidation of SOD (as well as the mediator) was determined from the optical absorbance at wavelengths chosen to minimize interference from the signals of the solvents, titrant, and mediator.[34] Alternately or in addition, the percent oxidation was determined from the amplitude of the EPR signal of SOD in comparison with a sample of fully oxidized SOD, taking into account the sample concentrations. The percent oxidation was plotted as a function of the reduction potential at equilibrium. The data were fit with the Nernst equation with allowance for multiple (or fractional) electrons per redox event, $E = E^\circ + 0.059/n \log(\mathrm{Ox}/(100 - \mathrm{Ox}))$, where E is the measured ambient potential in V, n is the number of electrons, Ox is the measured percent oxidized SOD, and E° is the reduction midpoint potential, as well as the Nernst equation assuming a single electron event, $E = E^\circ + 0.059 \log(\mathrm{Ox}/(100 - \mathrm{Ox}))$. The standard errors from the fits are quoted, and we estimate the experimental uncertainty associated with our E° values to be $\approx\pm$15 mV. For each titration, the potential obtained with the Nernst equation assuming a one-electron event was within error of the potential obtained using the more general form.

Although the mediators and the potential responded rapidly to titrant additions, they equilibrated slowly with SOD, with approximate half-times ranging from 15 min to 2 h and reaching equilibrium after up to 6 h. The system was deemed to have equilibrated when the change in potential with time became equal to the drift rate of $\approx$8 mV/h, determined in the absence of titrants. Because such long periods were required to achieve equilibrium, with most of the 15 mediators tried,[35] few mediators were sufficiently stable in aqueous solution to be useful. Finally, only potential mediators with an E° close to that of SOD are appropriate for use as mediators in a titration. Thus, although many mediators were tried, only two were effective for titrations of Fe-SOD and only one was effective for Fe-sub-(Mn)SOD. Oxidative titrations were performed using O_2 as the titrant, and reductive titrations were performed using either dithionite or methylviologen.

Results

Production of Metal-Exchanged SODs. The data in Table 1 demonstrate that removal of Mn from Mn-SOD is complete and that Fe binds almost stoichiometrically to (Mn)SOD on a per subunit basis. EPR and NMR data[21] demonstrate that Fe binds specifically in a single well-defined site, and the fact that reconstitution with a mixture of equal concentrations of Fe^{2+} and Mn^{2+} results in SOD with 40% of native Mn-SOD activity indicates that Fe^{2+} competes for the active site. However, Fe-sub-(Mn)SOD exhibits no detectable catalytic activity at pH 7.8[36] based on the standard assay.[16]

Table 1. Fe Contents, Mn Contents, and Activities of Proteins

sample	Fe content[a]	Mn content[b]	activity[c] (%)
Mn-SOD	0	0.98	100
apo(Mn)SOD	0	0	0
Fe-sub-(Mn)SOD	0.95	0	0

[a] Fe content was measured colorimetrically[46] and is expressed on a per subunit basis. The uncertainty is estimated to be 0.03/subunit. [b] Mn content was measured by EPR and is expressed on a per subunit basis. The detection limit is less than 1 μM Mn or 0.005/subunit, and the uncertainty is approximately 3%. [c] Catalytic activity was measured in the standard xanthine oxidase indirect assay at pH 7.8[16] which has a detection limit corresponding to less than 0.1% of native SOD activity and an uncertainty of $\approx$100 units/mg of protein for activities in the range of 5000 units/mg.

Activity with Respect to the Two Half-Reactions. The disproportionation of $O_2^{\bullet-}$ proceeds via alternating oxidation of $O_2^{\bullet-}$ with reduction of Fe^{3+} to Fe^{2+} and reduction of $O_2^{\bullet-}$ with reoxidation of Fe^{2+} to Fe^{3+} (eq 1).[2,3] To clarify the nature of the inactivity of Fe-sub-(Mn)SOD, we have assessed its ability to undergo each of the two half-reactions. Fe-sub-(Mn)-SOD's ability to reduce $O_2^{\bullet-}$ was evaluated via the reaction between (reduced) Fe^{2+}-sub-(Mn)SOD and $O_2^{\bullet-}$ to form (oxidized) Fe^{3+}-sub-(Mn)SOD, presumably by reaction 1b. The course of SOD oxidation was monitored via the visible absorbance of the active site Fe^{3+}. Thus, the results are not complicated by sites that have failed to bind Fe. Furthermore, optical and EPR comparisons of Fe-sub-(Mn)SOD samples allowed to fully reoxidize after the assays, and Fe^{3+}-sub-(Mn)-SOD prior to reduction, indicate that the Fe^{3+} sites remain intact and very little Fe is released. Spectra of steady-state $O_2^{\bullet-}$-oxidized SODs are shown in Figure 1. The upper panel shows that Fe^{2+}-sub-(Mn)SOD, like Fe^{2+}-SOD (lower panel), is oxidized by $O_2^{\bullet-}$ and therefore can reduce $O_2^{\bullet-}$.

Figure 2 shows that both SODs are oxidized by $O_2^{\bullet-}$ itself, and not the O_2 that results from spontaneous disproportionation of $O_2^{\bullet-}$, as oxidation by O_2 occurs much more slowly on a time scale of minutes and, moreover, is largely suppressed by the inclusion of glucose oxidase in the medium. By contrast, oxidation of SOD by $O_2^{\bullet-}$ is evident as sharp steps in the time courses as it occurs within the temporal resolution of our experiment, consistent with the published second-order rate constant of $5.5 \times 10^8\ s^{-1}\ M^{-1}$ at pH 8.[3] Since a single addition of approximately one stoichiometric equivalent of $O_2^{\bullet-}$ produced $\approx$80% oxidation of Fe-sub-(Mn)SOD, the latter evidently competes very effectively with spontaneous disproportionation at pH 7.8.

Fe^{2+}-sub-(Mn)SOD's ability to reduce $O_2^{\bullet-}$ demonstrates that it can both interact with substrate and transfer electrons to it and also suggests that Fe^{2+}-sub-(Mn)SOD can donate proton-(s) to substrate, assuming reaction (1b). However, whereas Fe-SOD reaches a steady-state oxidation level of $\approx$50% (Figure 1) consistent with comparable rates of SOD oxidation and reduction,[2] Fe-sub-(Mn)SOD reaches a steady-state oxidation level of $\approx$100%, indicating that oxidation of Fe^{2+}-sub-(Mn)-SOD by $O_2^{\bullet-}$ is much faster than rereduction of Fe^{3+}-sub-(Mn)-SOD (and oxidation of $O_2^{\bullet-}$).

When $O_2^{\bullet-}$ is added to degassed Fe^{3+}-SOD, the Fe^{3+} is reduced until a steady-state level of $\approx$50% is reached (Figure 3, bottom), confirming that Fe^{3+}-SOD can oxidize $O_2^{\bullet-}$ at a

(33) A somewhat higher concentration of benzylviologen was used because the effective mediating species near -240 mV is the benzylviologen dimer ($K_D = 52$ mM), whose E° was confirmed to be -260 mV under our conditions by cyclic voltammetry. We confirmed the literature values of the potentials of all the mediators used by cyclic voltammetry.

(34) Of the titrants, solvents, and mediators added in the course of titrations, only benzylviologen had a signal that significantly overlapped that of SOD.

(35) Benzylviologen, *p*-benzoquinone, DCIP, indophenol, *p*-aminophenol, 2,3,5,6-TMPD (tetramethylphenylaminediamine), *N*′,*N*′,*N*′,*N*′-TMPD, 1,5-anthroquinone, 2,6-anthroquinone, naphthoquinone, phenosaphranine, nitrofurazone, potassium ferrocyanide, methyl red, and methyl blue.

(36) 100% of the starting activity was recovered upon Mn reconstitution, demonstrating that the (Mn)SOD protein remains intact.

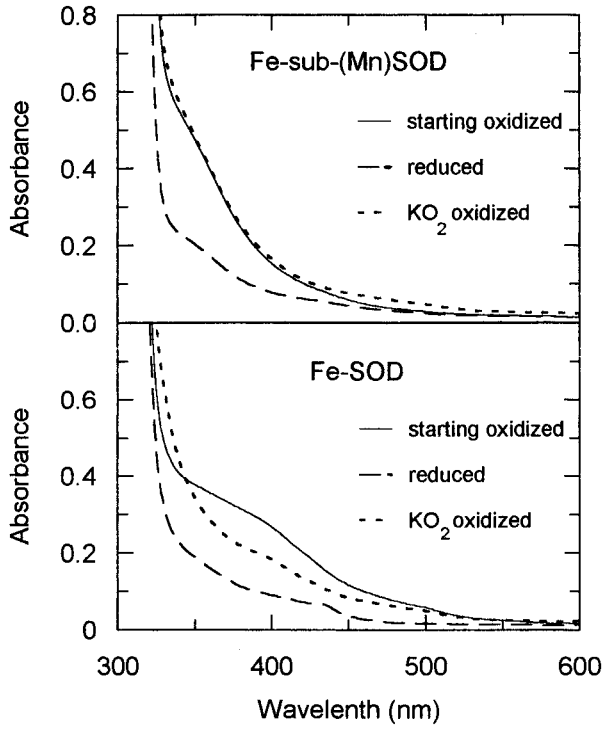

Figure 1. Steady-state oxidation of Fe^{2+}-sub-(Mn)SOD (top) and Fe^{2+}-SOD (bottom) by $O_2^{\bullet-}$ at pH 7.8. Optical spectra are shown of Fe-sub-(Mn)SOD and Fe-SOD fully oxidized before reduction (solid lines), fully reduced at the beginning of the assay (dashed lines), and after the SOD had reached a steady-state level of oxidation after numerous $O_2^{\bullet-}$ additions (dotted lines). The weak absorbance near 460 nm is due to DMSO.

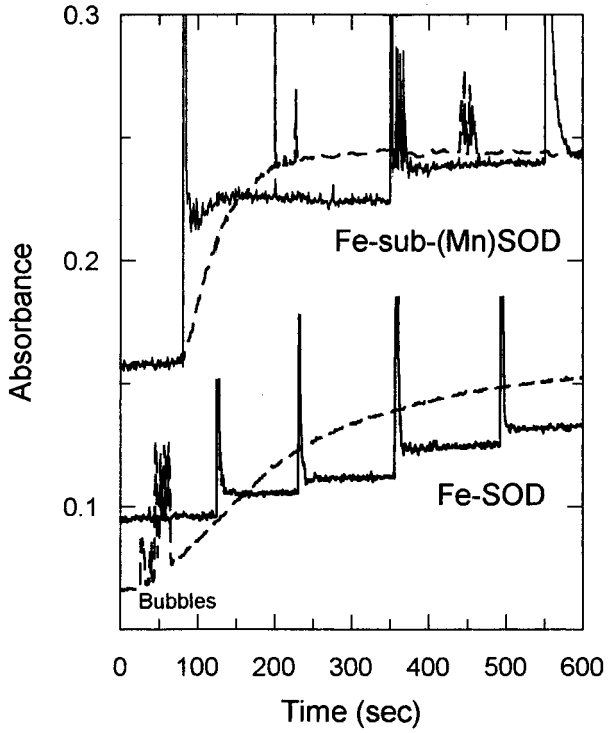

Figure 2. Time courses of the oxidation of Fe^{2+}-sub-(Mn)SOD (top) and Fe^{2+}-SOD (bottom) by $O_2^{\bullet-}$ or O_2 at pH 7.8. The spikes mark roughly stoichiometric additions of KO_2 in dry DMSO. These resulted in oxidation of SOD as shown by the solid line traces. Alternately, 60 mL of O_2 gas at atmospheric pressure was injected directly into the medium. The course of SOD oxidation by O_2 is shown by the dashed line traces. Passage of bubbles through the light path, effects of mixing, and lamp instabilities are evident as clusters of peaks and spikes in the traces. Vertical offsets were applied to individual traces in making the figure.

rate comparable to the rate at which it reduces $O_2^{\bullet-}$. Figure 4 shows that the catalase present in the reaction medium completely suppresses (third trace) reduction of SOD by H_2O_2 (bottom trace). Thus, any reduction of SOD is due to reaction with $O_2^{\bullet-}$, not H_2O_2 formed upon spontaneous disproportionation of $O_2^{\bullet-}$. In contrast, when $O_2^{\bullet-}$ is added to Fe^{3+}-sub-(Mn)SOD, the Fe^{3+} is not reduced, directly demonstrating that Fe^{3+}-sub-(Mn)SOD cannot accept an electron from $O_2^{\bullet-}$ (Figures 3 and 4). This can explain Fe-sub-(Mn)SOD's catalytic inactivity.

Since Figure 1 establishes that Fe^{2+}-sub-(Mn)SOD can transfer an electron to $O_2^{\bullet-}$, it is most likely that electron transfer per se is possible in the opposite direction too, but not observed either because it is not thermodynamically favorable and/or because $O_2^{\bullet-}$ binding is inhibited specifically in Fe^{3+}-sub-(Mn)-SOD.

Inhibition of $O_2^{\bullet-}$'s Interaction with Oxidized SOD by OH^-. $O_2^{\bullet-}$ binding to Fe^{3+}-SOD is believed to be competitively inhibited by OH^- binding to Fe^{3+}, with a pK of 8.5−9.[2,23,24] This and decreased Fe^{2+}-SOD affinity for substrate associated with a pK of ≈8.5[2,30] are believed to be responsible for the decrease in Fe-SOD activity above pH 8.5.[2] Since the pK of the oxidized state drops to 6.7 in Fe^{3+}-sub-(Mn)SOD,[21] reaction (1a) could be much more susceptible to OH^- inhibition. However, reaction (1b) is not expected to be inhibited at pH 7.8 in Fe-sub-(Mn)SOD because the pK of Fe^{2+}-sub-(Mn)SOD remains well above 7.8, at ≈9.2.[21] At pH 7.8, we expect that (1a) could be slowed by a factor of up to 14 by inhibition by OH^- [37] but that even then the reaction between Fe^{3+}-sub-(Mn)-SOD and $O_2^{\bullet-}$ should occur faster than $O_2^{\bullet-}$ disappears and thus be observable.[38]

At pH 6.7, inhibition of (1a) by OH^- should be decreased to less than a factor of 2 and 50−67% steady-state oxidation of Fe-sub-(Mn)SOD by $O_2^{\bullet-}$ is predicted.[39] Figure 5 (top) shows that, despite the more rapid spontaneous disproportionation of $O_2^{\bullet-}$ at pH 6.7, Fe^{2+}-sub-(Mn)SOD can compete for $O_2^{\bullet-}$ and become oxidized. However, essentially 100% steady-state oxidation is still observed, suggesting that the reaction between $O_2^{\bullet-}$ and Fe-sub-(Mn)SOD is inhibited by more than just OH^- binding. This is confirmed by Figure 5 (bottom), which shows no perceptible reduction of Fe^{3+}-sub-(Mn)SOD by $O_2^{\bullet-}$. Thus, either inhibition of the oxidized state by OH^- is not the only reason for the inactivity of Fe-sub-(Mn)SOD or the pK govern-

(37) Assuming $V = V_{max}[S]/(K_M(1 + ([I]/K_I)) + [S])$ and I signifies the competitive inhibitor, the maximum extent of competitive inhibition occurs at low [S], where $K_M \gg [S]$ and the rate is decreased by a factor of $f = 1 + ([I]/K_I) = 1 + 10^{(pH-pK)}$ in our case, since pK is the pH at which the inhibitor OH^- binds to 50% of the sites. Thus, for pH = 7.8, pK = 6.7, and $[S] \ll K_M$, competitive inhibition should slow the rate of (1a) from k_{1a} to k_{1a}/f, $f = 14$. At greater [S], k_{1a} will be decreased less (f will be smaller). The formalism of competitive inhibition is applied here although available evidence strictly indicates only that binding of OH^- to the oxidized state of SOD is mutally exclusive with reaction of that state with substrate. Thus the kinetic mechanism of inhibition appears to be competitive, but competition between OH^- and $O_2^{\bullet-}$ for the same binding site has not been proven.

(38) The reaction rate for the native enzyme is estimated to be at least 2 orders of magnitude faster than the rate of spontaneous disappearance of $O_2^{\bullet-}$ at pH 7.8.

(39) The fractional level of steady-state oxidation is $K_{ox}/(K_{ox} + 1)$, where the equilibrium constant K_{ox} is k_{1b}/k_{1a}. Competitive inhibition of reaction 1a decreases k_{1a} to k_{1a}/f, where $1 \le f \le 1 + 10^{(pH - pK)}$. Since native Fe-SOD has $K_{ox} \approx 1$, competitive inhibition should produce a steady-state oxidation level of up to $f/(f + 1)$. At pH 6.7, $0.50 < f/(f + 1) < 0.67$ or 50−67% steady-state oxidation is anticipated due to competitive inhibition. Even assuming the pK value of 6.1 reported by Yamakura,[25] between 50% and 83% steady-state oxidation is expected.

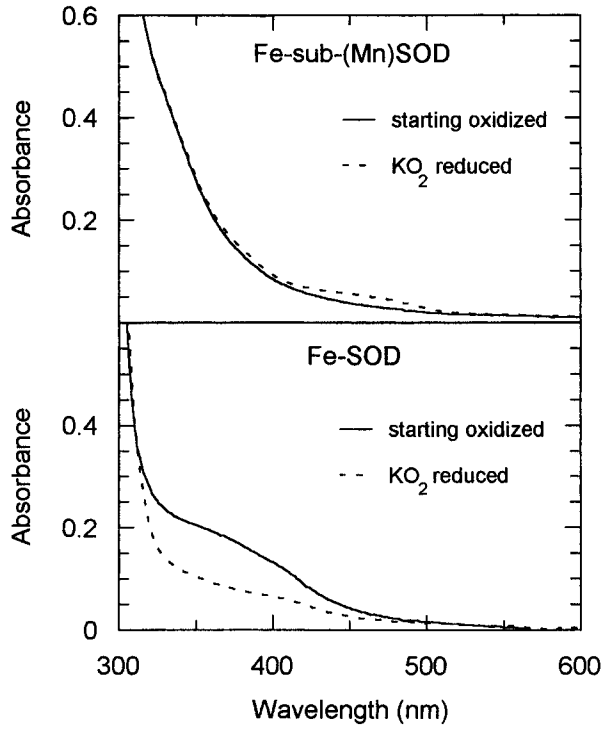

Figure 3. Steady-state reduction of Fe^{3+}-sub-(Mn)SOD (top) and Fe^{3+}-SOD (bottom) by $O_2^{\bullet-}$ at pH 7.8. Optical spectra are shown of Fe-sub-(Mn)SOD and Fe-SOD fully oxidized at the start of the assay (solid lines) and once the assay had reached a steady-state level of oxidation (dotted lines). Weak absorbance near 460 nm is due to DMSO.

ing inhibition is significantly lower than the pK of 6−7 describing the optical signal.

Comparison of the $E°$s of Fe-SOD and Fe-sub-(Mn)SOD. Alternately, the ability of Fe-sub-(Mn)SOD to reduce but not oxidize $O_2^{\bullet-}$ suggests that its $E°$ at pHs 7.8 and 6.7 may be lower than that of $O_2/O_2^{\bullet-}$. Indeed, although Fe-SOD can be reduced by ascorbate, consistent with Fe-SOD's $E°$ of 0.2−0.4 V,[28] Fe-sub-(Mn)SOD cannot.[40] This suggests that the $E°'$ of Fe-sub-(Mn)SOD is lower than ascorbate's $E°'$ of 0.058 V and, thus, substantially lower than the $E°'$ of Fe-SOD.

Measurement of the $E°$s of Fe- and Mn-SODs is very difficult and notoriously error-prone. This is largely because SOD does not equilibrate readily with most of the commonly used mediators.[2,41] Thus, some of the reported apparent $E°$s of SOD may be ascribed instead to the mediator. We have used specially designed glassware and mediators that are exceptionally long-lived in aqueous solution to maintain stable conditions for the 4−6 h necessary for SOD to equilibrate. Our criteria for $E°$ determinations were (1) that the same $E°$ value should be obtained with different mediators (which themselves have distinct $E°$s) or that a single mediator should produce different effects with the two different SODs, as a demonstration that the $E°$ obtained is that of SOD, not the mediator; (2) that the same $E°$ be obtained from data collected in reductive and oxidative titrations; and (3) that titrations exhibit Nernstian behavior. To our knowledge, only criterion 3 has been satisfied by previously published titrations of Fe-SOD.

Figures 6 and 7 show titrations of Fe-SOD and Fe-sub-(Mn)-SOD. An $E°$ of 223 ± 6 mV was obtained for Fe-SOD (Figure 6) from the combination of data from an oxidative titration with DCIP as the mediator (yielding an $E°$ of 235 ± 4 mV), a reductive titration with DCIP (yielding an $E°$ of 201 ± 2 mV), an oxidative titration with *p*-benzoquinone (yielding an $E°$ of 226 ± 2 mV), and a reductive titration with *p*-benzoquinone (yielding an $E°$ of 212 ± 16 mV). These values agree well with each other, indicating that they represent the $E°$ of Fe-SOD, not the mediator. The similarity of the $E°$ values obtained from oxidative and reductive titrations confirms that the titration points reflect Fe-SOD very close to equilibrium.

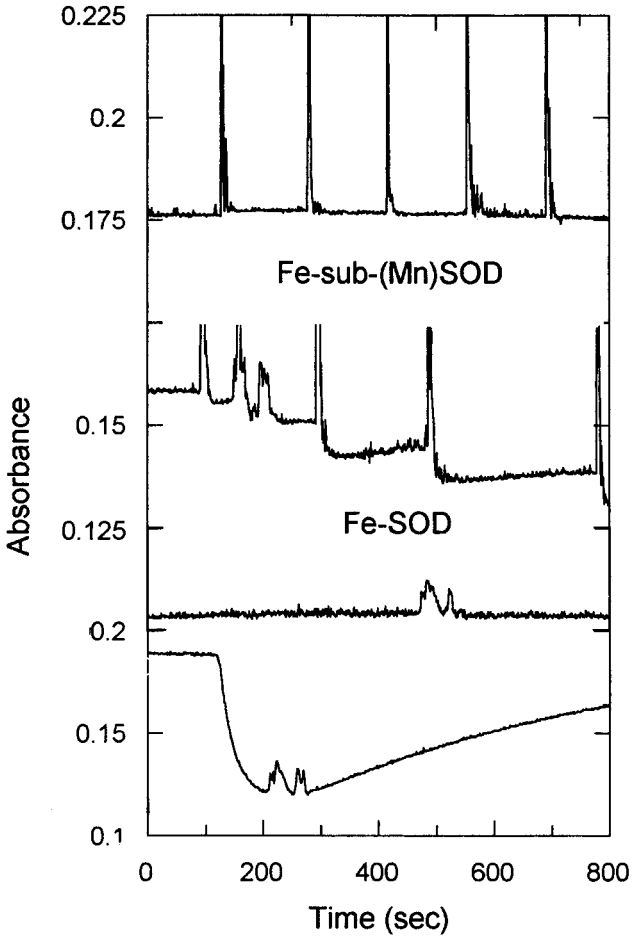

Figure 4. Time courses of the reduction of Fe^{3+}-sub-(Mn)SOD (top) and Fe^{3+}-SOD by $O_2^{\bullet-}$ (second) or H_2O_2 (third and fourth) at pH 7.8. The spikes mark roughly stoichiometric additions of KO_2 in DMSO in the upper two traces. Injection of 1.5 stoichiometric equivalents of H_2O_2 in the absence of catalase initiated the drop in absorbance of Fe^{3+}-SOD seen in the bottom trace. In the third trace, the H_2O_2 addition time was within 10 s of the addition time for the bottom trace, but the effects on SOD are suppressed by the presence of catalase. Lamp instabilities are evident as clusters of peaks in the traces.

Titration of Fe-sub-(Mn)SOD in the presence of benzylviologen yielded an $E°$ of −243 ± 2 mV (Figure 7). Benzylviologen was the only mediator found that would equilibrate with Fe-sub-(Mn)SOD and was stable in aqueous medium. Benzylviologen completely and relatively rapidly reduced Fe^{3+}-SOD, instead of equilibrating with it, as with Fe-sub-(Mn)SOD. Thus, we believe that the titration behavior observed for Fe-sub-(Mn)SOD specifically reflects the latter, not the benzylviologen. The degree of oxidation of Fe-sub-(Mn)SOD was determined by EPR and optical spectroscopy. The EPR signal of Fe^{3+}-sub-(Mn)SOD is centered near $g' = 4.3$, far from the signals of free radicals, and the optical absorption of the benzylviologen radical has a minimum at 350 nm. Thus, the absorbance at 350 nm was used to measure the amount of Fe^{3+}-

(40) Ascorbate's inability to reduce Fe^{3+}-sub-(Mn)SOD is unlikely to be due to failure to interact with the Fe^{3+} since ascorbate interacts with the Fe^{3+} of Fe^{3+}-SOD as well as the Mn^{3+} of Mn^{3+}-SOD.

(41) Verhagen, M. F. J. M.; Meussen, E. T. M.; Hagen, W. R. *Biochim. Biophys. Acta* **1995**, *1244*, 99−103.

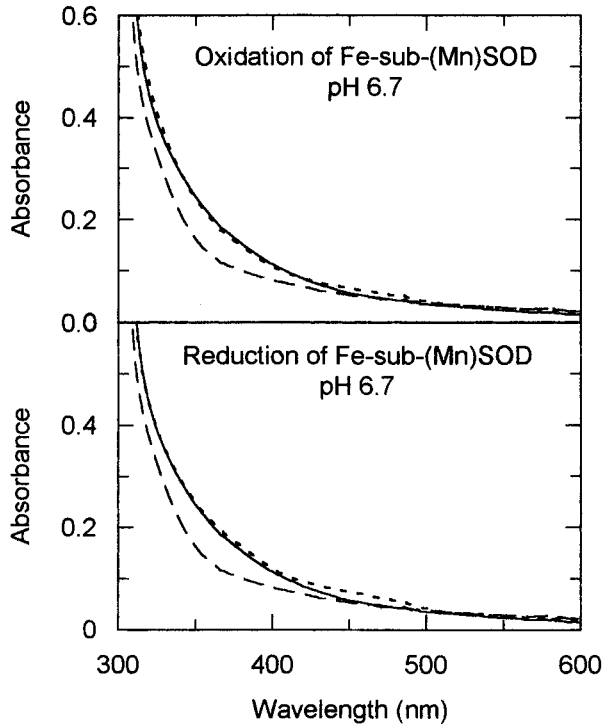

Figure 5. Oxidation of Fe^{2+}-sub-(Mn)SOD by $O_2^{\bullet-}$ at pH 6.7 (top) and reduction of Fe^{3+}-sub-(Mn)SOD by $O_2^{\bullet-}$ at pH 6.7 (bottom). (top) Optical spectra are shown of Fe-sub-(Mn)SOD fully oxidized before reduction (solid line), reduced at the beginning of the assay (dashed line), and after the SOD had reached a steady-state level of oxidation after multiple $O_2^{\bullet-}$ additions (dotted line). (bottom) Optical spectra of Fe-sub-(Mn)SOD fully oxidized before reduction (solid line) and after the SOD had reached a steady-state level of reduction after multiple $O_2^{\bullet-}$ additions (dotted line). Dithionite was added after the assay was complete to confirm that the SOD could be fully reduced (dashed line). The weak absorbance near 460 nm is due to DMSO. The pH did not change during the assay.

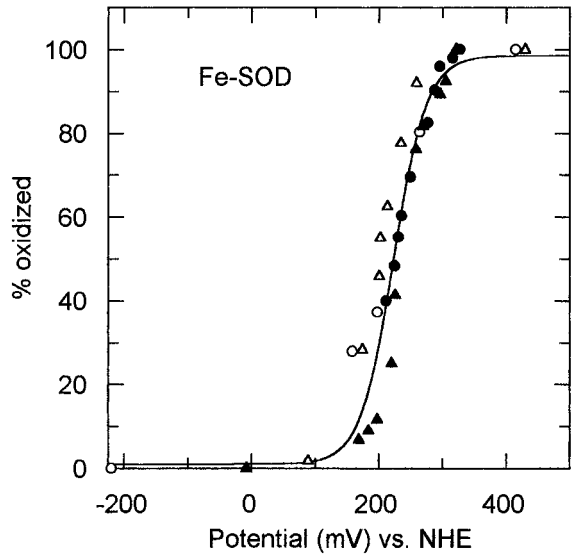

Figure 6. Potentiometric titrations of Fe-SOD. The percent oxidation of Fe-SOD evaluated by optical spectroscopy in the course of an oxidative titration by O_2 (▲) or a reductive titration by dithionite (△) both in the presence of DCIP, or an oxidative titration by O_2 (●) or a reductive titration by dithionite (○) both in the presence of benzoquinone, is plotted as a function of the reduction potential measured using an Ag|AgCl and Pt combination electrode. The best fit of the Nernst equation to the data is shown as a solid line ($E° = 223 \pm 6$ mV).

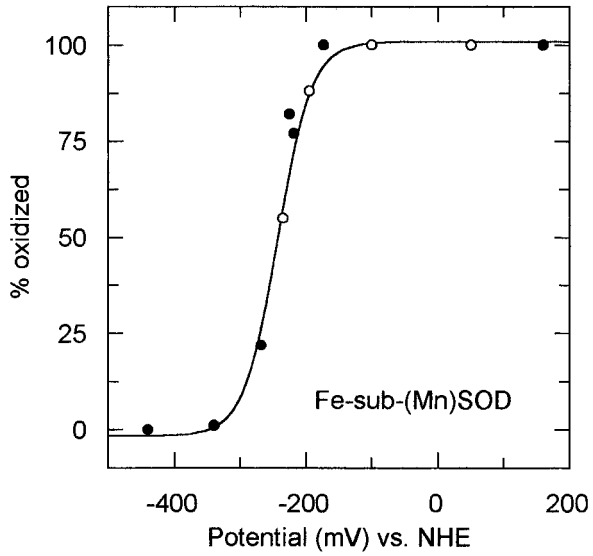

Figure 7. Potentiometric titrations of Fe-sub-(Mn)SOD. The percent oxidation of Fe-sub-(Mn)SOD evaluated by optical spectroscopy (○) or by EPR spectroscopy (●) is plotted as a function of the reduction potential measured using an Ag|AgCl and Pt combination electrode, in the presence of benzylviologen. The best fit of the Nernst equation to the data is shown as a solid line ($E° = -243 \pm 2$).

sub-(Mn)SOD present when benzylviologen was largely oxidized and could easily be accounted for. At low potentials, where substantial benzylviologen radical was present, the percent of oxidized Fe-sub-(Mn)SOD was determined by EPR. The agreement of the EPR and optical data on a single $E°$ confirms the noninterference of benzylviologen with the quantitation of Fe^{3+}-sub-(Mn)SOD (Figure 7), and the independence of the shape of the EPR signal on the presence of benzylviologen or the reduction potential indicates that the benzylviologen does not interact with the Fe^{3+} site (Supporting Information, Figure S2). Finally, samples from several different preparations of Fe-sub-(Mn)SOD were all consistent with a single $E°$. The potential of -243 ± 2 mV is almost 0.5 V lower than the $E°$ of Fe-SOD and able to account for the inactivity of Fe-sub-(Mn)-SOD with respect to oxidation of $O_2^{\bullet-}$, since this $E°$ is lower than that of $O_2/O_2^{\bullet-}$ ($=-160$ mV).

Although we have not yet succeeded in measuring the $E°$ of Mn-sub-(Fe)SOD, the fact that our Mn-sub-(Fe)SOD is colorless in air indicates that its bound Mn adopts the 2+ oxidation state instead of the 3+ oxidation state as in (Mn)SOD protein (also Yamakura et al.[25]) and suggests that the $E°$ of Mn-sub-(Fe)-SOD is significantly higher than that of Mn-SOD.

Discussion

Our data show that the inactivity of Fe-sub-(Mn)SOD can be explained by inability to oxidize $O_2^{\bullet-}$. This, in turn, can be explained by a much lower $E°$ for Fe in (Mn)SOD protein than in (Fe)SOD. It does not appear to be due to inhibition of $O_2^{\bullet-}$ binding to Fe^{3+}-sub-(Mn)SOD by OH^-. Thus, we propose that SOD proteins tend to apply $E°$ tuning appropriate to the native metal ion, to whatever metal ion is bound. When a metal ion with an $E°$ lower than the native ion is bound, the result would be a SOD with a lower-than-native potential. Since Mn compounds tend to have higher $E°$s than the analogous Fe compounds,[27] we surmise that (Mn)SODs tend to depress the potential of bound metal ions more than (Fe)SODs do, so that incorporation of Fe into (Mn)SOD results in a SOD with a low $E°$, as we have shown here. We have also obtained preliminary

evidence in support of the corollary that Mn-sub-(Fe)SOD should have a significantly higher-than-native $E°$.

Our arguments are applicable to either inner or outer sphere mechanisms for SOD, although the language used implies an inner sphere mechanism. An inner sphere mechanism is inferred for (1a) by the fact that competitive inhibitors F^- and N_3^- coordinate to Fe^{3+}. However, OH^- binding to oxidized SOD and reaction with $O_2^{\bullet-}$ could be mutually exclusive without OH^- and $O_2^{\bullet-}$ competing for a single binding site. Similarly, the requirement that the $E°$ of SOD be higher than that of $O_2/O_2^{\bullet-}$ in order for the enzyme to be active[42] applies regardless of the mechanism.

We also note that the enhancement of Fe-sub-(Mn)SOD activity at low pH[20,25] is consistent with a depressed $E°$, as well as inhibition by OH^-, since decreasing the pH would tend to increase the protonation of groups surrounding the active site and thus raise the $E°$ of SOD, but not that of $O_2/O_2^{\bullet-}$.

Since SODs must mediate both the reduction and the oxidation of their substrate, there is both an upper and a lower bound on the range of $E°$s which can support activity, and only SODs with $E°$s near the middle of the range are expected to turn over rapidly. Thus, SOD activity is exceptionally sensitive to the $E°$ of the metal ion, and the protein may be expected to have been refined by evolution to achieve a close to optimal $E°$.

Indeed, the difference between the $E°$s of Fe-SOD and Fe-sub-(Mn)SOD is very large and remarkable given that the (Fe)-SOD and (Mn)SOD proteins of *Escherichia coli* are expected to have very similar structures and the same amino acid ligand sets based on the similarity of their amino acid sequences and the strong structural homologies between all Fe- and Mn-SODs whose structures have been determined to date. Similarly, electrostatic potential calculations carried out on the structures of *E. coli* Fe-SOD and *Thermus thermophilus* Mn-SOD neglecting contributions from ligand residues indicate relatively small differences in the potential at the metal ion in the two proteins.[43] However, the large difference between the $E°$s of Fe-SOD and Fe-sub-(Mn)SOD suggests that it stems from some difference in the ligand sphere.

Since the identities of the ligand amino acids have been found to be invariant in all SODs investigated so far, we are investigating the possibility that the degree of protonation of a ligand, most importantly the coordinated solvent, is different in at least one oxidation state of Fe-SOD and Fe-sub-(Mn)-SOD. Density functional calculations have suggested that the $E°$ of the Mn of human Mn-SOD would be 1.3 V lower if the coordinated solvent were OH^- than if it were H_2O.[44] Thus, different coordinated solvent protonation in the two proteins could account for the large difference between the $E°$s we observe. Moreover, a difference in the degree of protonation could be effectively distributed over several residues in the active site in addition to the coordinated solvent, making it very difficult to detect via such indirect indicators as the metal-to-O distance measured in crystal structures. Such a distributed proton might nonetheless produce a comparable effect on $E°$ to that of a discrete additional proton on the coordinated solvent (or another ligand), as computations of the localization of the electron density added upon reduction of SOD show that it is extensively delocalized over the metal ion and the ligands.[44]

It is also possible that the conformation of the active site is altered upon binding the non-native metal ion so that the homology of the structures of the native Mn- and Fe-SODs does not extend to the metal-exchanged SODs, in at least one oxidation state.[21]

Concluding Remarks

Our data show that Fe-sub-(Mn)SOD retains ability to reduce $O_2^{\bullet-}$ but fails to catalyze the dismutation because it is unable to oxidize $O_2^{\bullet-}$. Furthermore, we show that this cannot be due solely to competitive inhibition by OH^- and that the $E°$ of Fe-sub-(Mn)SOD is significantly lower than that of Fe-SOD. Therefore, we propose that Fe-sub-(Mn)SOD (and Mn-sub-(Fe)-SOD) is unable to catalyze the complete catalytic cycle, at least in part, because the metal ion $E°$ is too low (or high) as a result of placement of the low potential metal ion in the protein that more strongly depresses $E°$ (or the high potential ion in the protein that depresses $E°$ less). This simple chemically rational proposal can explain observations dating back to 1976.[45] Our evidence that the (Fe)SOD and (Mn)SOD proteins have very different effects on the $E°$ of a bound metal ion, despite their superimposable backbone structures and identical amino acid ligand sets, suggests that the SOD proteins will be excellent systems for elucidating mechanisms by which proteins tune the $E°$s of bound metal ions in a given structural context and metal binding site.

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Supporting Information Available: Two figures, one showing optical spectra of Fe-SOD collected at a range of reduction potentials and the other showing EPR spectra of Fe-sub-(Mn)SOD at a range of reduction potentials (2 pages). See any current masthead page for ordering and Internet access instructions.

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(42) Note that in vivo the relevant reduction potential is the $E°$ of $O_2/O_2^{\bullet-}$ minus RT/F $\ln([O_2^{\bullet-}]/[O_2])$, where R is the gas constant, T is the absolute temperature, and F is the Faraday constant.

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