Redox Properties of Human Manganese Superoxide Dismutase and Active-Site Mutants†

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ABSTRACT: The redox potential of human manganese superoxide dismutase (MnSOD) has been difficult to determine because of the problem of finding suitable electron mediators. We have found that ferricyanide and pentacyanoaminoferrate can be used as electron mediators, although equilibration is very slow with a half-time near 6 h. Values of the midpoint potential were determined both by allowing enzyme and mediators to equilibrate up to 38 h and by reductive titration adding dithionite to enzyme and mediator. An overall value of the midpoint potential was found to be 393 ± 29 mV. To elucidate the role of His30 and Tyr34 in the active site of human MnSOD, we have also measured the redox properties of the site-specific mutants His30Asn (H30N) and Tyr34Phe (Y34F) and compared them with the wild-type enzyme. Crystal structures have shown that each mutation interrupts a hydrogen bond network in the active site, and each causes a 10-fold decrease in the maximal velocity of catalysis of superoxide dismutation as compared with wild type. The present study shows that H30N and Y34F human MnSOD have very little effect, within experimental uncertainty, on the redox potential of the active-site metal. The redox potentials determined electrochemically were 365 ± 28 mV for H30N and 435 ± 30 mV for Y34F MnSOD. These results suggest that the role of His30 and Tyr34 is more in support of catalysis, probably proton transport, and not in the tuning of the redox potential.

Superoxide dismutases have midpoint potentials that lie between +200 and +400 mV at pH 7 and are optimized to catalyze efficiently both the oxidative and reductive reactions of $O_2{\cdot}^-$ dismutation (*1*−*5*). The standard midpoint potential of free manganese in solution ($Mn^{2+/\ 3+}$) is 1510 mV, and that of $Fe^{2+/\ 3+}$ is 770 mV (reviewed in ref *6*). Therefore, superoxide dismutases tune the midpoint potential of their respective ligated metals to optimize electron transfer during both reactions of superoxide dismutation. In general, MnSOD[1] must depress the midpoint potential E_m of $Mn^{2+/\ 3+}$ by 1000 to 1300 mV, relative to the free metal potential in aqueous solution, for the E_m to lie halfway between the two redox couples involved in the catalytic dismutation, eqs 1 and 3 (*5*, *6*).

$$O_2{\cdot}^- \rightleftarrows O_2 + e^- \quad E_m = -160\ \text{mV} \tag{1}$$

$$Mn^{2+}SOD \rightleftarrows Mn^{3+}SOD + e^- \qquad -160\ \text{mV} < E_m < +890\ \text{mV} \tag{2}$$

$$H_2O_2 \rightleftarrows O_2{\cdot}^- + 2H^+ + e^- \quad E_m = +890\ \text{mV} \tag{3}$$

Optimal catalytic activity requires optimization of all steps in the mechanism. Since MnSOD acts alternatively as an oxidant (eqs 1 and 2) and a reductant (eqs 2 and 3) during the disproportionation of superoxide, efficient catalysis is achieved when the midpoint potential of the enzyme is about halfway between the respective potentials of the couples $O_2{\cdot}^-/O_2$ and $O_2{\cdot}^-/H_2O_2$.

Measurements of the redox potential of MnSOD and FeSOD have been difficult because of the problem of equilibrating the enzyme with the electrodes. The recent successful measurement of the redox potential of *Escherichia coli* FeSOD and Mn-substituted FeSOD using mediators (*7*) has stimulated these studies of human MnSOD which could not equilibrate with the redox electrode in the absence of mediators. Previous studies (*4*, *8*, *9*) demonstrate that oxidative or reductive titrations of MnSOD without added mediators between the enzyme and the electrode give random potential values that do not correlate with the oxidation state of the enzyme. To alleviate this problem, a mediator is used to transfer electrons between the electrode and the metal ion at the active site. In the present study, a coulometric technique was used for the prescreening of potential mediators (*10*, *11*). The midpoint potentials of wild-type and mutant MnSOD enzymes were determined electrochemically (*12*). Further work showed that coulometric titration of *E. coli* MnSOD using cyclic voltammetry was fairly limited and complex because of the low stability of the enzyme in the presence of a relatively strong electric current (*9*).

The active-site cavity of human MnSOD contains an extensive hydrogen bond network that involves the aqueous ligand of manganese which is hydrogen bonded to Gln143; Tyr 34 and His30 also participate in this network with their side chains adjacent to the metal (Figure 1). Replacement of Gln 143 by site-specific mutagenesis causes a very

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[1] Abbreviations: MnSOD, manganese superoxide dismutase; DCIP, 2,6-dichlorophenolindophenol; NHE, normal hydrogen electrode.

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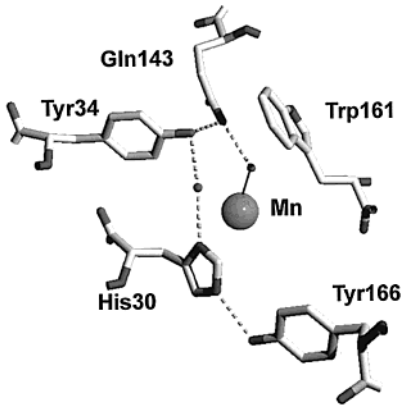

FIGURE 1: The active-site region of human MnSOD showing a hydrogen bond network, from the crystal structure of Borgstahl et al. (*25*). Two water molecules are shown as small spheres. Amino acid ligands of the manganese (His26, His74, His163, Asp159) are not shown.

significant decrease by 2 to 3 orders of magnitude in catalysis of superoxide decay (*13*, *14*). However, replacement of Tyr 34 by Phe (Y34F MnSOD) and replacement of His 30 by Asn (H30N MnSOD) had much smaller effects on catalysis. For each mutant, k_{cat} decreased by about 10-fold, and k_{cat}/K_m was unchanged for Y34F (*15*) or decreased 7-fold for H30N (*16*). The roles in catalysis of Tyr 34 and His 30 are not determined, although the kinetic data have been cited to suggest that the hydrogen bond network supports proton transfer to form product hydrogen peroxide (*15*, *16*). Catalysis by MnSOD involves both proton and electron transfer, and it has not been shown to date whether or how these are coupled. To approach these issues, we have determined the reduction potential of human wild-type MnSOD as well as the two site-specific mutants H30N and Y34F. These two mutations had very little effect on the redox potential of the active site despite their proximity to the metal, and we suggest that they are involved in sustaining proton transfer during catalysis rather than tuning the redox potential.

MATERIALS AND METHODS

Enzymes. Human MnSOD was cloned and overexpressed in *E. coli* using a modification of the protocol from Beck et al. (*17*, *18*). The construct expressed human MnSOD in the *E. coli* strain QC 774 (*sod* A^-/B^-) as a mature protein tagged with an extra methionine at the amino terminus. Culture conditions included either 100 μM $MnCl_2$ (for M9 media) or 1 mM $MnCl_2$ (for 2× YT media). Protein yields were on average 70 mg of pure protein per 50 g of wet bacterial pellet. Purity of the enzyme was determined on SDS−PAGE, which showed one intense band. The PCR-based site-directed mutagenesis for the H30N and Y34F mutants was described earlier (*15*, *16*).

Every batch of pure enzyme was extensively dialyzed against EDTA to remove adventitious metals. We found that dialyzing the enzyme more than three times for 12 h did not lower any further the total manganese content. Atomic absorption spectroscopy was used to measure the total manganese content in each batch of enzyme. The concentration of enzyme was taken as the manganese concentration determined in this manner. The protein concentration was determined using the Lowry assay and compared to the metal content to calculate the manganese content per monomer of enzyme.

Potentiometric Measurements. Redox potential measurements were performed at 25 °C using a computer controlled diode-array spectrophotometer (Hewlett-Packard 8453), a computer controlled combination electrode (Microelectrodes, Inc.), and a custom-made anaerobic cell based on the optical cell described by Stankovich (*11*). The combination electrode (Ag/AgCl and Pt) was inserted in the main port of the cell, while an auxiliary port was used to degas the cell and introduce purified nitrogen. Oxygen was excluded from the nitrogen line via a trap filled with methyl viologen reduced with dithionite in 100 mM KOH, while a second trap filled with indicating desiccant was used to retain humidity before reaching the cell. A second auxiliary port was used to add mediators, oxidizing and reducing agents, and to monitor enzyme concentration. All potential values are reported versus the normal hydrogen electrode.

Mediators. A successful mediator needed to have an intrinsic midpoint potential near that of the enzyme (initially estimated at 350 mV). Ideally, the mediator had to have signature peaks of absorbance in the visible range but not near that of the enzyme (450 to 600 nm). This condition was not essential but desirable to enhance the amount of data retrievable from the absorbance spectra of the enzyme with the mediator. Potential mediators that satisfied these conditions were examined by both cyclic voltammetry and redox titration to check their intrinsic midpoint potentials and relevant extinction coefficients at signature peaks of absorbance. Of 13 potential mediators tested,[2] only two, potassium ferricyanide $K_3Fe(CN)_6$ (Fe(III)) (Mallinckrodt) and sodium pentacyanoaminoferrate $Na_3Fe(CN)_5NH_3$ (Fe(II)) (Aldrich), were effective for titrations of human MnSOD enzymes. In addition, these two mediators approached equilibrium with the enzyme very slowly, with half-times around 6 h. Because such long periods of time were required to approach equilibrium, oxidative and reductive titrations were extremely hard to perform and complicated by enzyme degradation. As a consequence, single point experiments (where enzyme and mediator were allowed to equilibrate in the absence of a titrating agent) were favored over potentiometric titration (see below).

Single Point Experiments. Possible mediators for the redox titration of human MnSOD were tested by single point experiments in which mediator and enzyme were allowed to equilibrate from opposite redox states. A 3-mL solution containing enzyme (at 0.5 to 1 mM concentration of monomers in 100 mM phosphate buffer/100 mM KCl, pH 7.8) was introduced to the anaerobic cell and then sealed with the combination electrode and degassed. An absorbance spectrum was taken as a reference point. The enzyme was then partially reduced with degassed hydrogen peroxide, and a degassed solution containing the mediator was added to the appropriate final concentration (enzyme/mediator from 1:1 to 1:10). The approach to equilibrium was monitored by two methods: optically, by using the absorbance spectrum of Mn^{3+}SOD between 400 and 700 nm (and also of the

[2] The following were tested and rejected as mediators of MnSOD: ferrocene, DCIP, as well as chelated metals including complexes of Cu, Co, Fe with EDTA, diethylenetriaminepentaacetic acid (DTPA), citrate.

mediator at known optical signature(s)) and electrochemically, using the potential recorded by the electrode. Equilibrium was considered attained when the rate of change of the potential with time fell below 1 mV per 20 min (drift due to oxygen leakage into the system and/or protein degradation). The enzyme concentration ($\epsilon_{280} = 40\ 500\ M^{-1}\ cm^{-1}$) and catalytic activity (*14*) were checked before and after each experiment. The fraction of enzyme in the oxidized state was determined from the optical absorbance at 480 nm ($\epsilon_{480} = 600\ M^{-1}\ cm^{-1}$), from which the absorbance of the enzyme in the reduced state ($\epsilon_{480} \leq 30\ M^{-1}\ cm^{-1}$) was deducted. The percent oxidation of the mediator at equilibrium was also calculated from the optical spectra. The percent oxidation of the enzyme was plotted versus the ambient potential (E_h), and E_m was determined from the Nernst equation (eq 4) assuming a single electron transfer ($n = 1$) per active enzyme monomer.

$$E_h = E_m + 59.2 \log(\text{ox/red}) \quad (4)$$

E_h is the measured ambient potential at equilibrium in millivolts (mV), E_m is the midpoint potential obtained from the equation (in mV), ox/red is the ratio of oxidized to reduced enzyme at equilibrium, and $59.2 = 2.303RT/nF$ where $T = 298$ K. Values of the midpoint potential with standard errors were determined by a least-squares fit (Enzfitter, Biosoft) of the Nernst equation to titration data.

Redox Titration. Ferricyanide was used as a mediator for the reductive titration of human MnSOD with dithionite, in the same anaerobic cell as described above. Three milliliters of enzyme at 0.5 to 1 mM monomeric concentration were mixed with $Fe(CN)_6^{3-}$ with an excess of enzyme of 10:1 and degassed. Human MnSOD was titrated by adding 10% aliquots of dithionite as the reducing agent. At each addition of titrant, the system was allowed to equilibrate for up to 8 h, depending on the rate of potential change. Titration was completed when the enzyme was 100% reduced and the potential dropped below 200 mV (versus NHE). Oxidative titration with permanganate was attempted, but the equilibration rate between enzyme and mediator was too slow to allow accurate reading of the potential during reoxidation of the enzyme. As a consequence, the oxidative process was complicated by enzyme degradation and formation of manganese dioxide, and the data could not be fit to the Nernst equation.

RESULTS

Extinction Coefficient. When freshly isolated from *E. coli* (as described in Methods), human MnSOD is mostly oxidized ($90 \pm 5\%$). However, after a few days in storage buffer, the enzyme becomes partially reduced and stabilizes around an oxidation state of about 80% Mn^{3+} SOD. Previous work has discussed why MnSOD becomes partially reduced in an apparent oxidative environment and how the enzyme can be reoxidized (*3*, *5*). Unlike FeSOD, which is readily oxidized in the presence of bubbled oxygen gas, MnSOD is rather unaffected and is only partially reoxidized over a period of many days by this method. We were able to obtain a completely oxidized sample of MnSOD using $KMnO_4$ and found $\epsilon_{480} = 600 \pm 20\ M^{-1}\ cm^{-1}$. This value is slightly higher than that previously reported (*18*), and the difference is probably due to a more complete oxidation of MnSOD in the current work.

Table 1: Midpoint Potentials (E_m), Peak Absorbance, and Extinction Coefficients (ϵ, oxidized form) of Mediators Used to Measure the Redox Properties of Human Wild-Type MnSOD and Mutants[a]

mediator	midpoint potential without enzyme[b] E_m (mV)	midpoint potential with enzyme[c] E_m (mV)	peak absorbance (nm)	extinction coefficient ϵ ($M^{-1}\ cm^{-1}$)
$Fe(CN)_6^{3-/4-}$	435 ± 13	427 ± 13	421	971
$Fe(CN)_5NH_3^{2-/3-}$	403 ± 12	398 ± 18	397	1200

[a] Values of midpoint potentials are the average and standard error of three to six experiments. [b] Obtained by electrochemical titration of the mediator by addition of dithionite and permanganate as the reductive and oxidative agents. No enzyme was present. The solution at 25 °C contained 100 mM KCl and was buffered by 100 mM potassium phosphate with pH adjusted to 7.8 with HCl. [c] Obtained by equilibration titration in the presence of 700 μM wild-type human MnSOD; no titrant (dithionite or permanganate) was added. Conditions as listed in the legend to Figure 2.

Mediators. Titration of mediator alone was performed to determine solubility and stability in phosphate buffer, extinction coefficients, and midpoint potentials. Of 13 potential mediators tested,[2] only the ferrocyanide (Fe(II))/ferricyanide (Fe(III)) and pentacyanoaminoferrate (Fe(II))/Fe(III) redox couples were suitable to measure the redox potential of human MnSOD; that is, these are the only two mediators that achieved redox equilibration with enzyme. The extinction coefficients at optical signatures of $Fe(CN)_6^{3-}$ and $Fe(CN)_5NH_3^{2-}$ were measured in 100 mM KH_2PO_4/100 mM KCl, pH 7.8, and are reported in Table 1. Those values were in good agreement with reported values (*19*).[3]

Single Point Experiments. The two successful mediators, ferricyanide and pentacyanoaminoferrate, were used separately to calculate the intrinsic redox potential of human MnSOD and variants by single point equilibrium experiments. There was a slow redox equilibration between fully oxidized $Fe(CN)_6$ and partially reduced wild-type enzyme (Figure 2). From the spectral data, we observed a reoxidation to Mn^{3+}SOD determined from the absorbance at 485 nm, and a corresponding reduction of the mediator $Fe(CN)_6^{3-}$ from the absorbance at 421 nm (Figure 3), demonstrating that ferricyanide could participate in electron transfer with the metal at the active site. The half-time to equilibrium was around 6 h. The ambient potential and the ratio of concentrations of oxidized to reduced enzyme were substituted into the Nernst equation to derive the midpoint potential (E_m) of human MnSOD. Four experiments were conducted using ferricyanide as a mediator, giving a midpoint potential of 407 ± 21 mV for wild-type human MnSOD. Ferricyanide was also used to determine the midpoint potential of Y34F and H30N MnSOD. Three experiments were conducted for each mutant in which the enzyme was allowed to equilibrate with the mediator. Equilibration times were again slow with half-times up to 8 h. The midpoint potentials are reported in Table 2.

Similar experiments were conducted using pentacyanoaminoferrate as a mediator (data not shown). In this case, the oxidation state of the mediator was followed at 397 nm. The equilibration time between pentacyanoaminoferrate and

[3] M. Stankovich, personal communication.

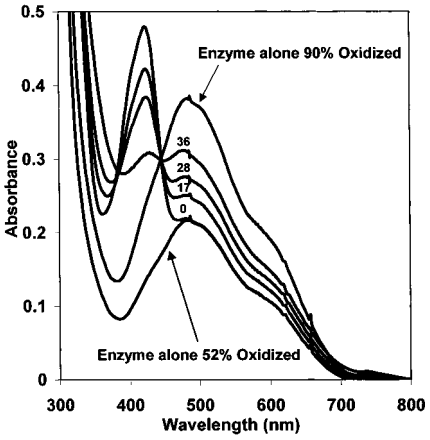

FIGURE 2: Absorbance of human wild-type MnSOD (maximum at 485 nm) and mediator ferricyanide (maximum at 421 nm) showing the equilibrium titration at pH 7.8 and 25 °C. The enzyme was partially reduced with H_2O_2 prior to the addition of fully oxidized $K_3Fe(CN)_6$. Time zero is at the addition of ferricyanide, after which enzyme and mediator were allowed to interact for up to 38 h. Selected traces are at 0, 17, 28, and 36 h. MnSOD was present at 700 μM, $Fe(CN)_6^{3-/4-}$ at 370 μM; the solution contained 100 mM KCl and was buffered by 100 mM potassium phosphate with pH adjusted with HCl.

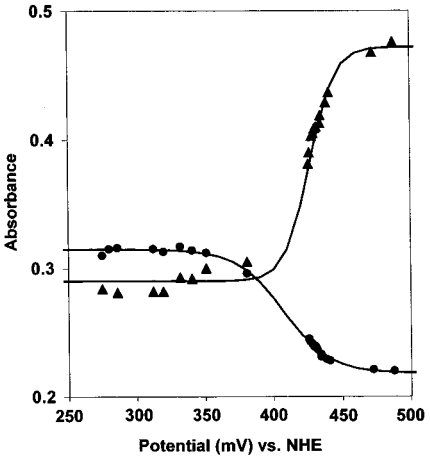

FIGURE 3: Signature peaks during the equilibration of (▲) ferricyanide at 421 nm and (●) MnSOD at 485 nm versus potential. The solid lines are least-squares fits of the Nernst equation (assuming $n = 1$) to the data of Figure 2 yielding midpoint potentials E_m of 407 ± 21 mV for wild-type human MnSOD and 427 ± 13 mV for the ferricyanide/ferrocyanide couple.

enzyme was longer than for ferricyanide; consequently, the amount of data retrievable using pentacyanoaminoferrate as a mediator was less due to enzyme degradation. The midpoint potential (E_m) found for wild-type human MnSOD from two separate experiments using pentacyanoaminoferrate as a mediator was 372 and 383 mV. This result is about 25 mV lower than by using ferricyanide but is within experimental uncertainties. These experiments demonstrate that both $Fe(CN)_6^{3-/4-}$ and $Fe(CN)_5NH_3^{2-/3-}$ can act as mediators with the active site metal of human MnSOD to determine the midpoint potential of the enzyme.

Redox Titration. To check for the reversibility and Nernstian behavior of the intrinsic redox potential of human MnSOD obtained by single point equilibration, we conducted full redox titrations using dithionite and permanganate as reducing and oxidizing agents, respectively. The enzyme was equilibrated with ferricyanide (10:1, enzyme/mediator) and

Table 2: Midpoint Potentials of Human MnSOD and Mutants Obtained by Equilibrium and Redox Titrations[a]

	equilibrium titration E_m (mV)[b]	redox titration E_m (mV)[c]
wild-type MnSOD	407 ± 21 372, 383[d]	395 ± 19
H30N	365 ± 28	*e*
Y34F	435 ± 30	*e*

[a] Values of midpoint potentials are the average and standard error of three to six experiments. [b] Enzyme and mediators allowed to equilibrate over a time course of up to 38 h. Conditions as listed for Figure 2. [c] Reductive titration by addition of dithionite to oxidized enzyme and mediator. Conditions as listed for Figure 4. [d] Last two values determined from two experiments using pentacyanoaminoferrate as mediator, all remaining values determined using ferricyanide. [e] These values not determined.

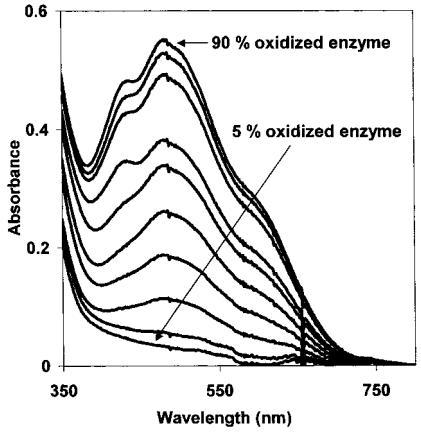

FIGURE 4: Reductive titration of human MnSOD with dithionite using $Fe(CN)_6^{3-/4-}$ as a mediator. Oxidized human MnSOD (1 mM) was mixed with oxidized $Fe(CN)_6$ (100 μM). The redox potential was recorded 1 to 8 h after each addition of dithionite. A limited number of traces are shown for clarity. The solution at 25 °C contained 100 mM KCl and was buffered by 100 mM potassium phosphate with pH adjusted to 7.8 with HCl.

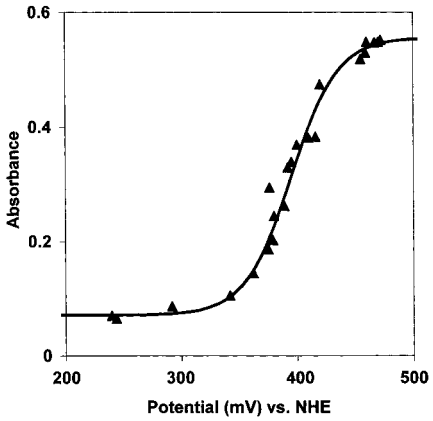

FIGURE 5: Absorbance from Figure 4 for human wild-type MnSOD at 485 nm versus potential. The solid line is a least-squares fit of the Nernst equation to the data ($n = 1$) giving a midpoint potential E_m of 395 ± 19 mV.

titrated by stepwise addition of titrant. The fraction of enzyme in the oxidized state was calculated from the spectral data and correlated to the measured ambient redox potential. Initially, the enzyme was 90% oxidized and gradually reduced until the potential dropped below 250 mV versus NHE (Figure 4). The Nernst equation was then fit to the data, and the midpoint potential was derived (Figure 5). Following this reductive titration, an attempt was made to

reoxidize the enzyme by stepwise addition of permanganate. However, the reoxidation of human MnSOD was complicated by simultaneous enzyme degradation and formation of a substantial amount of manganese dioxide obstructing the light beam. As a consequence, the data obtained during reoxidation of the sample with permanganate could not be fit to the Nernst equation. The activity of the enzyme was determined periodically using stopped-flow spectrophotometry. Micro samples were taken at each time point to evaluate the extent of enzyme degradation, which was factored in when interpreting the spectral data. On average, 10 to 30% of the enzyme was degraded during the redox titration, based on the absorption of the enzyme at 280 nm and activity measurements.

The E_m for human MnSOD obtained by the reductive titration and iterative fits to the Nernst equation was 395 ± 19 mV, which is in good agreement with the E_m calculated from single point equilibrium experiments (407 ± 21 mV). Unfortunately, we were unable to efficiently reoxidize the enzyme with permanganate. Therefore, the reversibility of the Nernstian behavior could not be checked.

DISCUSSION

The midpoint redox potential (E_m) of wild-type human MnSOD was found in this study by electrochemical titration to be $E_m = 393 \pm 29$ mV (pH 7.8, when combining all the data from the two different mediators). This places the redox potential of human MnSOD between that of the oxidation of superoxide to oxygen (−160 mV, eq 1) and the reduction of superoxide to hydrogen peroxide (+850 mV, eq 3), which is thermodynamically favorable for catalysis. The redox potentials for MnSOD from *Bacillus stearothermophilus* and *E. coli* were +260 and +310 mV (pH 7) (*20*), data obtained from coulometric titrations using DCIP which we found not to equilibrate with human MnSOD. A value of $E_m = +220$ mV (pH 7.8) for FeSOD from *E. coli* was reported by Vance and Miller (*7*), and Barrette et al. (*21*) reported $E_m = +270$ mV (pH 7) for *E. coli* FeSOD. The relationship of such redox potentials and the active-site geometry of MnSOD has been investigated using density functional and electrostatic calculations (*22*). Steady-state constants have been reported for Fe- and Mn-containing superoxide dismutases, and they do not differ appreciably in activity: for *E. coli* FeSOD, $k_{cat} = 26$ ms^{-1} and $k_{cat}/K_m = 330$ μM^{-1} s^{-1} (*23*); for human MnSOD, $k_{cat} = 40$ ms^{-1} and $k_{cat}/K_m = 800$ μM^{-1} s^{-1} (*18*).

The midpoint potentials for the site-specific mutants Y34F and H30N MnSOD were similar within experimental uncertainties to that of wild-type MnSOD (Table 2). The crystal structures of these mutants are available and show minimal changes in the vicinity of the metal. For Y34F at 1.9 Å resolution, the positions of the active-site ligands are virtually identical with the wild type (*15*). The most significant change in the structure of this mutant is the absence of the phenolic hydroxyl of residue 34 resulting in the interruption of a hydrogen bond network linking the aqueous ligand of the manganese with Tyr 166 from the adjacent subunit (Figure 1). These observations are consistent with the measured catalytic activity of Y34F MnSOD showing that k_{cat}/K_m for $O_2^{\bullet -}$ dismutation is identical to that of wild type (*15*). What is altered in catalysis by Y34F MnSOD is the maximal velocity or turnover number k_{cat}, which is decreased by an order of magnitude as compared to wild type. This is attributed to decreased proton transfer to the active site caused by interruption of the hydrogen bond network (*15*). Apparently, the decrease in k_{cat} for Y34F occurs with little change in the redox potential at the metal. That is, the function of Tyr34 in MnSOD is not significant tuning of the redox potential (or promoting stability (*15*)) but supporting proton transfer to the active site, and this proton transport appears to depend on maintaining the hydrogen bond network. This contrasts with Fe-substituted *E. coli* MnSOD for which Vance and Miller (*7*) measured $E_m = -240$ mV and attribute the lack of activity of this enzyme not with alterations in proton transfer but in the midpoint potential falling out of the range between the potentials of the two half-reactions of superoxide. Cabelli et al. (*24*) provide an insightful discussion of the role of proton transfer and its relation to electron transfer in both catalysis and product inhibition by superoxide dismutase.

These comments are reinforced by the data for H30N MnSOD. Here again, the redox potential is very close to that of wild-type MnSOD (Table 2). The crystal structure of H30N MnSOD at 2.3 Å shows that the environment around the metal is unchanged as compared with wild-type enzyme (*16*). The structure of the H30N mutant also shows that the replacement of His30 with Asn caused an interruption in the hydrogen bond network but at another location as compared with the interruption of Y34F MnSOD. Nevertheless, the data are rather similar; for H30N the value of k_{cat} is decreased 10-fold, although in this case the value of k_{cat}/K_m is decreased about 6-fold as compared to that of wild-type MnSOD (*16*). These data are in strong contrast to Q143N MnSOD and Q143A MnSOD in which both k_{cat} and k_{cat}/K_m for $O_2^{\bullet -}$ dismutation are decreased by two to three orders of magnitude. In addition, these and other Q143 mutants are Mn^{2+} in the resting state, indicating that the redox potential may be significantly increased as compared with wild type (*13, 14*).

The midpoint potential of human MnSOD was also measured by reductive titration. In these experiments, ferricyanide was emphasized as a mediator for its superior stability and spectral signatures over pentacyanoaminoferrate. The equilibration of ferricyanide with MnSOD is novel since this mediator has been shown not to work with both Fe- and MnSOD from *E. coli* (*9*, *23*). This is probably further evidence that the redox potential in the human enzyme is significantly different than its bacterial homolog. The oxidative titration of human MnSOD with MnO_4^- was inconclusive because of enzyme degradation and the formation of manganese dioxide. However, the reductive titration with dithionite was in close agreement with the single point equilibrium experiments where ferricyanide and pentacyanoaminoferrate were used in equilibrium titrations.

The redox potential measurements of human MnSOD were difficult and one more example of the challenge involved in electrochemical measurements with nonelectron carrier proteins. Nevertheless, two different approaches using two different mediators gave consistent results. Three of the four main criteria for determining a reduction potential were fulfilled: First, the same E_m was obtained using two distinct mediators, each with different spectral and redox characteristics; second, the same E_m was obtained from single point titrations and reductive titrations; and third, the reductive

titration followed Nernstian behavior. Reversibility of the Nernstian behavior could not be verified.

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