Articles

Mutation of Tyrosine 34 to Phenylalanine Eliminates the Active Site p*K* of Reduced Iron-Containing Superoxide Dismutase[†]

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ABSTRACT: We have compared the magnetic resonance properties and pH dependence of wild-type and mutant Fe-containing superoxide dismutase (Fe-SOD) in which the conserved active site tyrosine (Tyr 34) is replaced by phenylalanine. The EPR spectrum of the oxidized state and the NMR spectrum of the paramagnetically shifted resonances of the reduced state indicate that in both states the active site is relatively unperturbed by the mutation. Similarly, the mutant Fe-SOD retains approximately 41% of wild-type catalytic activity on a per Fe basis. However, replacement of Tyr 34 by Phe abolishes both NMR spectroscopic signatures of the active site p*K* of 8.5 of (reduced) Fe^{2+}-SOD. Neither accessibility to base-catalyzed exchange nor the chemical shifts of active site residues are affected by pH in the range of 6.5−10.5 in Y34F Fe^{2+}-SOD. Thus, the active site p*K* of 8.5 of Fe^{2+}-SOD most likely corresponds to deprotonation of Tyr 34. The widespread chemical shift changes associated with the p*K* could reflect Tyr 34's participation in the active site hydrogen bonding network and the network's propagation of the effects of deprotonating Tyr 34 to the Fe^{2+}. Deprotonation of Tyr 34 can also explain the dramatic decrease in active site accessibility to base-catalyzed exchange as the result of electrostatic repulsion between the exchange catalyst OH^- and the (Tyr 34)$^-$ ion formed at high pH. Similar electrostatic repulsion between (Tyr 34)$^-$ and the substrate $O_2^{\bullet-}$ is also consistent with the observed increase in K_M above pH 9 [Bull, C., & Fee, J. A. (1985) *J. Am. Chem. Soc. 107*, 3295−3304].

Fe-containing superoxide dismutases (Fe-SODs)[1] catalyze the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 in a two-step reaction:

$$O_2^{\bullet-} + Fe^{3+}SOD \rightarrow O_2 + Fe^{2+}SOD \qquad (1a)$$

$$O_2^{\bullet-} + Fe^{2+}SOD + 2H^+ \rightarrow H_2O_2 + Fe^{3+}SOD \qquad (1b)$$

(Lavelle et al., 1977; Bull & Fee, 1985). The Fe-SOD from *Escherichia coli* is a dimer of identical monomers which each contain a single Fe in the active site (Carlioz et al., 1988). The Fe is coordinated by three His ligands and one monodentate Asp$^-$, as well as a coordinated solvent molecule (presumably OH^- in oxidized Fe^{3+}-SOD) (Stallings et al., 1991; Lah et al., 1995). The active site also contains a Tyr residue (Tyr 34) which is conserved in all Fe- and Mn-SODs, but not in the (unrelated) Cu,Zn-SODs (Smith & Doolittle, 1992). Tyr 34, Gln 69, and the ligands are connected to each other, surrounding aromatic side chains, and backbone atoms by a network of hydrogen bonds (Stoddard et al., 1990a; Ludwig et al., 1991; Lah et al., 1995). In addition, the side chains of Tyr 34 and (conserved) His 30 separate the active site from a channel out to solvent (Stallings et al., 1991). Binding of the anionic substrate to the buried Fe has been proposed to be aided by electrostatic steering by charged residues in the channel [Benovic et al., 1983; Sines et al., 1990; but see Fee et al. (1986)].

We would like to identify the sources of the two protons required for SOD activity because protons are crucial to superoxide dismutase activity. The p*K*s[2] of these protons are important determinants of eq 1b's thermodynamic favorability. In addition, the relationship between proton transfer and electron transfer is central to the reactivity of Fe-SOD. Protonation of $O_2^{\bullet-}$ (pK_a = 4.7; Ingraham & Meyer, 1985) is much less favorable than protonation of O_2^{2-}. Similarly, $O_2^{\bullet-}$ is much more difficult to reduce than $O_2H^{\bullet}$ (Ross & Ross, 1977). Thus, protonation significantly favors reduction and *vice versa*, so Fe-SOD is most likely to link the two in order to facilitate both. Finally, chemical considerations have been used to argue that the rate-limiting

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[1] Abbreviations: Asp, aspartate; Arg, arginine; DSS, 4,4-dimethyl-4-silapentanesulfonate sodium salt; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorbance fine structure; His, histidine; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOD, superoxide dismutase; T_1, longitudinal relaxation time; T_2, transverse relaxation time; Tyr, tyrosine; WEFT, water-eliminated Fourier transform; WT, wild-type (not mutated); Y34F, mutant in which Tyr 34 is replaced by Phe.

[2] We use the notation p*K* to indicate the pH at which a residue is half-deprotonated and half-protonated. In interactive systems such as proteins, this is not the same as the pK_a (=$\Delta G°/2.303RT$), which is pH-dependent for any residues whose ionized state interacts with those of other residues, as the extent of ionization of the latter will depend on pH.

step in SOD catalysis is proton transfer leading to dissociation of H_2O_2 from the metal ion (Bull & Fee, 1985). Since Fe-SODs are irreversibly inactivated by H_2O_2, and Mn-SOD is reversibly inhibited by formation of a state believed to correspond to a long-lived peroxo complex (Bull et al., 1991), proton transfer that promotes dissociation of H_2O_2 helps to protect enzyme activity against inhibition as well as accelerating the reaction.

Indirect information on the proton donors has been gleaned from the pH dependence of Fe-SOD activity (Fee et al., 1981a,b; Terech et al., 1983; Bull & Fee, 1985). Overall catalytic activity decreases at high pH with a p*K* near 9 which affects K_M but not k_{cat} (Fee et al., 1981a,b; Terech et al., 1983; Bull & Fee, 1985). The apparent increase in K_M has been ascribed to competitive inhibition of (oxidized) Fe^{3+}-SOD by OH^-, which coordinates to Fe^{3+} (Tierney et al., 1995), presumably in place of $O_2^{\bullet -}$. A proton is taken up upon reduction of Fe^{3+}-SOD, probably by the coordinated OH^-, and the resulting coordinated H_2O of Fe^{2+}-SOD is believed to be one of the proton donors to substrate (Lah et al., 1995). Because Fe^{3+}-SOD has a p*K* of 9 affecting the active site (Fee et al., 1981a,b; Bull & Fee, 1985) and a single proton is taken up throughout the pH range of 7−10, Fe^{2+}-SOD is also inferred to have a p*K* near 9 (Bull & Fee, 1985). The proposed p*K* of Fe^{2+}-SOD was ascribed to an active site amino acid (Neiderhoffer et al., 1987), possibly the second proton donor. Because the rate constants of SOD's two half-reactions are comparable, the p*K* affecting activity reflects both oxidation states of the enzyme (Bull & Fee, 1985) and the p*K* of Fe^{2+}-SOD as well as that of Fe^{3+}-SOD should be associated with an increase in K_M.

We have recently used NMR spectroscopy to demonstrate that the active site of Fe^{2+}-SOD has a p*K* of 8.5 at 30 °C at low ionic strength, consistent with the predicted p*K* near 9 (Sorkin & Miller, 1997). The active site p*K* of 8.5 is associated with widespread chemical shift changes as well as a dramatic decrease in accessibility to OH^--catalyzed exchange with solvent for at least one active site proton (Sorkin & Miller, 1997). However, because the chemical shifts of many different protons all responded to the p*K*, it was not possible to identify the residue responsible.

Tyr 34 has been proposed to be one of the proton donors and the origin of the p*K* of Fe^{2+}-SOD (Bannister et al., 1987; Terech et al., 1983; Niederhoffer et al., 1987; Beyer et al., 1991; Stallings et al., 1991; Lah et al., 1995; Hsu et al., 1996). A p*K* near 9 is typical of Tyr residues, Tyr 34 is conserved among all Fe- and Mn-SODs (Beyer et al., 1991; Smith & Doolittle, 1992), the phenolic O of Tyr 34 is only 5.4 Å away from the Fe^{3+} in *E. coli* Fe^{3+}-SOD [Lah et al., 1995; also see Parker and Blake (1988)], and this Tyr appears to donate a hydrogen bond to the coordinated substrate analog (N_3^-) in Mn-SOD from *Thermus thermophilus* (Lah et al., 1995). Other potential proton donors are the H_2O/OH^- molecule coordinated to the metal ion (Stallings et al., 1991; Lah et al., 1995), Arg 170 (Chan et al., 1990), and the conserved Gln 69 in combination with Tyr 34 (Stoddard et al., 1990b).

In the current paper, we have exploited site-directed mutagenesis to test the popular hypothesis that Tyr 34 is partly responsible for the pH dependence of Fe-SOD activity, and the source of the recently-observed p*K* of the active site of Fe^{2+}-SOD.

EXPERIMENTAL METHODS

Expression and Purification of Y34F Fe-SOD. The sodB′ gene for *E. coli* Fe-SOD with the Y34F mutation, under control of the native Fe-SOD promotor on the plasmid pTZ19U sodB′ Y34F, was generously provided by J. A. Fee. The sodB′ gene was constructed from the WT sodB gene using oligonucleotide-directed mutagenesis (J. A. Fee personal communication). The complete nucleotide sequence was obtained for one strand of the sodB′ gene, which was confirmed to carry a mutation in the TAT codon for Y34, converting it to the TTT codon for Phe. The plasmid was transformed into the *E. coli* strain QC774 in which both chromosomal SOD genes have been inactivated (Carlioz & Touati, 1986). The strain was grown in defined medium containing the same salts and half the concentrations of the bases and amino acids as in Muchmore et al. (1989) but using 10 times the concentrations of micronutrients, and Y34F protein was expressed and purified using exactly the same methods as were used for WT Fe-SOD (Sorkin & Miller, 1997; Slykhouse & Fee, 1976). However, the yields of purified Y34F Fe-SOD were only 4 mg/L of culture, as opposed to 20 mg/L for WT Fe-SOD.

Protein Characterizations. Protein concentrations were measured using the published extinction coefficient of $1.01 \times 10^5\ M^{-1}\ cm^{-1}$ at 280 nm for WT Fe-SOD (Slykhouse & Fee, 1976), for both WT and Y34F Fe-SOD. Since Fe-SOD contains nine Tyr and eight Trp, mutation of one Tyr to Phe is expected to decrease the ϵ_{280} slightly, by approximately $3 \times 10^3\ M^{-1}\ cm^{-1}$, taking into account only the contributions of the Tyr and Trp (Pace et al., 1995), neglecting the effects of protein structure on Tyr and Trp absorbance, and assuming negligible contributions at 280 nm from Fe^{3+}. Thus, the resulting underestimate of the Y34F Fe-SOD protein concentration is expected to be less than 3%. The Fe contents of the samples were determined using the ferrozine assay of Carter (1971). The catalytic activities of Y34F and WT Fe-SOD were assessed using the indirect assay of McCord and Fridovich under standard conditions (McCord & Fridovich, 1969).

Sample Preparation. Samples of Fe-SOD to be used for pH titrations were first dialyzed extensively against deionized water as low ionic strength enhances the stability of Fe-SOD at high pHs. NMR samples contained 15−30 mg of Y34F Fe-SOD in 0.5 mL of 90% H_2O, 10% ^2H_2O, or 0.7−1.4 mM Fe-SOD dimers. A stock solution of NMR pH indicator molecules and DSS in 90% H_2O, 10% ^2H_2O were added to produce DSS and indicator concentrations of 0.3−2 mM (Sorkin & Miller, 1997); the sample was degassed by repeated evacuation and equilibration with Ar in the NMR tube, reduced by addition of a 2−3× molar excess of dithionite, and sealed with a septum and stopcock (Wilmad no. 507-OF-7) or flame-sealed. The NMR pH indicators used were imidazole, 2,4-dimethylimidazole, trimethylamine, and dimethylamine.

pH titrations were performed by injecting degassed 0.1 M NaOH or H_3PO_4 in 90% H_2O, 10% ^2H_2O through the septum of the NMR tube to change the pH, and collecting two NMR spectra at each pH. In addition to a super-WEFT spectrum of the Fe^{2+}-SOD active site resonances (Inubishi & Becker, 1983), a spin echo spectrum of the pH indicator molecules was collected to determine the pH (Sorkin & Miller, 1997). The chemical shifts of the active site Fe^{2+}-SOD resonances were then plotted as functions of the pH and fit with a Henderson–Hasselbalch equation with allowance for cooperativity:

$$\frac{\delta_A - \delta_{obs}}{\delta_A - \delta_B} = \frac{K^n}{K^n + 10^{-n(pH)}} \tag{2}$$

where δ_A and δ_B are the chemical shifts of the acid and base forms (obtained from the fit), δ_{obs} is the observed chemical shift at a given pH, K is the acid dissociation constant obtained from the fit, and n is the Hill coefficient obtained from the fit.

Spectroscopy. EPR spectra of WT and Y34F Fe^{3+}-SOD were obtained at 9.477 GHz at 100 K using 2 mW nominal microwave power, 10 G modulation amplitude, a time constant of 328 ms, and 2500 G wide scans obtained in 20 min. EPR samples were buffered at pH 7.5 at 22 °C with 25 mM HEPES and contained 50 mM NaCl but no cryoprotectant. NMR spectra of Fe^{2+}-SOD were obtained at 300 MHz for protons on an AMX 300 at 30 °C as described in Sorkin and Miller (1997). In brief, super-WEFT (Inubishi & Becker, 1983) spectra were collected with a rapid repetition rate to emphasize the rapidly-relaxing resonances from the active site of Fe^{2+}-SOD. The water resonance was saturated during the 35 ms delay between pulses as well as during the 15 ms relaxation delay between repetitions.

We measured the apparent selective T_1 of the resonance at 42 ppm, T_{1app}, which included the contributions of true $1/T_1$ relaxation as well as exchange with solvent, using a long selective pulse to invert that resonance followed by a variable delay and then a hard 90° observation pulse, with 300 ms of water saturation per repetition. The amplitudes of the resonance at 42 ppm (M) obtained from water counter-selective jump–return spectra (Plateau & Gueron, 1982; Hore, 1983) collected with (M_{ps}) and without (M_o) prior saturation of water were used along with T_{1app} to calculate the selective T_1 and k_{ex} at two pHs, using eqs 3 (Waelder et al., 1975; Krishna et al., 1979; Spera et al., 1991).

$$\frac{M_o - M_{ps}}{M_o} = k_{ex}T_{1app} \tag{3a}$$

$$1/T_{1app} = 1/T_1 + k_{ex} \tag{3b}$$

The T_1 so obtained was then used to calculate additional values of k_{ex} from $M = M_{ps}$ obtained from super-WEFT spectra at a range of pHs and $M = M_o$ obtained at low pH where exchange and thus saturation transfer is negligible (Sorkin & Miller, 1997).

In our pH range, the observed exchange rate with solvent is related to the rate constant for base-catalyzed exchange, k_{OH}, the concentration of OH^-, and the protection factor P, assuming the EX2 limit (Englander & Kallenbach, 1984; Englander et al., 1996).

$$k_{ex} = k_{OH}[OH^-]/P \tag{4a}$$

$$\log(k_{ex}) = \log(k_{OH}/P) + \log K_w + pH \tag{4b}$$

Thus, the log of the exchange rate is expected to vary linearly with pH, with a slope close to 1 in the event of a pH-independent protection factor.

RESULTS AND DISCUSSION

Integrity of the Active Site. Purified Y34F Fe-SOD was found to contain between 0.87 and 0.90 Fe per monomer, compared to the WT Fe-SOD Fe stoichiometry of 0.92–0.94 Fe per monomer. Considering that the ϵ_{280} of the Y34F Fe-SOD may be slightly lower than that of WT Fe-SOD, these Y34F stoichiometries may be slight overestimates. Nonetheless, it is clear that most of the active sites contain Fe, and only those that do are observed in our NMR spectra of the active site.

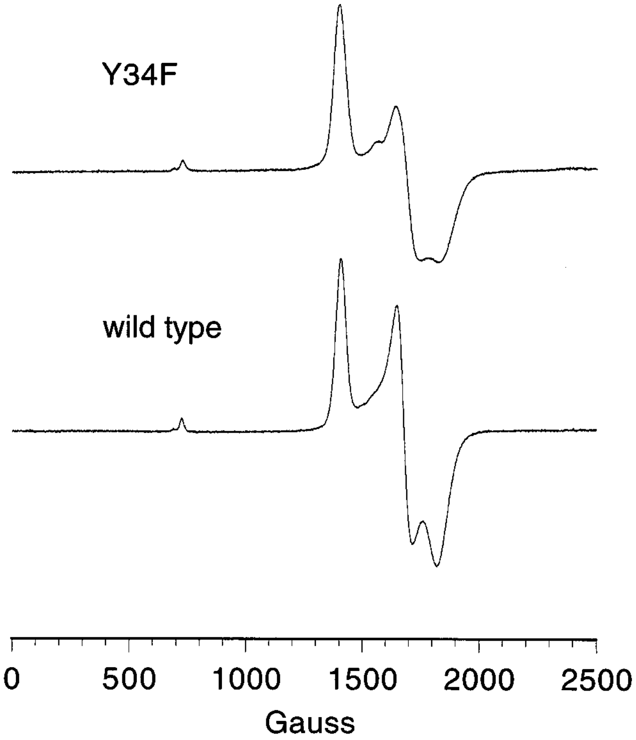

FIGURE 1: EPR spectra of oxidized Y34F Fe^{3+}-SOD (top) and WT Fe^{3+}-SOD (bottom). The samples and spectroscopic parameters are described under Experimental Methods.

In order to assess the integrity of the active site, as well as the degree to which Fe is bound specifically in the active site of Y34F Fe-SOD, we performed EPR spectroscopy and optical spectrophotometry on the oxidized enzyme, and NMR spectroscopy on the reduced enzyme.

The EPR spectrum of Y34F Fe^{3+}-SOD is shown in Figure 1. The Fe^{3+} signal appears slightly less rhombic than that of WT Fe^{3+}-SOD, and displays apparent g values of 9.33, 4.84, 4.02, and 3.71, similar to the values of 9.36, 4.82, 4.03, and 3.72 observed in WT Fe^{3+}-SOD. The differences between these and the published values of 9.98, 4.99, 4.12, and 3.77 may be due to sample conditions or spectrometer calibration (Slykhouse & Fee, 1976). Both Fe-SOD samples also contain a small signal at $g = 4.3$ which most likely corresponds to nonspecifically bound Fe^{3+}. Thus, the EPR spectrum of Y34F Fe^{3+}-SOD is essentially the same as that of WT Fe^{3+}-SOD. The optical spectra of Y34F and WT Fe^{3+}-SOD are also essentially the same; thus, the electronic structure and coordination geometry of the Fe^{3+} of Y34F Fe^{3+}-SOD appear qualitatively similar to those of WT Fe^{3+}-SOD.

The electronic and spin properties of Fe^{2+} in WT and Y34F Fe^{2+}-SOD can be compared indirectly via NMR spectra[3]

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[3] Because all the resonances outside the diamagnetic region of ≈12 ppm to −1 ppm almost certainly correspond to protons near or connected through bonds to Fe^{2+}, we refer to them as the active site spectrum. Some residues near Fe may have a negligible paramagnetic chemical shift (and yet still be paramagnetically relaxed), if they arise from protons close to Fe but positioned in a direction close to the magic angle relative to the principle axes of the g tensor of Fe^{2+}.

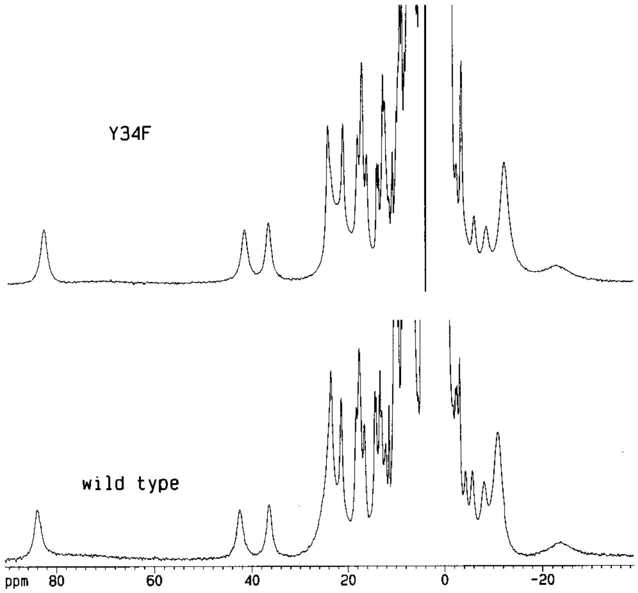

FIGURE 2: ^{1}H NMR spectra of reduced Y34F Fe^{2+}-SOD (top) and WT Fe^{2+}-SOD (bottom). The samples were at pHs 6.3 and 6.1, respectively, and contained indicator molecules and DSS as described under Experimental Methods.

(Figure 2). The paramagnetic contributions to chemical shift provide indirect information on the Fe^{2+} unpaired electron spin distribution among the valence orbitals, and the ^{1}H NMR line widths of active site residues are very sensitive to active site structure as they depend on the inverse sixth power of the protons' distances to Fe^{2+} (LaMar et al., 1973; Bertini & Luchinat, 1986). Thus, although the majority of the paramagnetically shifted ^{1}H resonances of Fe^{2+}-SOD have not yet been assigned, the strong parallels between the spectra of WT and Y34F Fe^{2+}-SOD, with respect to both line positions and widths, indicate that the electronic structure of Fe^{2+} is similar in the two proteins and moreover that the environments of individual protons in the two active sites are very similar too.

Catalytic Activity. Under the standard assay conditions of McCord and Fridovich (1969), Y34F Fe-SOD displays substantial catalytic activity. Whereas our WT Fe-SOD has an activity of 6100 ± 200 units/mg of protein, Y34F Fe-SOD consistently displays activities of 2400 ± 200 units/mg of protein, which are stable in the assay for at least 2 min, similar to WT Fe-SOD. Thus, on a per Fe basis, Y34F Fe-SOD retains ≈ 41% of WT activity. The standard assay is conducted under conditions corresponding to a low superoxide concentration of roughly nanomolar (Bull et al., 1982), and reveals only the ratio k_{cat}/K_M. Nonetheless, the relatively large amount of activity displayed by the mutant indicates that Tyr 34 is not essential to SOD activity and has a relatively minor effect on k_{cat} or K_M near neutral pH (compensating large effects on k_{cat} and K_M are deemed less likely). It remains possible that Tyr 34 contributes to a non-rate-limiting step or becomes more important to enzyme activity under different conditions than those employed for the standard assay. Alternately, Tyr may be required for an additional activity not measured by the standard assay. The effect of the Tyr 34 to Phe mutation on the catalytic properties of Fe-SOD will be examined in more detail in a forthcoming manuscript.

Overall, the similarities between the EPR and NMR spectra of WT and Y34F Fe-SOD, as well as the substantial activity of Y34F Fe-SOD in the standard assay, indicate that the active site is only minimally perturbed by the Tyr to Phe mutation, in either oxidation state. Thus, it is legitimate to interpret any observed differences between Y34F and WT Fe-SOD in terms of the amino acid replacement itself, not secondary effects of changes in the active site structure.

Relation of Tyr 34 to the pK of Reduced SOD. Despite the apparent integrity of the active site of Y34F Fe^{2+}-SOD at pH 7.4, its pH dependence is very different from that of WT Fe^{2+}-SOD. In contrast to WT Fe^{2+}-SOD, in which virtually every active site resonance changes chemical shift between pHs 7 and 10, by up to 4.5 ppm, the high- and low-pH spectra of Y34F can be overlain (Figure 3), indicating qualitatively insignificant chemical shift changes. This is confirmed by plots of the chemical shifts of individual resonances as functions of pH, such as the one in Figure 4. The chemical shift of each of the Y34F Fe^{2+}-SOD active site resonances falls between the chemical shift asymptotes of the analogous WT resonance. However, the chemical shifts of the paramagnetically shifted ^{1}H resonances provide no indication of a pK between pH 6.5 and 10.5 affecting the active site of Y34F Fe^{2+}-SOD.

In WT Fe^{2+}-SOD, not only the active site chemical shifts but also the protection factor describing exchange with solvent of one of the active site protons displayed a pK of 8.5 (Sorkin & Miller, 1997). At moderately high pH, the WT resonance at 43 ppm exchanges with solvent on a millisecond time scale and therefore becomes partially saturated when water is presaturated (Sorkin & Miller, 1997). The analogous resonance in Y34F Fe^{2+}-SOD, at 42 ppm, also exchanges with water on a millisecond time scale at moderately high pH. Since the active site spectrum and the pattern of exchange with solvent of Y34F are almost identical to those of WT Fe^{2+}-SOD, we tentatively assign the Y34F resonance at 42 ppm to the same ligand δNH as the resonance at 43 ppm of WT Fe^{2+}-SOD [see Ming et al. (1994)].

The intensity of the resonance at 43 ppm in WT Fe^{2+}-SOD decreases with increasing pH in the presence of saturated water, but only up to approximately pH 8.5, beyond which the resonance retains roughly 50% of its intensity (Figure 5). Thus, the rate of exchange increases with pH below pH 8.5, but not above it. The analogous resonance at 42 ppm in Y34F Fe^{2+}-SOD decreases in intensity with increasing pH similar to the WT behavior below pH 8.5. However, in Y34F Fe^{2+}-SOD, the resonance continues to lose intensity with increasing pH above pH 8.5 as well, until the resonance is no longer visible (Figures 5 and 3).

The measured peak intensities of the resonance at 42 ppm in Y34F Fe^{2+}-SOD were used to calculate the rate of exchange with solvent of the corresponding His δNH, at a range of pHs, using eqs 3. The value of the selective T_1 used was 10.4 ms, the average of the measured selective T_1s of 10.3 ± 0.3 and 10.5 ± 0.6 ms obtained at pHs 6.4 and 8.3, respectively. Because the values of T_1 obtained at a pH well below the active site pK of WT Fe^{2+}-SOD and at a pH very close to the pK are within error of one another, the T_1 is shown not to vary with pH so that a single average value can be applied throughout the pH range.

The logarithms of the exchange rates obtained for Y34F Fe^{2+}-SOD are plotted as a function of pH in Figure 6, along with the analogous data from WT Fe^{2+}-SOD for comparison (Sorkin & Miller, 1997). Y34F Fe^{2+}-SOD displays the linear increase in the log of the exchange rate with a slope close to 1 that is predicted for simple base-catalyzed exchange in

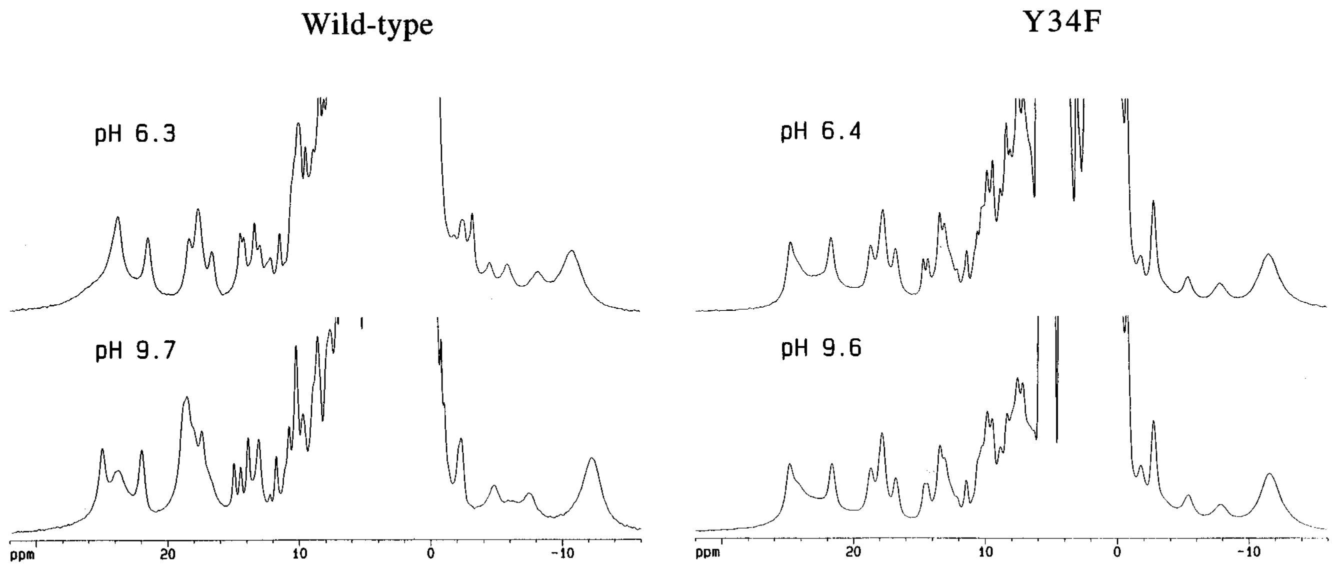

FIGURE 3: ^{1}H NMR spectra of WT Fe^{2+}-SOD (left) and Y34F Fe^{2+}-SOD (right) at two pHs, below the active site pK of WT Fe^{2+}-SOD (top) and above it (bottom). Only the region between 32 and −16 ppm is shown.

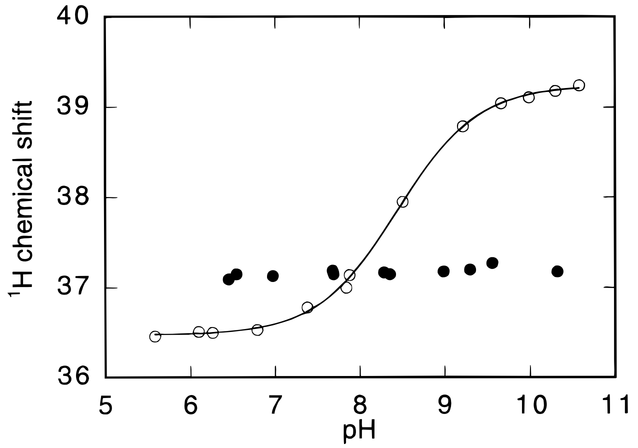

FIGURE 4: pH dependence of the chemical shift of the Y34F Fe^{2+}-SOD resonance at 37 ppm (●) compared with that of the analogous resonance of WT Fe^{2+}-SOD (○) (Sorkin & Miller, 1997). Both samples contained indicator molecules and DSS as described under Experimental Methods.

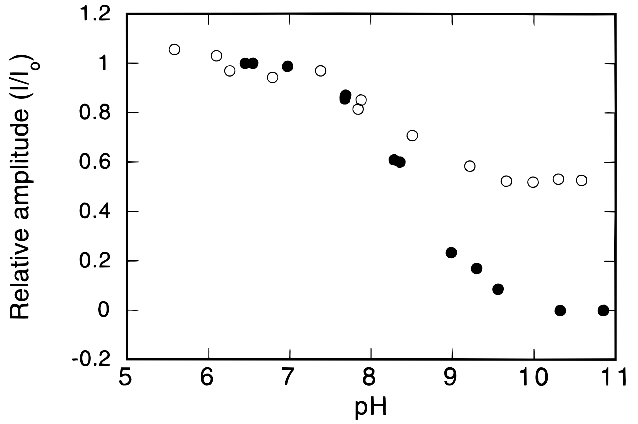

FIGURE 5: pH dependence of the amplitude of the resonance at 42 ppm in Y34F Fe^{2+}-SOD (●) and the analogous resonance (at 43 ppm) in WT Fe^{2+}-SOD (○).

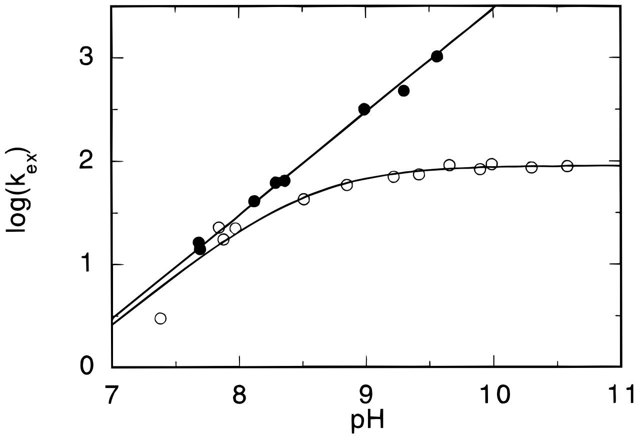

FIGURE 6: pH dependence of the log of the exchange rate of the resonance at 42 ppm in Y34F Fe^{2+}-SOD (●), compared with that of the analogous resonance (at 43 ppm) in WT Fe^{2+}-SOD (○) (Sorkin & Miller, 1997). Exchange rates were calculated from the peak intensities shown in Figure 5, using eqs 3. The line accompanying the Y34F data is the best fit of eq 4b, which assumes a slope of 1 and yields $k_{OH}/P = (2.96 \pm 0.12) \times 10^7$ M^{-1} s^{-1} from the intercept of −6.53. When the log of k_{ex} vs pH is fit to a straight line without assuming a value for the slope, the slope obtained is 0.96 [$\log(k_{ex}) = -6.23 + 0.96 \pm 0.023$ pH]. The curve accompanying the WT data is the best fit of eq 6b of Sorkin and Miller [$k_{ex} \approx [10^{-14}/(10^{-pK} + 10^{-pH})](K_{op}{}^{A}k_{OH})$ (Sorkin & Miller, 1997)] with allowance for a pH-dependent protection factor of $P = 1/K_{op}{}^{A}$ at low pH, and $P \approx \infty$ at high pH, which yields values of $k_{OH}/P = (2.7 \pm 0.3) \times 10^7$ M^{-1} s^{-1} and pK = 8.5 ± 0.1.

the EX2 limit (Englander & Kallenbach, 1984; Englander et al., 1996). This contrasts sharply with the WT behavior, which has been interpreted in terms of an equilibrium between two forms of Fe^{2+}-SOD related by a pK of 8.5, the high-pH form of which has a protection factor more than 100 times larger than that of the low-pH form (Sorkin & Miller, 1997). Unlike WT Fe^{2+}-SOD, there is no change in the exchange behavior of Y34F Fe^{2+}-SOD above pH 8.5 and no evidence for a pK between 6.5 and 10 affecting protection from exchange for the proton that resonates at 42 ppm.

In summary, we have monitored two independent signatures of the pK of 8.5 of the active site of reduced Fe^{2+}-SOD: the pH dependence of the active site chemical shifts and the pH dependence of the protection factor against exchange of one of the ligand His protons. Both become pH-independent upon replacement of Tyr 34 by Phe, although the active site remains basically intact. Although it is possible that Tyr 34 serves to propagate the effects of deprotonating some other amino acid that cannot itself affect the active site, the simplest interpretation of our results is that the pK of 8.5 of the active site of Fe^{2+}-SOD represents deprotonation of Tyr 34. The p*K* of 8.5 is slightly lower than usual for Tyr; however, this could reflect electrostatic stabilization of tyrosinate by the nearby positively charged Fe^{2+}.

Deprotonation of Tyr 34 could lead to the widespread chemical shift changes associated with the active site p*K* of WT Fe^{2+}-SOD via Tyr 34's participation in the hydrogen bond network that also includes ligands to Fe^{2+}. Deprotonation of Tyr 34 could cause redistribution of the protons in the network and alter the hydrogen bonding and polarization of a ligand, which would in turn subtly alter the ligand's effect on the electronic state of Fe^{2+} and thus the paramagnetic contributions to chemical shift.

Our comparison of the pH dependence of base-catalyzed exchange in WT and Y34F Fe^{2+}-SOD provides information on the specific property of Tyr 34 that is responsible for the pH dependence of the active site His exchange rate. If Tyr's negative charge at high pH is responsible for the difference between WT and Y34F's pH dependencies, then the two enzymes should resemble one another at low pH when both Phe and Tyr are neutral, but behave differently at high pH. If the distinguishing property of Tyr 34 is its ability to donate a hydrogen bond, then WT and Y34F Fe^{2+}-SOD should differ at low pH where only Tyr can donate a hydrogen bond, but resemble one another at high pH where neither Tyr^{-} nor Phe can do so. If Tyr's O is required for its ability to accept a hydrogen bond, then Y34F should differ from WT Fe^{2+}-SOD at both low and high pHs. We find that the exchange rates and their pH dependencies in Y34F and WT Fe^{2+}-SOD are very similar at low pH, but are completely different at high pH, where Y34F Fe^{2+}-SOD's exchange rate continues to increase in proportion to the OH^{-} concentration but WT Fe-SOD's becomes pH-independent. Thus, it appears that Tyr 34's negative charge when it is ionized is responsible for protection against OH^{-}-catalyzed exchange in WT Fe^{2+}-SOD. This could be due to simple electrostatic repulsion between tyrosinate and the exchange catalyst OH^{-} (or, equivalently, decreased attraction between OH^{-} and the smaller net positive charge of the active site when Tyr 34 is ionized).

Since OH^{-} is a substrate analog, the fact that access of OH^{-} to the active site decreases upon ionization of Tyr 34 suggests that formation of the tyrosinate anion of Tyr 34 could similarly decrease the active site's affinity for $O_2^{\bullet -}$. The similarity of the exchange rates of WT and Y34F at pHs below the p*K* is consistent with the similarity of the two SODs' activities at pH 7.8. Indeed, our assignment of the p*K* of Fe^{2+}-SOD to Tyr 34 together with Bull and Fee's conclusion that the p*K* of Fe^{2+}-SOD as well as that of Fe^{3+}-SOD should contribute to the pH dependence of activity (Bull & Fee, 1985) indicates that Tyr 34's ionization is responsible for the increase in K_M at high pH, along with OH^{-} binding to Fe^{3+}. However, since we find that both the activity and the exchange rate of Y34F resemble those of WT near neutral pH, our data make no statement as to the possible roles of Tyr 34 in catalytic activity under those conditions, despite the strong conservation of Tyr 34 among Fe- and Mn-SODs. This issue is being addressed by direct enzyme kinetic measurements at a range of pHs.

CONCLUSIONS

Our observation that replacement of Tyr 34 with Phe abolishes two independent signatures of the active site p*K* of 8.5 of Fe^{2+}-SOD confirms that the same event is responsible for both. It is possible that the p*K* of 8.5 represents deprotonation of another group and that Tyr 34 is responsible only for propagating its effects. However, in this case, one would expect that some effects of the p*K* due directly to the residue responsible, or propagated by other means, would persist in Y34F Fe-SOD. Y34F Fe^{2+}-SOD's lack of any p*K* affecting observable active site chemical shifts between pHs 6.5 and 10, as well as its complete loss of the enhanced protection from OH^{-}-catalyzed exchange at pHs above 8.5, argues strongly that Tyr 34 is directly responsible for the p*K* of 8.5 of WT Fe^{2+}-SOD. Since the p*K* of Fe^{2+}-SOD contributes to the pH dependence of the K_M of overall activity, Tyr 34 is inferred to share the responsibility for the pH dependence of the K_M. Furthermore, our exchange rate measurements suggest that the increase in the K_M of Fe-SOD at high pH reflects in part electrostatic repulsion or decreased attraction between the reduced active site and negatively charged $O_2^{\bullet -}$ upon ionization of Tyr 34 to Tyr^{-}.

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