Spectroscopic Measurement of a Long-Predicted Active Site p*K* in Iron-Superoxide Dismutase from *Escherichia coli*†

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Received December 11, 1996; Revised Manuscript Received February 18, 1997⊗

ABSTRACT: The accepted mechanism of Fe-containing superoxide dismutase (Fe-SOD) activity and inhibition by anions implies the existence of a group with a p*K* of 8.6−9.0 in the active site of reduced Fe-SOD [Bull, C. & Fee, J. A. (1985) *J. Am. Chem. Soc. 107*, 3295−3304]. We have performed pH titrations of reduced Fe-SOD by NMR spectroscopy and observe a p*K* of 8.5 at 30 °C which is the only p*K* affecting the active site between pH 5.5 and 10.5. Thus, we present the first spectroscopic evidence of the predicted p*K*. Although the p*K* is associated with chemical shift changes for almost all of the resonances of the active site, resonance line widths and the T_1 of a ligand proton are not significantly affected by the p*K*, indicating that there is no significant conformational change and only relatively minor effects on the electronic spin properties of Fe^{2+}. The changes in chemical shift are probably caused by changes in hydrogen bonding to a ligand and attendant subtle perturbation of the Fe^{2+} paramagnetism upon loss of the proton with the p*K* of 8.5. The p*K* is also associated with a dramatic restriction of the exchange of at least one ligand proton. Thus, active site accessibility to solvent and OH^- decreases by more than 2 orders of magnitude upon loss of the proton with the p*K* of 8.5. Since OH^- is a competitive inhibitor of Fe-SOD, and thus a substrate analog, this dramatic and unusual decrease in accessibility to OH^- is consistent with the increase in the K_M for $O_2^{\bullet-}$ that is associated with a p*K* near 9.

Iron-containing superoxide dismutase (Fe-SOD)[1] converts $O_2^{\bullet-}$ to O_2 and H_2O_2 in a two-step mechanism (Lavelle et al., 1977; Bull & Fee, 1985)

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

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

where SOD signifies the enzyme and Fe indicates the bound iron ion, which cycles between its +3 and +2 oxidation states.

The Fe-SOD from *Escherichia coli* is a homodimer (Carlioz et al., 1988) and each monomer has an active site containing a single Fe ion ligated by three His residues and a monodentate Asp^-. OH^- occupies a fifth coordination site, and substrate analogs bind at a sixth (Lah et al., 1995). The four amino acid ligands are absolutely conserved in the amino acid sequences of all Mn- and Fe-SODs sequenced to date, as is a Tyr in the active site (Tyr 34, numbering of Fe-SOD from *E. coli*). The only ionizable groups in the active site which are likely sources of the two protons required for catalysis are Tyr 34 and the coordinated solvent. These and the metal ligands appear to be connected by a network of hydrogen bonds which stabilizes the ligands and connects the potential proton donors to bulk solvent (Stoddard et al., 1990; Ludwig et al., 1991; Lah et al., 1995).

The p*K*s[2] of the proton donors contribute to the energetics of reaction 1b and are important to the mechanism of superoxide dismutation by Fe-SOD activity decreases at high pH with a p*K* of 8.8−9 at 25 °C (Fee et al., 1981b; Bull & Fee, 1985) due to an increase in K_M with no change in k_{cat} (Bull & Fee, 1985). Because the rates of the two half reactions are comparable (Bull & Fee, 1985), this p*K* represents the net effect of pH on both half reactions. A p*K* of 8.6−9 also affects the Fe^{3+} site of (oxidized) Fe^{3+}-SOD, causing changes in its optical and EPR spectra (Fee et al., 1981a) and inhibiting substrate analog binding to Fe^{3+} (Bull & Fee, 1985). This p*K* is believed to represent coordination to Fe^{3+} of a second OH^- (Tierney et al., 1995), which acts as a competitive inhibitor and thus increases the apparent K_M of Fe^{3+}-SOD (Scheme 1).

A proton is taken up by Fe^{3+}-SOD upon reduction, throughout the pH range of 7−10 (Bull & Fee, 1985). The group that takes up a proton upon Fe^{3+}-SOD reduction could be one of the two proton donors to substrate upon Fe^{2+} reoxidation (reaction 1b; Scheme 1). This group has been proposed to be the OH^- coordinated to Fe^{3+}, which would then become H_2O upon protonation in Fe^{2+}-SOD (Stallings et al., 1991). Because a *single* proton is taken up upon

† This work was supported by PRF Grant ACS-PRF 28379-G4 and the American Cancer Society Grant IRG 11-33. The NMR spectrometer used was purchased with the generous assistance of NIH Grant RR 04794.

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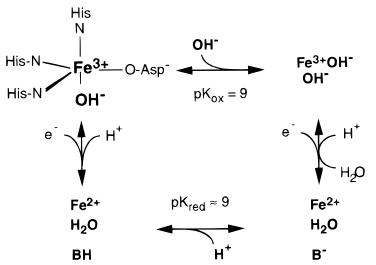
⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1997.

[1] Abbreviations: Asp, aspartate; Arg, arginine; DSS, 4,4-dimethyl-4-silapentane sodium sulfonate; EPR, electron paramagnetic resonance spectroscopy; EXAFS, extended X-ray absorbance fine structure spectroscopy; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; His, histidine; NMR, nuclear magnetic resonance spectroscopy; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SOD, superoxide dismutase; Tyr, tyrosine; WEFT, water-eliminated Fourier transform.

[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.

S0006-2960(96)03047-4 CCC: $14.00

Scheme 1: Cartoon of the Events Associated with Proton Uptake or Release from the Active Site of Fe-SOD, Adapted from Stallings et al. (1991)[a]



[a] The amino acid ligands are only depicted in the low pH oxidized state for the sake of simplicity, but are believed to remain coordinated in the other states as well (Lah et al., 1995).

reduction both above and below the p*K* of ≈9 affecting the Fe^{3+} of Fe^{3+}-SOD and because both Fe-SOD overall catalytic activity and Fe^{3+}-SOD display p*K*s near 9, (reduced) Fe^{2+}-SOD is also inferred to have a p*K* near 9[3] (Bull & Fee, 1985). The ligands to Fe^{2+} were shown not to have a p*K* near 9 by Mössbauer spectroscopy (Niederhoffer et al., 1987). Thus, it was proposed that the hypothetical p*K* near 9 should represent ionization of an amino acid side chain in the active site (Niederhoffer et al., 1987), called BH (Stallings et al., 1991; Scheme 1), and that BH could be the second proton donor.

Tyr 34 is a favorite candidate for BH (Niederhoffer et al., 1987; Lah et al., 1995). The phenoxy O of Tyr 34 is 5.4 Å from Fe^{3+} in Fe^{3+}-SOD (Lah et al., 1995), and Tyr 34 has been proposed to supply a proton required for catalysis or substrate binding in both Fe-SODs and Mn-SODs (Terech et al., 1983; Bannister et al., 1987; Parker & Blake, 1988; Beyer et al., 1991; Stallings et al., 1991).

In order to identify the sources of the protons donated to the substrate and thus understand the mechanism by which Fe-SOD catalyzes its reaction, it is imperative to (1) determine whether the predicted p*K* near 9 does, in fact, occur in Fe^{2+}-SOD and (2) identify this putative p*K*'s significance to catalytic activity. We have used ^{1}H NMR spectroscopy of Fe^{2+}-SOD in anaerobic samples to obtain the first spectroscopic evidence for a p*K* of 8.5, which is consistent with the predicted value near 9. Our NMR spectra reveal that the p*K* is associated with subtle but pervasive changes in the active site spectrum and a dramatic change in hydrogen bonding or access of OH^- to at least one residue in the active site. A forthcoming report seeks to identify the group responsible for the p*K* near 9.

METHODS

Fe-SOD was overexpressed from the *E. coli* sodB gene on the plasmid pHS1-8 in *E. coli* strain QC774 in which both chromosomal SOD genes have been inactivated (Carlioz & Touati, 1986). Cultures were grown microaerobically or anaerobically in order to stabilize Fe^{2+} in solution and thus achieve higher dissolved iron content and bioavailability than is possible with (highly insoluble) Fe^{3+}. Fe-SOD was purified essentially as in Slykhouse and Fee (1976) except that neither hydroxylapatite nor isoelectric focussing were found to be necessary to obtain higher than 95% purity based on SDS−PAGE. Purified enzyme specific activities were all greater than 6500 units/mg of protein, and Fe content was 2.2 Fe/protein dimer based on the absorbance at 350 nm and the published extinction coefficients (Slykhouse & Fee, 1976).

NMR Samples. NMR samples contained approximately 30 mg of Fe-SOD in 0.5 mL, resulting in dimer concentrations of approximately 1.4 mM. ^2H_2O was included to a final concentration of 10% v/v. Because Fe-SOD is stabilized at high pH by very low ionic strengths, Fe-SOD was dialyzed extensively against deionized water prior to pH titrations. A stock solution of NMR pH indicator molecules and DSS was added to produce the final concentrations of the indicators listed in Table 1 and $^1/_3$ mM DSS. Samples of Fe^{2+}-SOD were prepared by degassing Fe^{3+}-SOD plus indicators in an NMR tube with a gas-tight septum and valve seal (Wilmad 507-OF-7). A 2−3 times molar ratio of similarly degassed sodium dithionite solution was used to completely reduce such degassed Fe-SOD, based on its complete loss of color.

pH Titrations. Following the examples of Valcour and Woodworth (1986) and others (Rabenstein & Fan, 1986), we have assembled a selection of small molecules to serve as internal NMR pH indicators (Table 1). For each of the indicators used, we determined the pK_a' in a 10 mM solution in 90% ^1H_2O, 10% ^2H_2O at 30 °C. No attempt was made to correct for the isotope effect of the 10% ^2H_2O. The accuracy of these measurements was limited to ≈0.02 pH unit due to systematic error in pH meter calibration.

Because each indicator's chemical shift(s) varies with pH near the pK_a', the ambient pH of Fe-SOD samples containing the indicators could be determined by reading the indicators' chemical shifts [relative to internal DSS at 0 ppm (De Marco, 1977)] and comparing these with standard chemical shift *vs* pH titration curves obtained under aerobic but otherwise similar conditions. We estimate a possible 0.04 pH unit error due to the effects of ionic strength on the indicators' pK_as (Perrin & Dempsey, 1983). We only used indicators which do not interact with Fe-SOD, based on comparisons of their NMR line widths, chemical shifts, and integrated signal areas in samples with and without Fe^{3+}-SOD present. The indicators used do not to affect the specific activity of Fe-SOD.

The Fe^{2+}-SOD samples were titrated from pH 5.6 to 10.6 and back to pH 6.3 by injection through the sample tube septum of degassed 0.1 M KOH or HCl in 90% ^1H_2O, 10% ^2H_2O. At each point in the titration, two NMR spectra were collected, one optimized for detection of the NMR pH indicators in order to measure the pH and one optimized for observation of Fe^{2+}-SOD active site resonances (below).

Because the titration medium contained only Fe^{2+}-SOD, the indicators, DSS, and the small amount of sodium dithionite required to reduce Fe-SOD, the HCl and KOH added in the course of the titration could make a significant contribution to overall ionic strength. Therefore, samples were titrated both up to high pH and back again to low pH in order to compare the spectra of Fe^{2+}-SOD obtained on the return titration (at higher ionic strength) with the spectra obtained in the ascending titration beginning at low ionic strength. Fewer points were taken in the return titration because the protein is more unstable to addition of HCl. After

[3] If the p*K* of ≈9 of oxidized Fe^{3+}-SOD were replaced by a p*K* of 8 in reduced Fe^{2+}-SOD, then between pH 8 and ≈9 one less proton would be taken up than below 8 or above 10. Similarly, if reduced SOD's p*K* were 10, then between pH ≈9 and 10 one more proton would be taken up than below pH ≈9.

Table 1: pH Indicators used, the Concentrations, and Their pK_a's

indicator compound	concentration (mM)	literature pK	resonance observed	measured pK	$\Delta\delta^a$ (ppm)
imidazole	2.0	6.95[b]	C(2) proton	6.98	−0.912
			C(4), C(5) protons	6.98	−0.348
2,4-dimethylimidazole	1.0	8.47[c]	C(5) proton	8.41	−0.293
			C(2) methyl protons	8.41	−0.250
trimethylamine	0.3	9.80[b]	methyl protons	9.85	−0.708
dimethylamine	0.3	10.77[b]	methyl protons	10.84	−0.438

[a] The difference between the chemical shift asymptotes: high pH − low pH. [b] Determined at 298 K (Perrin & Dempsey, 1983). [c] Determined in 100% ^2H_2O with 100 mM KCl at 303 K (Valcour & Woodworth, 1986).

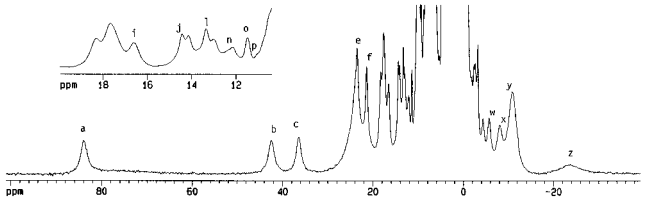


FIGURE 1: Full super-WEFT spectrum of Fe^{2+}-SOD in 90% ^1H_2O at pH 7.0, with an expansion of the region between 10 and 20 ppm, showing lettering of peaks discussed in the text. Sweep width, 65 000 Hz; scans, 8000. This sample was dissolved in 50 mM HEPES, pH 7.0, and 100 mM NaCl, reduced with dithionite, and flame sealed. The pH was determined from the chemical shifts of HEPES, which serves as the internal pH indicator for this sample.

the most extended titration, the sample retained 85% of the starting specific activity.

The pH dependence of the chemical shift of each sufficiently well-resolved resonance of Fe^{2+}-SOD was fit to a Henderson−Hasselbalch equation with allowance for cooperativity (Markley, 1975)

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

in which δ_A and δ_B are the chemical shifts of the acid and base forms (the asymptotes 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.

NMR Conditions. All data were collected on an AMX 300 equipped with a 5 mm probe head and variable temperature control. Spectra were collected at a nominal temperature of 30 °C which was determined to actually be 30.3 °C. Chemical shifts were referenced to internal DSS. The indicator molecules were observed using a spin echo sequence containing 30 ms delays to suppress the signal of Fe^{2+}-SOD. The resonances of Fe^{2+}-SOD's active site were observed using the super-WEFT pulse sequence (Inubishi & Becker, 1983) in combination with a rapid repetition rate to suppress more slowly relaxing resonances. Unless stated otherwise, the resonance of water was suppressed by selective saturation during the 15 ms relaxation delay and the 35 ms delay between super-WEFT pulses.

To observe protons that exchange relatively quickly with water, the jump−return water counter-selective pulse sequence (Plateau & Gueron, 1982; Hore, 1983) was used either alone or with presaturation of water to determine rates of exchange with water (Krishna et al., 1979). The height of peak **b** (42.5 ppm, Figure 1) was measured as a function of pH, normalized to the height of peak **c** (36.4 ppm, Figure 1), which does not exchange rapidly with solvent, in order to correct for instrumental instabilities which affect spectral amplitudes. Selective T_1s were measured using selective inversion of resonance **b** and a nonselective 90° read pulse, with solvent presaturation during the variable recovery delay between the inversion and read pulses and during the relaxation delay between scans, for a total of 300 ms, at the same power as was used in the jump−return plus or minus saturation experiments. The height of peak **b** was normalized to the height of peak **a** (83.9 ppm, Figure 1), which was not inverted by the selective pulse, in order to correct for instrumental instabilities.

RESULTS

pK of 8.5 Affects Many Chemical Shifts. In order to focus on pKs that affect the active site, we have used NMR spectroscopy, because the signals from active site residues can readily be distinguished from those of the rest of the protein on the basis of their striking paramagnetic chemical shifts. The resonances outside 12 to −1 ppm in the NMR spectrum are shown to correspond to protons close to Fe^{2+} in space or connected to it through bonds because these resonances are all absent from the spectrum of Fe^{3+}-SOD (Miller & Sorkin, 1997), although Fe^{3+}-SOD has a very similar structure to that of Fe^{2+}-SOD (Lah et al., 1995) and differs primarily with respect to the oxidation state and spin properties of the Fe. Thus, we refer to the resonances outside 12 to −1 ppm as the active site spectrum.[4]

In Fe^{2+}-SOD, the resonances at ≈84, 42, and 37 ppm (**a**, **b**, and **c**, Figure 1) have been collectively assigned to the δNH protons of the His ligands to Fe^{2+} (Ming et al., 1994). Since these protons are observable despite being close to Fe^{2+} through space as well as connected to it through bonds, at least a few signals should be visible from each of the other amino acid residues in the active site as well, despite paramagnetic relaxation. Thus, although we have not specifically assigned any additional resonances, we can be confident that at least some of the resonances of Tyr 34 and the other amino acids close enough to Fe^{2+} to be remotely plausible proton donors to substrate (His 30 and Tyr 76) should be included in the active site spectrum, and if any of these residues titrate with pH, changes in their chemical shifts should be evident.

Spectra were collected at a range of pHs, and the region from 32 to −18 ppm of selected spectra is shown in Figure 2. Most of the paramagnetically shifted resonances move as the pH is changed. However, the spectral changes are fully reversible and thus not due to the increase in ionic

[4] Some resonances 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+} (and thus susceptible to close to zero dipolar paramagnetic chemical shift).

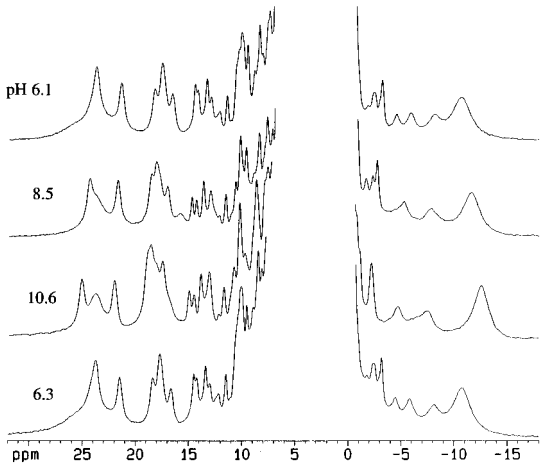


FIGURE 2: Selected NMR spectra from a pH titration of Fe^{2+}-SOD up to high pH and back. The pH 6.3 spectrum is collected upon return to low pH. For each spectrum 4000−12000 scans were collected. Sample conditions and spectroscopic methods are described in the Methods.

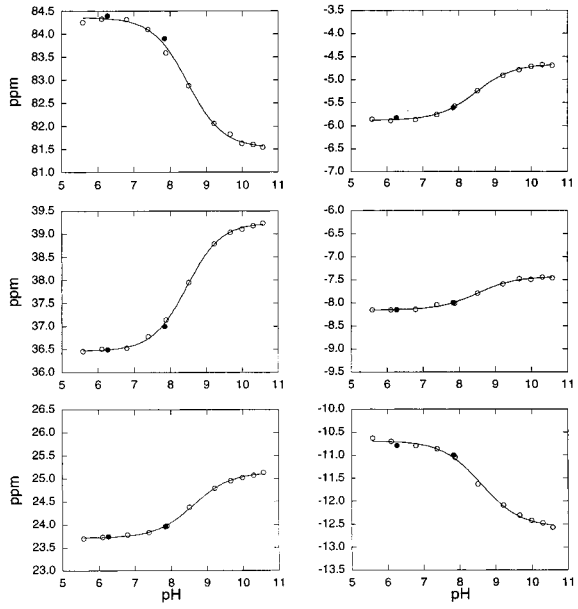


FIGURE 3: pH titration curves for three downfield resonances and three upfield resonances of Fe^{2+}-SOD. These resonances are **a**, **c**, and **e** (left column, top to bottom) and **w**, **x**, and **y** (right column, top to bottom). The chemical shifts are plotted as functions of pH with open circles representing data obtained in the titration from pH 5.6 up to pH 10.6 and filled circles representing data collected on the return to low pH. The data are accompanied by the best fit of eq 2, incorporating the parameters listed in Table 2.

strength that results from the additions of acid and base. Many of the resonances overlap during at least part of the titration so it is possible to obtain full titration curves for only approximately half of them. Nonetheless, it is clear from Figure 2 that pH affects almost all of the active site resonances and more than a single residue.

Plots *vs* pH of the chemical shifts of a few resolved resonances are shown in Figure 3, along with fits to the Henderson−Hasselbalch equation (eq 2). For each of the resonances that was sufficiently well-resolved to permit full analysis, the agreement between the fit and the data is excellent, indicating that a single deprotonation (or OH^- binding) event can explain the chemical shift change. The titrations are all fully reversible, as evidenced by the excellent superposition of chemical shifts obtained in the ascending titration (open symbols) and the return titration to low pH (filled symbols). The p*K*s obtained from two independent titrations of two different samples were all within the error of one another except those of resonance **c** (Table 2). The p*K*s obtained from all the different resonances analyzed only ranged from 8.4 to 8.6 and the Hill coefficients ranged from 0.78 to 0.99 (Table 2). No evidence was obtained for another p*K* in the pH range of 5.5−10.5, for resonances between 84 and 11.5 ppm, and for resonances between −4 and −25 ppm. Thus, a single deprotonation event of p*K* 8.50 ± 0.07 seems to affect the active site spectrum.

The fact that more than 25 resonances titrate, with no evidence for more than one p*K* between 5.5 and 10.5, contrasts with the more commonly observed independence of the p*K*s of different amino acids, or collective titrations involving only two or occasionally three residues (Forman-Kay et al., 1992; Oda et al., 1994). At least some of the spectroscopic pH dependencies observed in Fe^{2+}-SOD must represent indirect effects because the chemical shifts of the δNH protons of the three ligand His all respond to the p*K* of 8.5, although all three His remain protonated and coordinated to Fe^{2+} based on signal areas and chemical shifts. Furthermore, the indirect effects cause the ligand His' chemical shifts to change almost as much as the other most

Table 2: p*K*s Obtained for Different Resonances, in Different Titrations, the Observed Changes in Chemical Shift and the Line Widths

resonance	chemical shift (ppm)[a]	pK^b 1st titration	pK^b 2nd titration	$\Delta\delta$ (ppm)[c] 1st titration	$\Delta\delta$ (ppm)[c] 2nd titration	line width (Hz)[d]
a	83.9	8.48 ± 0.05	8.55 ± 0.05	−2.84	−2.86	500
b	42.5	8.35 ± 0.11	8.50 ± 0.10	0.77	0.82	460
c	36.4	8.45 ± 0.02	8.52 ± 0.04	−2.77	−2.60	380
e	23.6	8.62 ± 0.03	8.65 ± 0.06	1.42	1.32	180
f	21.4	8.50 ± 0.06	8.50 ± 0.06	0.61	0.57	190
i	17.7	8.54 ± 0.04	8.53 ± 0.04	0.95	0.92	160
j	14.5	8.44 ± 0.02	8.49 ± 0.05	0.58	0.55	90
l	13.3	8.41 ± 0.03	8.45 ± 0.05	0.58	0.53	90
o	11.5	8.59 ± 0.09	8.56 ± 0.04	0.34	0.31	70
w	−5.7	8.48 ± 0.04	8.46 ± 0.07	1.22	1.19	220
x	−8.0	8.51 ± 0.06	8.59 ± 0.06	0.72	0.77	320
y	−10.8	8.60 ± 0.06	8.56 ± 0.09	−1.88	−1.89	660
z	−23.7	8.47 ± 0.04	8.47 ± 0.06	4.62	4.49	1600

[a] The chemical shift at pH 7.0 in Figure 1. [b] p*K* and error obtained from a fit of eq 2 to the chemical shift *vs* pH does not include uncertainty in pH determinations. [c] The difference between the chemical shift asymptotes δ_A and δ_B obtained from fit of eq 2: high pH − low pH. [d] The line widths were measured at pH 7.0 in the spectrum in Figure 1, either manually as the width at half height or, in the case of **f**, by Lorentzian line shape fitting. Resonance **e**'s line width was measured at high pH in order to avoid resonance overlap which sets in below pH 9.

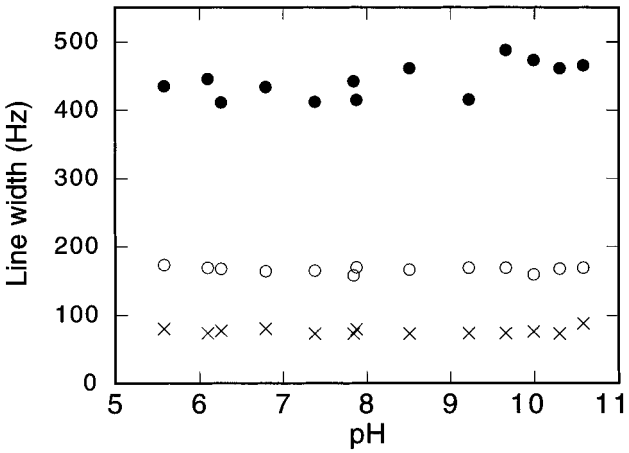


FIGURE 4: pH dependence of the line widths of three resonances, **b** (●), **f** (○), and **o** (×). Line widths were measured manually as the width at half height (**b** and **o**) or by deconvolution of Lorentz line shapes (**f**) in spectra collected at a range of pHs and processed using identical parameters. Spectra were collected and pHs measured as described in the Methods section.

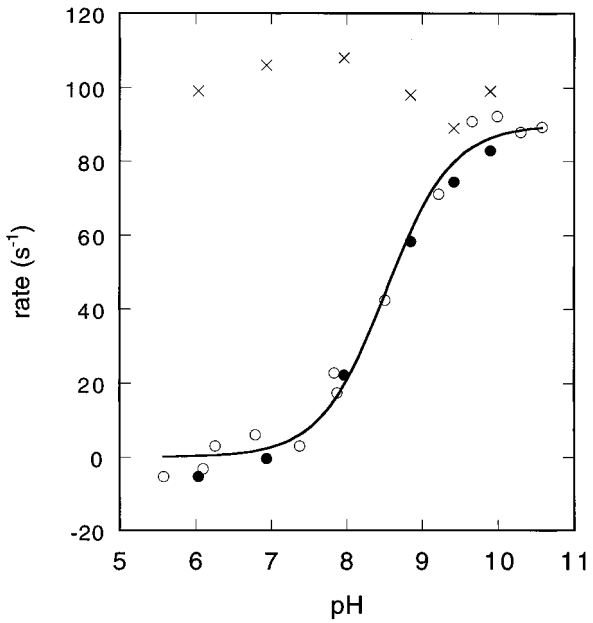


FIGURE 5: pH dependence of the selective $1/T_1$ relaxation rate (×), solvent exchange rate obtained from jump−return data using eq 3 (●), and solvent exchange rate obtained from super-WEFT data and eq 3 (○) for resonance **b**. The solid curve is the best fit to the data of eq 6b, obtained with p$K = 8.53 \pm 0.11$, $K_{op}{}^{A}k_{OH} = (2.7 \pm 0.3) \times 10^7$ M^{-1} s^{-1}. Spectra were collected and pHs measured as described in the Methods.

strongly affected resolved resonance. Thus, large chemical shift changes cannot be used to distinguish resonances of the primary titrating group from resonances whose chemical shift changes represent indirect effects, and the resonances that shift a lot do not provide any clues to the identity of the group that releases a proton.

Significance of the pK. NMR not only permits measurement of the p*K* but also provides valuable insights into events associated with it. Because the resonances whose chemical shifts change the most are also qualitatively the broadest resonances, it appears that the chemical shift changes observed are at least partially due to changes in the paramagnetic contribution to chemical shift. Therefore, to determine whether the chemical shift changes could reflect a change in active site conformation or a change in the paramagnetism of Fe^{2+}, we have characterized the dependence on pH of the magnetic relaxation properties of a few active site protons.

The two resolved resonances at 21.4 and 11.5 ppm (**f** and **o**, Figure 1) have modest chemical shifts and line widths, suggesting that they do not belong to ligands. Therefore, their line widths should reflect only through-space mechanisms of paramagnetic $1/T_2$ relaxation. None of **f**, **o**, or the ligand His δNH resonance **b**'s line widths change significantly over the pH range (Figure 4), in agreement with the fact that no pH-dependent line width changes are visually apparent for any of the resonances in Figure 2. Selective T_1s obtained for resonance **b** at six pHs indicate that **b**'s T_1 is also essentially pH independent (Figure 5). This is consistent with the fact that all the factors that contribute to T_1 also contribute to T_2, and further supports the conclusion that the widespread chemical shift changes do not reflect changes in protons' distances from Fe^{2+}, or a large change in the paramagnetism of Fe^{2+}.

Effect of pH on the Rate of Exchange of a Hydrogen Bonded Ligand Proton. The p*K* near 9 of Fe^{2+}-SOD has been proposed to be related to proton donation and/or substrate binding (Bull & Fee, 1985). Hydrogen bonding could be an important element of both of these proposed functions, and a change in hydrogen bonding could also provide an explanation for the observed widespread chemical shift changes. Essentially all the exchangeable protons in the active site are believed to participate in a hydrogen bond network (Stoddard et al., 1990; Ludwig et al., 1991; Lah et al., 1995). This is borne out by our NMR data, as only resonances **n** and **p** (12.1 and 11.0 ppm, Figure 1) are subject to saturation transfer from H_2O at low pH and even the ligand His side chain δNHs, which normally exchange very rapidly, exchange more slowly than their $1/T_1$ relaxation rates at low pH in Fe^{2+}-SOD.

At increasing pHs, resonance **b** decreased in amplitude in spectra obtained using water presaturation. This was shown to be due to increasingly rapid exchange with saturated water, not deprotonation, as the signal retained full intensity even at high pH if it was observed by jump−return excitation without water presaturation. The ratio of the signal intensity in the presence of water presaturation to that in the absence of water presaturation yields the rate of exchange with saturated solvent provided the apparent T_1 is known (Krishna et al., 1979):

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

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

where M_{ps} and M_o are the peak intensities observed with and without prior saturation of water, respectively, k_{ex} is the exchange rate and T_{1app} is the "apparent" T_1 value measured without taking into account the effect on signal recovery of exchange with water (Waelder et al., 1975; Krishna et al., 1979; Spera et al., 1991). The true T_1 is then calculated from T_{1app} and the exchange rate. This method requires that saturation transfer from water be predominantly mediated by exchange, not by cross relaxation with other resonances (Krishna et al., 1979). The fact that only two other active site resonances appear significantly saturated upon saturation of water suggests that other resonances will not transfer water

saturation on to the His δNH. Moreover, since most of the other protein resonances are not saturated by water, cross relaxation between them and the His δNH under study can contribute to the latter's recovery upon exchange with a saturated water proton. Thus, the appropriate T_{1app} in eq 3a is the *selective* T_{1app} obtained by inverting only the resonance under study, despite the more common and convenient use of nonselective T_{1app}.

Both the selective T_{1app} and the signal intensities in jump return spectra with and without saturation of water were measured at a range of pHs, and the exchange rates and selective $1/T_1$s obtained from eqs 3a and b are given in Figure 5. Since the T_1 was found to be pH independent, exchange rates could also be calculated from the super-WEFT spectra collected over the whole pH range, using the peak amplitude at low pH where exchange is negligible as M_o (Figure 5). Because the method is not able to measure exchange rates significantly slower than $0.1 \times 1/T_1$, the data at pHs, below 7.0 simply indicate exchange rates slower than 10 s^{-1}. At high pHs the measured rate is still well within an order of magnitude of $1/T_1$ so the experimental values are reliable and the rate can be concluded to reach a plateau between pHs 9.4 and 10.

Extensive studies of proton exchange in proteins have shown that at moderate to high pHs such as ours, the exchange rate observed can be considered to represent the product of an equilibrium constant K_{op} for formation of some "open" conformation in rapid equilibrium in which the proton under study is fully accessible to solvent and OH^- [the so-called EX2 regime of Hvidt and Nielsen (1966), the rate constant of base-catalyzed exchange in effect for the open population (k_{OH}) and the OH^- concentration (Englander & Kallenbach, 1984; Englander, 1996).

$$k_{ex} = K_{op} k_{OH} [OH^-] \tag{4a}$$

and

$$\log(k_{ex}) = \log(K_{op} k_{OH}) + \log(K_w) + \text{pH} \tag{4b}$$

Thus $\log(k_{ex})$ *vs* pH is expected to be linear with a slope of 1 in a pH range in which K_{op} is pH independent. The logs of the reliably measured exchange rates are plotted *vs* pH in Figure 6, and between pH 7.0 and 8.5 the data agree qualitatively with eq 4b.

Above pH 8.5, however, the log of the exchange rate does not continue to increase linearly with increasing pH. This could represent the onset of the EX1 regime when the observed exchange rate becomes limited by the rate of opening (Englander & Kallenbach, 1984) or it could reflect a pH-dependent rapid equilibrium between two forms of Fe^{2+}-SOD related to one another by a p*K*. Because the rate constant for base-catalyzed exchange in the open form is considered to depend on the chemical identity of the group under study, modified only by its immediate covalent neighbors, it should be the same for the acid and base forms of the protein (Molday et al., 1972; Bai et al., 1993). By contrast, K_{op} embodies the fraction of time that the exchanging proton is fully accessible to solvent and OH^-. Thus, differences between the observed exchange rates of the proposed acid (A) and base (B) forms are ascribed to different K_{op}s, $K_{op}{}^A$ and $K_{op}{}^B$, respectively. It is easily shown (see Supporting Information) that the observed exchange rate

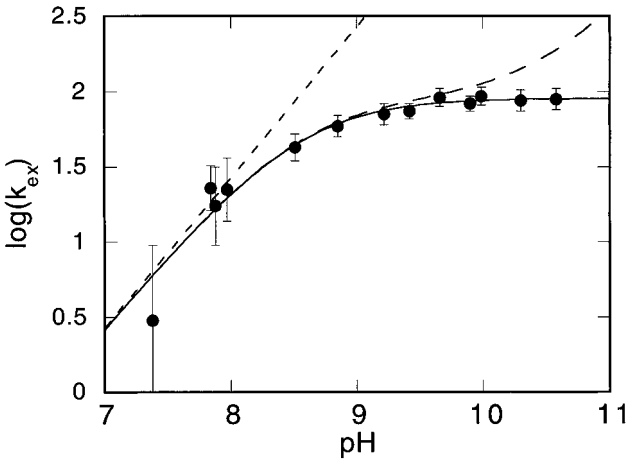


FIGURE 6: Log of the solvent exchange rate, k_{ex}, *vs* pH for resonance **b** (●) accompanied by the best fit of eq 6b to the exchange rate [solid line, p*K* of 8.53 ± 0.11, $K_{op}{}^A k_{OH} = (2.7 \pm 0.3) \times 10^7$ M^{-1} s^{-1}] as well as the low pH asymptote with the limiting theoretical slope of 1 and the value of $K_{op}{}^A k_{OH}$ obtained from the fit (short-dashed line) and the curve obtained using the p*K* and $K_{op}{}^A k_{OH}$ values obtained in the fit but assuming $K_{op}{}^B = 0.01\ K_{op}{}^A$ (long-dashed line). The ends of the error bars are obtained by taking the logs of (k_{ex} − error) and (k_{ex} + error), where the error was determined independently for each value of k_{ex} from the signal-to-noise of resonances **b** and **a** (used for normalization) and the uncertainty in T_{1app}.

can be calculated:

$$k_{ex} = \frac{K_w\, k_{OH}}{K + [H^+]}\left(K_{op}{}^A + \frac{K}{[H^+]} K_{op}{}^B\right) \tag{5a}$$

and

$$\frac{d(\log k_{ex})}{d(\text{pH})} = \frac{[H^+]}{K + [H^+]} + \frac{K K_{op}{}^B}{[H^+] K_{op}{}^A + K K_{op}{}^B} \tag{5b}$$

At limiting low and high pHs, the slope of log k_{ex} *vs* pH tends to 1 (Supporting Information). Between these limits, the slope is always less than 1 for $K_{op}{}^A > K_{op}{}^B$ as in our case. In the context of this model, our slope of approximately zero at high pH indicates that $K_{op}{}^B \ll K_{op}{}^A$ and that our data do not extend into the region in which the expected slope of 1 is recovered. The horizontal separation between the two limiting log k_{ex} *vs* pH straight lines can be shown to be equal to log ($K_{op}{}^A/K_{op}{}^B$) in pH units (see Supporting Information). Thus, from Figure 6, $K_{op}{}^B < 0.01\ K_{op}{}^A$.

If $K_{op}{}^B$ is considered negligible relative to $K_{op}{}^A$, then eq 5a simplifies to

$$k_{ex} = \frac{K_w}{K + [H^+]} K_{op}{}^A k_{OH} \tag{6a}$$

or

$$k_{ex} \approx \frac{10^{-14}}{10^{-pK} + 10^{-\text{pH}}} (K_{op}{}^A k_{OH}) \tag{6b}$$

When eq 6b is fit to the data in Figure 5, we obtain a p*K* of 8.53 ± 0.11, $K_{op}{}^A k_{OH} = (2.7 \pm 0.3) \times 10^7$ M^{-1} s^{-1} and the solid curves shown in Figures 5 and 6. The fit to the data is excellent (Figures 5 and 6) and is contrasted with the dependence on pH of the log of the exchange rate predicted

for the cases of a single pH-independent K_{op} or $K_{op}{}^{A} = 100K_{op}{}^{B}$ in Figure 6.

DISCUSSION

In order to measure the internal pH of the NMR sample at each point in the titration without ever exposing the sample to air, we have used a system of small molecules with pH-sensitive chemical shifts and p*K*s in the range of interest, as internal NMR pH indicators (Rabenstein & Fan, 1986; Valcour & Woodworth, 1986). This method is ideal for anaerobic samples, and the pH indicators constitute a system of buffers which ensures a regular change in pH with acid or base added. The combination of pH indicators used also permits relatively precise measurement of the pH. The inaccuracy of $\approx\pm0.07$ pH unit is dominated by an estimated possible 0.06 pH unit error in the pK_as of the indicators in the protein solutions used. The contribution of errors in measuring chemical shifts is negligible by comparison. The use of indicators makes NMR pH titrations practical for a wide range of air sensitive samples, pressurized samples or samples, with reagents in equilibrium with the gas phase.

Our NMR pH titrations of the active site of Fe^{2+}-SOD yield a single p*K* with a value of 8.50 ± 0.07. Measurements of the p*K* affecting overall activity yielded values of 8.8−9.0 (at 22−25 °C, Fee et al., 1981b; Bull & Fee, 1985) and 9.4 (at 4 °C, Bull & Fee, 1985). Optical and EPR pH titrations of Fe^{3+}-SOD yielded a p*K* of 9.0, whereas the pH dependence of azide binding to Fe^{3+}-SOD yielded a p*K* of 8.6 (at room temperature, Fee et al., 1981a) and 9.4 (at 4 °C, Bull & Fee, 1985). Thus, the p*K* predicted for Fe^{2+}-SOD should be lower at higher temperature and should be slightly less than 8.6−9.0, at 30 °C. Our measured value of 8.5 is consistent with this and is the only p*K* affecting the active site between pHs 5.5 and 10.5. Therefore, we conclude that we have measured the p*K* near 9 that has been predicted for Fe^{2+}-SOD for over 10 years (Bull & Fee, 1985).

All three His δNH protons are visible in NMR spectra even at the highest pH, ruling out deprotonation of any of the His ligands with the p*K* of 8.5. This is consistent with Mössbauer data (Niederhoffer et al., 1987) and chemical intuition (Sundberg & Martin, 1974; Brodsky et al., 1984).

Possible Explanations for the Widespread Changes in Chemical Shift. The widespread chemical shift changes and the pH dependence of exchange with solvent can provide information on the nature and significance of the event with the p*K* of 8.5. Such widespread chemical shift changes in response to a single p*K* could reflect (1) a perturbation of the paramagnetism of Fe^{2+}, including reorientation of the principle axes of the **g** tensor that would alter Fe^{2+}'s contributions to the chemical shifts of all the protons nearby, (2) a conformational change in the active site (and thus altered paramagnetism felt by many protons), and/or (3) the response of many residues to the loss of a proton from a hydrogen bond network that involves them all.

1. Subtle Changes in Paramagnetism Are Probable. Although most of the chemical shift changes observed are small enough to be explained by changes in the diamagnetic component, the facts that (1) almost all the paramagnetically shifted resonances respond to the p*K* and (2) the resonances that shift the most are qualitatively those with the broadest lines (Table 2) immediately suggest that the pH dependent spectral changes are due at least in part to changes in the paramagnetic component of the chemical shift. Thus, either the Fe^{2+} itself or the positions or bonding interactions relative to Fe^{2+} of the protons in the active site appear to be subtly affected by the p*K*.

EPR and Mössbauer spectra of Fe^{2+}-SOD revealed no effect on the metal ion of changing the pH from 7 to 11 (Niederhoffer et al., 1987). However, the EPR spectra were obtained on the NO adduct of Fe^{2+}-SOD, and both the EPR and Mössbauer data were collected at low temperature. Recent studies of Mn^{3+}-SOD have shown that the coordination environment of the metal ion can change significantly with temperature (Whittaker & Whittaker, 1996), and older work on Fe^{3+}-SOD in the presence of excess N_3^- indicated a coordination change upon freezing (Slykhouse & Fee, 1976). Thus, the state of Fe^{2+}-SOD's metal site may not be the same after freezing to low temperature as at the temperatures at which the enzyme turns over. Therefore, the small change in Fe^{2+} paramagnetism required to account for at least a portion of our relatively modest chemical shift changes could have escaped detection in the other experiments.

2. There Is No Change in Active Site Conformation and Paramagnetic Effects are Very Slight. Since conformational changes and changes in hydrogen bonding are commonly associated with chemical shift changes of up to 2 ppm in diamagnetic systems (Baker & Kintanar, 1996), these could also contribute significantly to the chemical shift changes we observe. Moreover, because any changes in paramagnetic chemical shift must be relatively small, they might be due to changes in the protons' positions and bonding relative to Fe^{2+} with only minor changes in the properties of Fe^{2+} itself. These possibilities can be tested because the distance to Fe^{2+} and the spin coupling to Fe^{2+} also affect the paramagnetic contribution to proton relaxation.

Paramagnetic $1/T_2$ relaxation is mediated by Curie, contact, and dipolar mechanisms (Bertini & Luchinat, 1986). Curie relaxation depends on the **g** values, the spin of Fe^{2+}, and the distance r to Fe^{2+} to the minus sixth power. The dipolar contribution is also proportional to $1/r^6$, as well as the electronic spin correlation times, and the contact contribution reflects the degree of through-bond spin coupling between Fe^{2+} and ligand nuclei (LaMar et al., 1973; Bertini & Luchinat, 1986). The magnetic field dependence of Fe^{2+}-SOD's line widths indicates that the Curie contribution dominates even for the ligand His δNHs (Lansing and Miller, unpublished observations) consistent with the large size of Fe-SOD and the short electronic spin correlation times typical of Fe^{2+} (Bertini & Luchinat, 1986). Thus, for nonligand protons, the line width is proportional to $1/r^6$ and extremely sensitive to the distance to Fe^{2+}. The fact that no significant line width changes were observed argues strongly that there is no active site conformational change associated with the p*K* of 8.5.

$1/T_1$ relaxation contains negligible contributions from the Curie mechanism (Bertini & Luchinat, 1986) and thus provides a better vantage point for assessing the pH dependence of dipolar and contact relaxation. Since the line width measurements indicate that the distances to Fe^{2+} are essentially pH independent, then the dipolar contribution to $1/T_1$ must be essentially pH independent. Thus, the pH independence of $1/T_1$ for the ligand His δNH **b** cannot reflect compensating opposite changes in the contact and dipolar contributions, and even the contact contribution must be

essentially pH independent. We conclude that the spin, the average **g** value, and the spin correlation times of Fe^{2+} as well as the degree of through bond spin coupling between Fe and at least one of the ligands cannot have changed very much (although changes in either the **g** anisotropy of Fe^{2+} and/or the orientation of the principle axes of the **g** tensor remain possible). Thus, any perturbations of the paramagnetism of Fe^{2+} are very slight.

3. A Possible Change in Active Site Hydrogen Bonding and/or Access to Solvent or OH$^-$. We have also addressed the possibilities that the p*K* of 8.5 might be associated with changes in active site accessibility or hydrogen bonding by measuring the rate of exchange of one of the hydrogen-bonded active site protons, the δNH proton of a ligand His corresponding to resonance **b**. Active site hydrogen bonds serve to stabilize the structures and tune the reactivities of metal ion sites (Kiefer et al., 1995; Van Dyke et al., 1996). Active site accessibility is also particularly crucial to Fe-SOD activity, as both $O_2^{\bullet-}$ and the protons required for turnover must bind at rates of at least k_{cat}, or 26 000 s^{-1} (Bull & Fee, 1985). The fact that all three of Fe^{2+}-SOD's ligand His exchangeable protons are observable with water presaturation (and one exchanges only on a time scale of hours) indicates that they are involved in hydrogen bonds (Englander & Kallenbach, 1984; Perrin et al., 1990), consistent with the crystallographic predictions (Stoddard et al., 1990; Stallings et al., 1991).

The transient local unfolding model holds that exchange with solvent is retarded for protons buried in folded proteins because they are only exposed to solution a fraction of the time, when the local structure transiently unfolds to a fully accessible open conformation (Englander & Kallenbach, 1984; Bai et al., 1995). Thus, the observed rate of exchange is related to the rate of opening when this is rate limiting (EX1), or the exchange rate of the exposed group times the fraction of time the group is freely accessible to solvent (EX2, Englander & Kallenbach, 1984). In the EX2 regime, the observed rate then yields the equilibrium constant for formation of the accessible open conformation, K_{op} (Englander & Kallenbach, 1984).

The observed increase in exchange rate with increasing pHs between 7.0 and 8.5 agrees with the theoretical prediction of a linear increase in the log of the observed exchange rate with the pH. However, above pH 8.5, our data indicate that either the "closed" conformation of Fe^{2+}-SOD is in relatively slow equilibrium with its related open conformation (EX1) or that the p*K* of 8.5 is associated with a greater than 100-fold decrease in the accessibility of the His δNH. In the former model, the p*K* that emerges from the exchange rate *vs* pH profile is simply the accidental consequence of the value of the opening rate required to explain the data, and the rate constant for base catalyzed exchange of the open conformation. Thus, its agreement with the p*K* affecting the chemical shifts is unexpected (though possible). In the second model, the significant parameters obtained from the data are the p*K* relating the low and high pH forms of Fe^{2+}-SOD, and the K_{op} of the low pH form. Because the chemical shifts' dependencies on pH provide independent evidence for a p*K* affecting the active site and the value of 8.50 $\pm$ 0.07 of this p*K* agrees excellently with the p*K* of 8.53 $\pm$ 0.11 that emerges from the pH dependence of the exchange rate, it is simplest to interpret both pH dependencies in terms of a single event with a p*K* of 8.5.

In most proteins, increasing the pH above 8.5 leads to increased observed exchange rates. However, this is usually due simply to the increased concentration of OH^- and thus the increased base-catalyzed exchange rate, with no change in the accessibility to solvent (and OH^-) until the protein begins to unfold at very high pH (Englander & Kallenbach, 1984). Our model for the pH dependence of Fe^{2+}-SOD incorporates this pH-dependent increase in the rate of base-catalyzed exchange. However, Fe^{2+}-SOD is stable at high pH and the solvent accessibility of an active site proton *decreases* (instead of increasing) by a factor of more than 100 as a result of the event with the p*K* of 8.5. This is unlikely to be the result of a conformational change as we have shown that the event with a p*K* of 8.5 does not involve a conformational change in the active site and the two residues believed to be most important in governing solvent access to the active site are derived from an α-helix that also provides one of the ligands to Fe (Lah et al., 1995). Thus, they are unlikely to undergo a significant conformational change without also affecting the active site. Instead, the observed dramatic decrease in accessibility of one residue in response to deprotonation of another could be due to a change in hydrogen bonding involving both of them, wherein loss of one proton from the hydrogen bonding network causes the others to be bound more tightly. Since the active site hydrogen bond network includes all the ligands to Fe, deprotonation of a group in the network could result in changes in the degree of hydrogen bonding to the ligands that in turn modulate the Fe^{2+}'s electronic spin properties. Thus, altered active site hydrogen bonding could produce subtle changes in the paramagnetic contributions to chemical shifts, as well as changes in the diamagnetic contributions to the chemical shifts of hydrogen-bonded active site amino acids.

Alternately, the additional negative charge (or loss of a positive charge) resulting from deprotonation of the residue with the p*K* of 8.5 could electrostatically decrease the accessibility of the active site to OH^-, the exchange catalyst. Since OH^- is also a substrate analog, decreased active site affinity for OH^- is consistent with the observed increase in K_M associated with the p*K* (Bull & Fee, 1985).

Both proposals are compatible with the popular hypothesis that the p*K* should correspond to deprotonation of the conserved active site Tyr 34. Crystallographic results suggest that Tyr 34 forms a hydrogen bond with the conserved Gln which in turn hydrogen bonds to the coordinated solvent (Stoddard et al., 1990; Ludwig et al., 1991; Lah et al., 1995). Thus, deprotonation of Tyr 34 could indirectly perturb the paramagnetism of Fe^{2+} enough to produce the modest chemical shift changes we observe. Tyr 34 is also positioned between the Fe and the channel proposed to provide access to solvent, so the tyrosinate ion formed upon deprotonation of Tyr 34 could reduce the active site's accessibility to OH^-. No direct evidence that Tyr 34 has a p*K* near 9 in Fe^{2+}-SOD has been published so far. However, now that we have observed the p*K*, we can test this hypothesis using mutant Fe-SOD in which Tyr 34 is replaced by Phe.

CONCLUDING REMARKS

We report the first spectroscopic observation of a p*K* near 9 in Fe^{2+}-SOD that has been predicted for over 10 years. This observation provides strong support for the mechanism

derived earlier by Bull and Fee (1985). The p*K* of 8.5 is associated with widespread changes in the chemical shifts of the active site residues. This complicates its assignment to a specific amino acid. However, this behavior provides important insights into the nature and significance of the deprotonation (or OH^- binding) event. We have ruled out a significant active site conformational change and gross alteration of the electronic spin properties of Fe^{2+}, in addition to deprotonation of a His ligand. However, we have obtained striking indirect evidence suggesting a possible change in the hydrogen bonding or solvent accessibility of the active site. Loss of a proton from either Tyr 34 or another group in the active site hydrogen-bond network could account for this as well as the observed widespread changes in chemical shift.

ACKNOWLEDGMENT

We thank Drs. J. A. Fee and D. Touati for the kind gift of the gene for Fe-SOD on the plasmid pHS1-8 as well as the *E. coli* strain QC774.

SUPPORTING INFORMATION AVAILABLE

Derivation of eq 5a and 5b and its corollaries (2 pages). Ordering information is given on any current masthead page.

REFERENCES

Bai, Y., Milne, J. S., Mayne, L., & Englander, S. W. (1993) *Proteins 17*, 75−86.
Bai, Y., Sosnick, T. R., Mayne, L., & Englander, S. W. (1995) *Science 269*, 192−197.
Baker, W. R., & Kintanar, A. (1996) *Arch. Biochem. Biophys. 327*, 189−199.
Bannister, J. V., Bannister, W. H., & Rotilio, G. (1987) *CRC Crit. Rev. Biochem. 22*, 111−180.
Bertini, L., & Luchinat, C. (1986) *NMR of Paramagnetic Molecules in Biological Systems*, Benjamin-Cummings, New York.
Beyer, W., Imlay, J., & Fridovich, I. (1991) *Prog. Nucleic Acid Res. Mol. Biol. 40*, 221−253.
Brodsky, N. R., Nguyen, N. M., Rowan, N. S., Storm, C. B., Butcher, R. J., & Sinn, E. (1984) *Inorg. Chem. 23*, 891−897.
Bull, C., & Fee, J. A. (1985) *J. Am. Chem. Soc. 107*, 3295−3304.
Carlioz, A., & Touati, D. (1986) *EMBO J. 5*, 623−630.
Carlioz, A., Ludwig, M. L., Stallings, W. C., Fee, J. A., Steinman, H. M., & Touati, D. (1988) *J. Biol. Chem. 263*, 1555−1562.
De Marco, A. (1977) *J. Magn. Reson. 26*, 527−528.
Englander, S. W. (1996) *Hydrogen Exchange & Macromolecular Dynamics*, in *Encyclopedia of NMR* (Grant, D. M., & Harris, R. K., Eds.) pp 2415−2420, John Wiley & Sons, New York.
Englander, S. W., & Kallenbach, N. R. (1984) *Q. Rev. Biophys. 16*, 521−655.
Fee, J. A., McClune, G. J., Lees, A. C., Zidovetzki, R., & Pecht, I. (1981a) *Isr. J. Chem. 21*, 54−58.
Fee, J. A., McClune, G. J., O'Neill, P., & Fielden, E. M. (1981b) *Biochem. Biophys. Res. Commun. 100*, 377−384.
Forman-Kay, J. D., Clore, G. M., & Gronenborn, A. M. (1992) *Biochemistry 31*, 3442−3452.
Hore, P. J. (1983) *J. Magn. Reson. 55*, 283−300.
Hvidt, A., & Nielsen, S. O. (1966) *Adv. Protein Chem. 21*, 287−386.
Inubishi, T., & Becker, E. D. (1983) *J. Magn. Reson. 51*, 128−133.
Kiefer, L. L., Paterno, S. A., & Fierke, C. A. (1995) *J. Am. Chem. Soc. 117*, 6831−6837.
Krishna, N. R., Huang, D. H., Glickson, J. D., Rowan, R., III, & Walter, R. (1979) *Biophys. J. 26*, 345−366.
Lah, M. S., Dixon, M. M., Pattridge, K. A., Stallings, W. C., Fee, J. A., & Ludwig, M. L. (1995) *Biochemistry 34*, 1646−1660.
LaMar, G. N., Horrocks, W. D., Jr., & Holm, R. H. (1973) *NMR of Paramagnetic Molecules*, Academic Press, New York.
Lavelle, F., McAdam, M. E., Fielden, E. M., Roberts, P. B., Puget, K., & Michelson, A. M. (1977) *Biochem. J. 161*, 3−11.
Ludwig, M. L, Metzger, A. L., Pattridge, K. A., & Stallings, W. C. (1991) *J. Mol. Biol. 219*, 335−358.
Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70−80.
Miller, A.-F., & Sorkin, D. L. (1997) *Comments Mol. Cell. Biophys. 9*, 1−48.
Ming, L.-J., Lynch, J. B., Holz, R. C., & Que, L., Jr. (1994) *Inorg. Chem. 33*, 83−87.
Molday, R. S., Englander, S. W., & Kallen, R. G. (1972) *Biochemistry 11*, 150−158.
Niederhoffer, E. C., Fee, J. A., Papaefthymiou, V., & Münck, E. (1987) in *Isotope and Nuclear Chemistry Division, Annual report*, pp 79−84, Los Alamos National Laboratory.
Oda, Y., Yamazaki, T., Nagayama, K., Kanaya, S., Kuroda, Y., & Nakamura, H. (1994) *Biochemistry 33*, 5275−5284.
Parker, M. W., & Blake, C. C. F. (1988) *J. Mol. Biol. 199*, 649−661.
Perrin, D. D., & Dempsey, B. (1983) *Buffers for pH and Metal Ion Control*, Chapman and Hall, London.
Perrin, C. L., Dwyer, T. J., Rebek, J., Jr., & Duff, R. J. (1990) *J. Am. Chem. Soc. 112*, 3122−3125.
Plateau, P., & Gueron, M. (1982) *J. Am. Chem. Soc. 104*, 7310−7311.
Rabenstein, D. L., & Fan, S. (1986) *Anal. Chem. 58*, 3178−3184.
Slykhouse, T. O., & Fee, J. A. (1976) *J. Biol. Chem. 251*, 5472−5477.
Spera, S., Ikura, M., & Bax, A. (1991) *J. Biomol. NMR 1*, 155−165.
Stallings, W. C., Metzger, A. L., Pattridge, K. A., Fee, J. A., & Ludwig, M. L. (1991) *Free Radical Res. Commun. 12*−*13*, 259−268.
Stoddard, B. L., Howell, P. L., Ringe, D., & Petsko, G. A. (1990) *Biochemistry 29*, 8885−8893.
Sundberg, R. J., & Martin, R. B. (1974) *Chem. Rev. 74*, 471−517.
Terech, A., Pucheault, J., & Ferradini, C. (1983) *Biochem. Biophys. Res. Commun. 113*, 114−120.
Tierney, D. L., Fee, J. A., Ludwig, M. L., & Penner-Hahn, J. E. (1995) *Biochemistry 34*, 1661−1668.
Valcour, A. A., & Woodworth, R. C. (1986) *J. Magn. Reson. 66*, 536−541.
Van Dyke, B. R., Saltman, P., & Armstrong, F. A. (1996) *J. Am. Chem. Soc. 118*, 3490−3492.
Waelder, S., Lee, L., & Redfield, A. G. (1975) *J. Am. Chem. Soc. 97*, 2927−2928.
Whittaker, M. M., & Whittaker, J. W. (1996) *Biochemistry 35*, 6762−6770.

BI963047Z