ABSTRACT: Fe-containing superoxide dismutase (FeSOD) is an excellent model for studies of the role of the protein in shaping redox catalytic activity in metalloenzymes. In order to use NMR spectroscopy to monitor the protons in FeSOD, we have assigned all the observable backbone resonances in the HN heteronuclear single quantum correlation spectrum (HN-HSQC), in both of the reduced and oxidized states. This task required $^2$H, $^13$C and $^15$N triple labeling and quadruple resonance techniques due to FeSOD’s molecular weight of 42 kDa and paramagnetic high-spin active site Fe. The unobserved and unassigned residues of FeSOD are accounted for by paramagnetic relaxation and slow back-exchange of solvent protons into backbone H$^N$ positions. Of FeSOD’s 192 residues, we have previously assigned resonances for 118 in the oxidized state and now 141 in the reduced state. For residues $> 14$ Å from Fe, resonances are observable in both oxidation states. Relatively small chemical shift changes were found to accompany Fe$^{2+}$ oxidation, but these extend throughout the protein. Specific binding of the substrate analog F$^-$ to Fe$^{2+}$ SOD was found to involve residues near Tyr34, consistent with participation of this residue in substrate binding. This represents the first location of substrate binding to Fe$^{2+}$ SOD. Additional residues at the dimer interface are sensitive to a variety of anions in both of FeSOD’s oxidation states and also Fe oxidation, consistent with either interaction with substrate or domain movement associated with substrate binding and metal ion oxidation. Copyright © 2000 John Wiley & Sons, Ltd.

KEYWORDS: NMR; non-heme iron; paramagnetic; superoxide dismutase; redox-linked changes; substrate binding

INTRODUCTION

Fe-containing superoxide dismutase (FeSOD) is an excellent model in which to study the role of the protein in shaping metal-centered redox catalysis, both because it embodies three essential ingredients of redox catalysis (electron transfer, proton transfer coupled to electron transfer and substrate binding) and because as redox-active metalloenzymes go, FeSOD is exceptionally simple, stable and small.

FeSOD and homologous MnSOD are members of a group of enzymes that forestall oxidative damage by scavenging O$_2$ and H$_2$O$_2$ and converting them to O$_2$ and H$_2$O (reviewed in Refs 1 and 2). FeSOD catalyzes the two-step $^1$ disproportionation of 2O$_2$ to O$_2$ and H$_2$O$_2$:

$$\text{SOD(Fe}^{3+}) + \text{O}_2^{••} + \text{H}^+ \longrightarrow \text{SOD(Fe}^{2+}) + \text{O}_2 + \text{H}_2\text{O}_2$$

(1a)

Each of FeSOD’s identical monomers contains a simple active site consisting of a single high-spin Fe ion with two His and an Asp as equatorial ligands, and a His and a coordinated solvent as axial ligands. The second-sphere residue Tyr34 is also universally conserved among FeSODs and MnSODs. The active site is buried but protein dynamics are believed to admit the substrate O$_2^{••}$ for reaction (1a). However, reaction (1b) is believed to proceed via an outer-sphere mechanism. Until now, this binding site for substrate has been unknown, but recently Sorkin has shown that (reduced) Fe$^{2+}$ SOD binds F$^-$, so the effects of this anion can be used to locate the reduced state binding site.

By contrast, the Fe$^{3+}$ of oxidized Fe$^{3+}$ SOD is known to coordinate substrate analogs F$^-$, N$_3^-$ and OH$^-$ with $K_d$s of 7 mM, 2 mM and 4 µM, respectively. Other small anions including SCN$^-$ ($K_D = 32$ mM)$^5$ also inhibit FeSOD and competitively inhibit N$_3^-$ binding.$^5$ Bull and Fee$^6$ concluded that these anions could occupy an ‘anion binding pocket’ in both FeSOD oxidation states. In contrast, Argese et al.$^9$ suggested that anions do not bind to a site, but inhibit FeSOD by electrostatic screening as a function of ionic strength. Our NMR data provide the first concrete evidence for the existence of a substrate binding
site distinct from the metal ion, that interacts with even non-coordinating inhibitors of FeSOD.

As a prerequisite for characterizing redox changes and substrate binding to FeSOD, we have assigned the $^1$H, $^{13}$N, $^{13}$C, $^{15}$C and $^{13}$O resonances of the backbone, including all the backbone amides visible in the conventional HN heteronuclear single quantum correlation spectrum (HN-HSQC) in both FeSOD’s Fe$^{3+}$ and now Fe$^{2+}$ oxidation states. Despite the fact that FeSOD is much smaller than most redox-active metalloenzymes, the 42 kDa dimer is larger than most proteins that have been assigned by NMR. At the time of writing we are aware of only five larger systems which have been substantially assigned. However, the two monomers of FeSOD are symmetry-related in the x-ray crystal structure, and we find that they are spectroscopically identical in both oxidation states.

FeSOD exhibits a $\tau_c$ of 22 ns, which results in rather broad $^1$H resonances in the proteated protein. In addition, paramagnetic effects further shorten the $T_1s$ and $T_2s$ of $^1$H’s less than 10 Å from an Fe$^{2+}$ ion in Fe$^{2+}$SOD and less than 14 Å from an Fe$^{3+}$ in Fe$^{3+}$SOD. Hence the active site resonances must be treated as a separate spectroscopic problem. NMR assignments of paramagnetically shifted active site resonances have been published for many non-heme, non-sulfur Fe-proteins (e.g. Refs 17–20) and backbone assignments have been obtained for several heme proteins, 21–23 and iron–sulfur proteins. 24–26 Ours are the first substantial backbone assignments of a non-heme, non-sulfur Fe-protein. Furthermore, since FeSOD has catalytic activity, not just electron transfer activity, our assignments provide valuable spectroscopic probes of aspects of redox catalysis such as substrate binding and release, proton transfer and conformational changes related to activity.

Chemical shift changes associated with oxidation state change have been observed in cytochromes and ferredoxins. These have so far been found to represent minor conformational changes accompanied by substantial changes in the paramagnetic contribution to chemical shift. 22,24,26,56,57 However, significant redox-linked changes in dynamics have been observed. 21,25,27–30 The structures of different oxidation states of metalloenzymes have also been compared and generally found to differ relatively little outside the active site, 31–33 but these comparisons were generally made in the crystalline state. Hence our solution-state comparison of two oxidations states of a larger metalloenzyme is unique. Our comparison of the chemical shifts and $^1$H solvent exchange rates of Fe$^{3+}$- and Fe$^{2+}$SOD indicates relatively subtle redox-linked long-range effects on conformation and dynamics in FeSOD and our analysis of chemical shift changes in response to anions identifies the location of substrate binding to Fe$^{2+}$SOD for the first time.

**EXPERIMENTAL**

**Sample preparation**

Uniformly $^2$H-, $^{13}$C- and $^{15}$N-labeled FeSOD was overexpressed from *Escherichia coli* BL21 harboring the plasmid pRLK3, a sodB-bearing derivative of pET-24a constructed by R. L. Koder, Jr, in the Miller laboratory, and grown in 100% $^2$H$_2$O $^{15}$N, $^{13}$C-M9 (containing 100 μM FeCl$_3$ and exclusively [U-$^2$H$_3$], $^{13}$C-glucose) supplemented with 0.05% Bioexpress (fully $^2$H-, $^{13}$C- and $^{15}$N-labeled, from Cambridge Isotope Laboratories). Overexpression of FeSOD was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 1 mM when the cultures reached an A$_{600}$ of 0.8, after 9 h in culture. Cells were harvested 1 h later when the cultures had reached an A$_{600}$ of 1.6 A. A 50 mg amount of perdeuterated FeSOD was purified from two 11 cultures as described previously 33,35 and had a specific activity of 5900 units mg$^{-1}$ protein based on the standard assay. 36 This activity is comparable to the usual activity of ~6000 ± 100 u mg$^{-1}$.

Perdeuterated FeSOD was purified and stored for over 6 months at 4°C in solutions made up in $^1$H$_2$O, resulting in exchange of most of the backbone amide $^1$Hs for $^2$Hs. Experiments were conducted on 17 mg of (~1.3 mM) Fe$^{3+}$SOD in 0.25 ml of 10 mM morpholinooethanesulfonic acid (MES), pH 6.0, 10 mM NaCl in 90% $^1$H$_2$O–10% $^2$H$_2$O (the standard buffer) in a 5 mm Shigemi tube, and a sample of 33 mg (~1.3 mM) Fe$^{2+}$SOD in 0.5 ml of the same solution at pH 6.5. To produce a sample of Fe$^{2+}$SOD, Fe$^{3+}$SOD was degassed and purged with Ar repeatedly (in the NMR tube) and then reduced by addition of a few microliters of 140 mM Na$_2$S$_2$O$_4$ in 100 mM NaOH. The sample was flame-sealed and the final internal pH determined to be 6.5 based on the NMR spectrum of internal MES. In order to be able to directly compare oxidized and reduced FeSOD at the same pH, a $^{15}$N-labeled Fe$^{3+}$SOD sample was prepared at pH 6.5. The spectrum of Fe$^{3+}$SOD at pH 6.5 was virtually superimposable on that of Fe$^{2+}$SOD at pH 6, so assignments made at pH 6 could be extended to pH 6.5 spectra with confidence. Samples were stored at 4°C and NMR experiments were conducted at 25°C.

**NMR experiments**

The backbone $^1$H, $^{15}$N, $^{13}$C, $^{15}$C and $^{13}$C were assigned based on constant-time 3D NMR experiments incorporating sensitivity enhancement and deuterium decoupling. 37 Except for the HNCOS, 3D NMR experiments were conducted at NCI-FCRDC. HNCOS and a variety of 2D spectra were collected at The Johns Hopkins University. Data were collected on Unity$^{TM}$ 380 MHz NMR spectrometers operating at 500 MHz for $^1$H using 5 mm Nalorac or Varian HCN probes with Z-axis pulsed field gradients and facility for $^2$Hdecoupling. The deuterium channel was custom built by one of the authors (R.A.B.). Table 1 lists the NMR spectra acquired and the parameters used.

NMR data were processed using nmrPipe and analyzed using ANSIG. 40 All chemical shifts are relative to $^1$H of 4,4-dimethyl 4-silapentane sodium sulfonate (DSS) at 0 ppm, including $^{13}$C and $^{15}$N, via the ratios of the gyromagnetic ratios of each of these nuclei with that of $^1$H. 41

The rates of exchange at 25°C of $^1$H’s were measured based on resonance volumes in HSQC spectra collected
Table 1. Summary of spectroscopic data used in assigning the backbone of FeSOD

<table>
<thead>
<tr>
<th>Experiment (SOD oxidation state)</th>
<th>F1 (15N)</th>
<th>F2 (15N)</th>
<th>F3 (1H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>SW (Hz)</td>
<td>Complex points</td>
<td>SW (Hz)</td>
</tr>
<tr>
<td>HNCA (red)</td>
<td>15 C (56 ppm)</td>
<td>3268</td>
<td>56</td>
</tr>
<tr>
<td>HNCA (ox)</td>
<td>15 C (56 ppm)</td>
<td>3268</td>
<td>56</td>
</tr>
<tr>
<td>HN(CA)CA/CO (red)</td>
<td>13 C (56 ppm)</td>
<td>3268</td>
<td>48</td>
</tr>
<tr>
<td>HN(CA)CA/CO (ox)</td>
<td>13 C (56 ppm)</td>
<td>3268</td>
<td>48</td>
</tr>
<tr>
<td>HN(CA)CB (red)</td>
<td>13 C (43 ppm)</td>
<td>7543</td>
<td>56</td>
</tr>
<tr>
<td>HN(CA)CB (ox)</td>
<td>13 C (43 ppm)</td>
<td>7543</td>
<td>56</td>
</tr>
<tr>
<td>HN(COCA)CB (red)</td>
<td>13 C (43 ppm)</td>
<td>7543</td>
<td>48</td>
</tr>
<tr>
<td>HN(COCA)CB (ox)</td>
<td>13 C (43 ppm)</td>
<td>7543</td>
<td>48</td>
</tr>
<tr>
<td>HNCO (red)</td>
<td>13 C (175 ppm)</td>
<td>1700</td>
<td>60</td>
</tr>
<tr>
<td>HNCO (ox)</td>
<td>13 C (175 ppm)</td>
<td>1700</td>
<td>60</td>
</tr>
<tr>
<td>HN(COCA)CB (red)</td>
<td>13 C (43 ppm)</td>
<td>7919</td>
<td>48</td>
</tr>
<tr>
<td>HSQC (both)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*Approximate carrier frequency.

at intervals after transfer of FeSOD from 10 mM MES in \(\text{H}_2\text{O}\) buffer into \(\text{D}_2\text{O}\) buffer by gel filtration over G10 resin pre-equilibrated with \(\text{H}_2\text{O}\) buffer. The \(\text{D}_2\text{O}\) buffer solution contained the same buffer and salt as the \(\text{H}_2\text{O}\) buffer and was adjusted to an uncorrected pH meter reading of 6.0.

\(15\text{N}\)-labeled samples of FeSOD were used to identify extremely slowly exchanging H N's and residues involved in substrate analog binding. Fe\(^{3+}\)SOD samples were prepared in 10 mM MES standard buffer with either 5 mM Na\(_3\)N, 10 mM KF, 100 mM KSCN, 100 mM KBr or no additions (the control) and characterized by NMR in the oxidized state. The Na\(_3\)N or KF concentration was increased to 100 mM in the relevant samples, and these were characterized again. All five samples were then reduced, flame sealed and characterized by NMR one more time. The pH was confirmed to remain within 0.1 pH unit of 6.0 throughout for most samples. Resonances that shifted upon addition of ligand were tentatively mapped on to the nearest new resonance appearing in the spectrum obtained in the presence of substrate analog. By this conservative analysis, all the chemical shift changes reported are the minimum change consistent with the spectra.

**RESULTS AND DISCUSSION**

**Assignment of the backbone HSQC resonances of Fe\(^{3+}\) SOD is limited by paramagnetism and slow exchange**

Perdeuterated FeSOD suspended in 90% \(\text{H}_2\text{O}\) was employed for all the 3D experiments used to obtain backbone assignments. The overall greater resolution and greater sensitivity obtained with such samples reflect doubling of the H N \(T_2\) relaxation times upon perdeuteration. The virtual absence of signals in the aliphatic region of the \(\text{H}^1\) 1D spectrum of Fe\(^{3+}\)SOD shows that deuteration is almost complete (Fig. 1).

Although 183 backbone amide resonances plus seven indoles and 21 pairs of side-chain amide resonances might ideally be observed, the standard HSQC of [U-\(\text{H}\), \(13\text{C}\), \(15\text{N}\)]Fe\(^{3+}\)SOD in \(\text{H}_2\text{O}\) reveals only 132. All the resonances observed have superimposable counterparts in the HSQC of (proteated) [\(1\text{H}\), \(15\text{N}\)]Fe\(^{3+}\)SOD. However, at least 36 amides visible in the spectrum of proteated Fe\(^{3+}\)SOD suspended in \(\text{H}_2\text{O}\) fail to exchange with \(\text{D}_2\text{O}\) even after prolonged incubation at 25°C (weeks) and their signals are missing from the spectrum of perdeuterated Fe\(^{3+}\)SOD, presumably because these positions retain the \(\text{H}\) incorporated biosynthetically. The slowly exchanging amides are very well protected indeed, as 19 of them failed to exchange even upon incubation of Fe\(^{3+}\)SOD at pH 9.5 for 2 weeks at 25°C and at pH 9.5 in 2 M urea for an additional 2 days at 37°C. The very
Plate 1 Comparison of the locations of residues that could not be assigned or observed in Fe$^{2+}$SOD (red, A) and residues not observed or assigned in Fe$^{2+}$SOD (green, B). (A) Fe$^{2+}$SOD residues for which at least one assignment was obtained are coloured blue and residues for which no assignments were obtained are coloured red. (B) Fe$^{2+}$SOD residues for which at least one assignment was obtained are coloured blue and residues for which no assignments were obtained are coloured green. Coordinates used are those of Fe$^{2+}$SOD, 1ISA, from Ref. 5.
Plate 2 Chemical shift differences for individual nuclei (A) and decomposition of r.m.s. chemical shift change at each residue (B) into maximum paramagnetic component (C) and minimum conformational component (D).

For each residue, the $H^o,N,C',C^\alpha$ and $C^\beta$ oxidized–reduced chemical shift changes (as available) are plotted in orange, green, red, yellow and blue, respectively in (A). (B), (C) and (D), respectively, show the r.m.s. of the various chemical shift changes at each residue (blue), their average, = maximum paramagnetic component (red) and the standard deviation = minimum conformational component $[(x^2) - (\langle x \rangle)^2]^{1/2}$, green.

Since the chemical shift changes were calculated as oxidized – reduced chemical shifts, they are the effect of oxidation, or $-1^*$ the effect of reduction.
Plate 3 Locations of residues with the greatest r.m.s. chemical shift differences between the oxidized and reduced states.

The r.m.s. of the changes in H, N, C', Cα, and Cβ chemical shifts upon oxidation (ox-red), for each residue, are shown plotted on the structure using red to denote r.m.s. changes of 0.84 ppm and higher and orange through yellow and green to blue for smaller changes. Note that a residue with no r.m.s. change in chemical shifts would appear in the same colour of blue as assigned residues in Plate 1, but no such residues were found (e.g. Ala104A near the top left corner experiences a 0.08 ppm r.m.s. chemical shift change). The changes in chemical shift could only be calculated for residues that were assigned in both oxidation states, other residues are coloured grey. For each residue, all the nuclei for which assignments were available in both oxidation states were included in the r.m.s., but this was as few as two chemical shifts in some cases. The changes in C' chemical shift are not taken into consideration for Gly86 and Asp105 in making this figure, but are shown in Plate 2. Gly86 and Asp105 have very large changes in their C' chemical shifts in the direction opposite to changes in their neighbours' C' chemical shifts and in the context of small positive and negative changes in their other nuclei's chemical shifts (Plate 2). Thus Gly86 and Asp105's chemical shift changes should be viewed with caution and their C' chemical shift changes were not included in the calculation of the r.m.s. change used this figure. Lys107's Cα chemical shift change also deviates sharply from those of its neighbours but in this case the C' and Cβ changes do also (Plate 2). These three residues are all more than 20 Å from the closest Fe.
Plate 4 Chemical shift differences in Fe$^{2+}$SOD amide proton (H) and nitrogen (N) resonances between the control and samples with anion added.

Each sample, including the control, contained 1 mM Fe$^{2+}$SOD dimers in 10 mM MES and 10 mM NaCl, pH 6.0. KF, NaN$_3$, KBr and KSCN were added to a final concentration of 100 mM to four different protein samples. HSQC spectra were collected on all samples as described in the Experimental section. The actual change in $^1$H chemical shift (control – salt added) has been multiplied by a factor of five, for ease of graphical representation of both $^{15}$N and $^1$H changes in the same ppm scale. For residues with no detectable changes, 0.01 ppm for $^1$H and -0.03 ppm for $^{15}$N were applied to reflect the uncertainty in chemical shift measurement and to distinguish them from peaks for which no assignments have been obtained.
Plate 5 Location of residues affected by addition of anions in FeSOD.
The chemical shift changes upon addition of anions for each residue are mapped on the structure of FeSOD using grey for regions where no backbone assignments have been made and blue for residues that are assigned. Residues Lys116, Ans117 and Phe118, which experience significant changes in amide $^1$H and/or $^{15}$N chemical shifts in both oxidation states upon addition of each of the four anions, namely F-, N$_3$-, Br- and SCN- are coloured in yellow. Residues 35 – 40 that are affected only in the reduced state of the enzyme upon addition of 100 mM F- are shown in red. The Fe atoms are shown as orange spheres.
Plate 6 Chemical shift changes of Fe³⁺SOD amide resonances between the control and samples with different anions added. Each sample, including the control, contained 1 mM Fe³⁺SOD dimers in 10 mM MES and 10 mM NaCl, pH 6.0. KBr and KSCN were added to a final concentration of 100 mM to two different protein samples. KF was added first to a concentration of 10 mM and further increased to 100 mM final concentration while NaN₃ was initially added to a concentration of 5 mM and also further increased to a total concentration of 100 mM. HSQC spectra were collected on all samples as described in the Experimental section. The actual change in ¹H chemical shift (control – salt added) has been multiplied by a factor of five, for ease of graphical representation of both ¹H and ¹⁵N changes in the same ppm scale. For residues with no detectable changes, 0.01 ppm for ¹H and −0.03 ppm for ¹⁵N were applied to reflect the uncertainty in chemical shift measurement and to distinguish them from peaks for which no assignments have been obtained.
slow exchange of many backbone H's is consistent with FeSOD’s excellent stability, and its $T_M$ above 70 °C (Wintrone P. and A.-F. Miller, unpublished work). In addition, each monomer of FeSOD contains a paramagnetic Fe$^{2+}$ ion which severely relaxes resonances of nuclei nearby and precludes their observation in the HSQC.

Of the 132 Fe$^{2+}$SOD resonances, we have assigned 117 to backbone NHs and have obtained assignments for the C's and C's of 24 more residues, via the NH of the following residue (Table 2, Fig. 2, Plate 1A). The assignments extend from residue number 1 to the C terminus comprising 141 or 73% of SOD’s residues and including all the surface loops, consistent with FeSOD’s excellent stability. The assigned chemical shifts have been deposited in the BioMagResBank under accession number 4341.

Eleven of the 16 non-Pro, non-$N$-terminal residues whose C and C were assigned via the NH of the succeeding residue correspond to NH$_i$C resonances that were visible even in the oxidized state. Thus, the resonances of these NHs are probably not missing due to paramagnetism, but due to slow exchange instead. Indeed, 10 of these 11 residues occur in helices or the β sheet, or are packed against the β sheet. The five other assigned residues’ NHs are probably obliterated by paramagnetism. The 10 assigned residues whose NHs are probably missing due to slow exchange can easily be accounted for among the at least 36 resonances observable in [1H; $^{15}$N]Fe$^{2+}$SOD but not deuterated Fe$^{2+}$SOD. In addition, 20 completely unassigned residues far from Fe occur in two strands of the β sheet, as well as the faces of helices packed against the β sheet and two stretches of unassigned α helix (see Plate 1A). Thus, 30 of the ≥36 resonances missing due to slow exchange can easily be rationalized on the basis of FeSOD’s secondary and tertiary structure.

Differential scanning calorimetry of MnSODs with mutations in the active site indicates that the mutants are more stable than the wild-type (but see Ref. 43). This implies that the active site of wild-type SOD represents a strained conformation which is enforced by highly favorable interactions elsewhere in the protein. The extremely slow H$^+$ exchange we observe well beyond the active site is consistent with the existence of such very stable interactions.

Since at least 25 unassigned resonances are absent from the HSQC due to slow exchange, up to 27 of the 50 non-Pro residues not assigned here may be absent from the HSQC due to paramagnetic relaxation. Indeed, many of the unassigned (red) residues in Plate 1 are close to the Fe$^{2+}$.

Nonetheless, we have assigned $^{13}$C nuclei as close as 9 Å from Fe$^{2+}$, and our assignments include a number of NHs with significantly paramagnetically shifted 1H chemical shifts. The amide protons of Asn140, Ala141 and Ser120 occur at 12.2, 11.5 and 11.1 ppm, respectively. Indeed, Ala141 is the analog in FeSOD of the active site Gln146 of MnSOD that is central to the active site hydrogen bond network and believed to participate in determining SOD’s metal ion specificity. The fact that this residue could be assigned in standard 3D sequences indicates that however coupled to the metal ion’s coordination sphere it may be in MnSOD, it is relatively unaffected by Fe’s paramagnetism in FeSOD. We presume that this difference is primarily due to the different identities of the side-chains in the two proteins, but it may also be indicative of subtle differences in the position of this stretch of backbone.

Table 2. Numbers of each type of nucleus assigned in this work and by Vathyam et al. 10

<table>
<thead>
<tr>
<th>Oxidation state</th>
<th>H$^N$</th>
<th>N</th>
<th>C$\alpha$</th>
<th>C$\beta$</th>
<th>C$'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced</td>
<td>117</td>
<td>117</td>
<td>114</td>
<td>141</td>
<td>111</td>
</tr>
<tr>
<td>Oxidized</td>
<td>95</td>
<td>95</td>
<td>118</td>
<td>118</td>
<td>94</td>
</tr>
</tbody>
</table>

Figure 2. Sample backbone walk. Representative strips from HNCA (left) and HN(CA)CB spectra (right) used to make backbone assignments. Each strip is labeled on top by the amino acid whose NH was detected and at the bottom with the 1H chemical shift as well as the 15N chemical shift. Light (dark) contours are positive (negative) and indicate an even (odd) number of attached aliphatic carbons.
Assignment of Fe\(^{3+}\) SOD

In the oxidized state, we assigned 95 of the 118 HN resonances observable in the HSQC to backbone amides. In addition, \(^{13}C\) resonances of 23 residues including seven of the eight prolines were assigned via the NH of the following residue, for a total of 118 assigned residues.\(^{10}\)

The smaller number of H\(^{1}\) resonances visible in the oxidized state is consistent with electronic differences between Fe\(^{3+}\) and Fe\(^{2+}\).\(^{45,46}\) High-spin Fe\(^{3+}\)\(^{2+}\)'s ionic ground state and distant excited states (and hence its slow electron spin relaxation) conspire to make it a more severe \(^{1}H\) relaxer than Fe\(^{2+}\), so that residues with the H\(^{1}\) less than 14 Å from Fe\(^{3+}\) are relaxed beyond detection [vs 10 Å from Fe\(^{2+}\); Plate 1(B)]. In addition, Fe\(^{3+}\)'s highly symmetric electronic state does not confer paramagnetic chemical shifting on nearby residues. Hence their broader resonances are obscured beneath sharper resonances of residues far from Fe\(^{3+}\).

Comparison of Fe\(^{3+}\) SOD's chemical shifts with those of Fe\(^{2+}\) SOD

Comparison of the chemical shifts of Fe\(^{3+}\) SOD and Fe\(^{2+}\) SOD (both at pH 6.5) reveals that virtually every resonance moves significantly (Plate 2). The average residue experiences a 0.24 ppm r.m.s. change in the \(^{1}H\), \(^{13}C\) and \(^{13}C\) chemical shifts, with a maximum r.m.s. value of only 1.6 ppm. Owing to the relatively small magnitude of chemical shift changes observed, these may represent conformational as well as paramagnetic effects, and neither can be safely assumed to dominate. However, the residues with the largest changes are those whose neighbors were unassignable in the oxidized state specifically, consistent with paramagnetic effects (Plate 3). Residues with comparable chemical shift changes for \(^{1}H\), the \(^{13}Cs\) and \(^{13}N\) are most likely responding to changes in dipolar paramagnetic contributions to chemical shift, since this depends on position relative to Fe but not on gyromagnetic ratio. Thus, the average change in chemical shift at each residue, \(\langle x \rangle\), provides a qualitative gauge of the maximum significance of paramagnetic contributions to chemical shift in the reduced state. Chemical shift changes due to conformational changes have a greater tendency to cancel out of the averages,\(^{47,48}\) but still contribute fully to the mean square chemical shift changes, \(\langle x^2 \rangle\), and the root mean square (r.m.s. \(\langle x^2 \rangle^{1/2}\)) at each residue. Thus, a minimum estimate of diamagnetic conformational chemical shift changes may be obtained from the standard deviation of the chemical shift changes: \(\sigma = (\langle x^2 \rangle - \langle x \rangle^2)^{1/2}\) calculated for each residue (Plate 2).

Large average chemical shift changes (of paramagnetic origin) are observed primarily for residues in helix 1 of the first domain and the N-terminal coil that flanks it (up to residue 64), as well as the 171–176 helix (Plate 3). Asn117 and Phe118 in an interface loop and Ser137 at the edge of the \(\beta\) sheet also show significant apparent paramagnetic effects and are adjacent to regions that are paramagnetically obliterated in the oxidized state.

However, overall, the effects are really quite modest, with the maximum average change being \(-0.90\) ppm, for Glu21 (C\(^{\alpha}\) 14 Å from Fe). This is partly a reflection of the fact that we cannot observe residues close to (and presumably most responsive to) the site of redox activity, in the oxidized state. All the effects we observe are relatively long-range (>14 Å), consistent with the slower \(1/r^3\) decay of dipolar chemical shift effects vs the \(1/r^6\) decay of dipolar relaxation.\(^{49,50}\)

Consistent with the crystallographic findings,\(^{3}\) our results show that redox-coupled conformational effects are probably fairly small. However, they are also virtually ubiquitous. Almost all residues display a >0.1 ppm standard deviation in chemical shift change (probably not due to paramagnetism), which we consider significant. Very large changes are observed at Gly86 and Asp105, due entirely to changes in \(^{13}C\) chemical shift. Although these latter results are viewed with scepticism, it is possible that they reflect formation of new hydrogen bonds involving the carbonyls of each of these residues upon reduction.\(^{49}\)

The possibility of dynamic changes was investigated via measurements of the H\(^{1}\) exchange rates of residues that exchange on a time-scale of multiple minutes to several days, by exchanging each of Fe\(^{3+}\) SOD and Fe\(^{2+}\) SOD into \(^{2}H\)\(_2\)O. Although a few residues’ exchange rates changed by a factor of three upon reduction, none of the exchange rates measured changed by as much as a factor 10. Hence for exchange in this time range, long-range redox-linked changes in dynamics are very minor in FeSOD.

Substrate analog binding to Fe\(^{3+}\) SOD

Studies of anion binding to Fe\(^{3+}\) SOD have been few because Fe\(^{3+}\) is EPR and optically silent. However, MCD spectroscopy revealed that neither N\(^{-}\), nor F\(^{-}\) change the coordination number of Fe\(^{3+}\) in Fe\(^{2+}\) SOD.\(^{7}\) More recently, Sorkin and Miller have found that F\(^{-}\) binds near the active site without coordinating to Fe.\(^{8,58}\)

Our comparison of the HSQCs of Fe\(^{2+}\) SOD in the absence and presence of different substrate analogs, F\(^{-}\), N\(^{-}\), Br\(^{-}\) and SCN\(^{-}\), reveals that many resonances shift (Plate 4). All four anions caused the chemical shifts of residues 4, 21, 36–37, 53, 116–119, 137–143, 165–170 and 184 to change by 0.64–0.11 ppm for \(^{13}N\) and 0.19–0.01 ppm for \(^{1}H\). However, the magnitudes and directions of chemical shift changes at each resonance depended on the anion added. Hence the response is not simply a function of ionic strength. Many of the responsive residues are in the dimer interface and are part of the crystallographically described substrate binding funnel\(^{3}\) (Plate 5). The response observed could reflect anion binding at this location or reorientation of the two monomers relative to one another in the presence of the anions.

Residues 14, 17, 19, 24, 35–40, 62, 84 and 91 are sensitive to the presence of F\(^{-}\) but not to any of the other anions (Plate 4 and 5). Of these, residues 35–40 stand out as being particularly strongly influenced, exhibiting average absolute value \(^{1}H\) chemical shift changes of 0.13 ppm.
in the presence of $\text{F}^-$ but only changes of 0.01, 0.01 and 0.01 ppm for $\text{N}_\text{a}^-$, $\text{SCN}^-$ and $\text{Br}^-$. Furthermore, the resonance of Asn37 could no longer be located in the presence of $\text{F}^-$ but was barely affected by the other anions. This suggests the existence of a second binding site, unique to $\text{F}^-$. Earlier work has shown that ionization of Tyr34 excludes anions from the active site of Fe$^{3+}$SOD$^{14,50}$ and that $\text{F}^-$ binds more weakly to Y34FeSOD than to WT.$^8$

Our finding that the chemical shifts of residues 35–40 are strongly and specifically influenced by $\text{F}^-$ therefore supports the conclusion that $\text{F}^-$ binds near Tyr34. Hence we report the first location of a substrate binding site for Fe$^{2+}$SOD.

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$\text{F}^-$ and $\text{N}_\text{a}^-$ coordinate directly to Fe$^{3+}$ in Fe$^{3+}$SOD as indicated by EPR and optical spectral changes.$^8,51–53$ However, other anions such as $\text{SCN}^-$ also compete with $\text{N}_\text{a}^-$ and substrate without coordinating to Fe$^{3+}$. Presumably they bind to the protein near the active site. To determine whether a protein-derived anion-binding site exists, and to locate it, we examined anion binding to Fe$^{3+}$SOD by NMR. Many residues distant from Fe$^{3+}$ were responsive to $\text{N}_\text{a}^-$, $\text{F}^-$, $\text{SCN}^-$ and $\text{Br}^-$ (Plate 6). Most of the changes occurred on going from 0 to 5 mM in the case of Na$\text{N}_\text{a}$ and from 0 to 10 mM in the case of KF, whereas almost no change occurred upon further increasing each of these to a concentration of 100 mM. Since 10 mM NaCl was present in all samples, the first step represented only a 50% increase in ionic strength whereas the second step represented more than a seven fold increase. Therefore, the chemical shift changes either are not simply functions of ionic strength, or they saturate very early.

The chemical shifts of Lys116, Asn117 and Phe118 change dramatically for all anions, as they did in the reduced state (Plates 4 and 6). Hence these residues appear to contribute to an anion binding site accessible to all four anions tested, independent of Fe oxidation state, consistent with it being composed entirely of protein residues. This site is located in the cleft between the two monomers (Plate 5) in the ‘funnel’ predicted to provide substrate access to the active site based on crystal structures.$^5$ Additional residues closer to Fe and therefore undetected in our HSQC's could also contribute to this outer-sphere binding site. Lys116, Asn117 and Phe118 are conserved among FeSODs but differently conserved among MnSODs, and approach 9 Å from the Fe of the other monomer. The fact that all four anions interact with them still allows that the effect be simply one of ionic strength, but the effects vary among the anions and are highly localized indicating at least that a specific patch of the protein has a special sensitivity to anions. The smaller magnitude of the effects observed for SCN$^-$ is consistent with its higher $K_p$. Hence our data support the interpretation of Bull and Fee$^3$ over that of Argese et al.$^9$ and we conclude that Lys116, Asn117 and Phe118 contribute to the anion binding site responsible for similar inhibition of both oxidation states.$^7$ The presence of a negatively charged ion in the anion-binding site near the mouth of the substrate-access funnel could prevent the physiological substrate, $\text{O}_2^{\cdot\cdot}$, from entering the active site.$^{54}$

Phe118 shows the most consistent, large change in chemical shift upon binding of each of the four anions, in both oxidation states, and it is also the residue that exhibits the maximum difference in chemical shift between the oxidized and reduced state of the enzyme. The $\delta^C$ of Phe118 is 15 Å from Fe but ring Cs are 9 Å from the other monomer’s Fe. Moreover, the aromatic ring of Phe118 from one monomer comes within 5 Å of the aromatic ring of the universally conserved Tyr34 of the other monomer. Phe118 appears to shield Tyr34 and is proposed to stabilize Tyr34 via weak polar interactions.$^{55}$ Hence Phe118 would be particularly sensitive to alterations in the interaction between SOD monomers. Moreover, the relationship between Phe118 and the active site implies that it or movement of the monomers relative to one another could play a role in FeSOD’s catalysis.

**CONCLUSIONS**

FeSOD is a member of a growing class of enzymes that includes protocatechuate 4,5-dioxygenase and lipoxygenase, enzymes involved in degradation of persistent carcinogens and biosynthesis of key signaling molecules.$^{59}$ Despite FeSOD’s paramagnetism and its much larger size than most proteins assigned by NMR, we have independently assigned the backbone of perdeuterated FeSOD in both oxidation states, including residues in the second sphere surrounding the active site. Residues not assigned were not visible in the HSQC because of either paramagnetic relaxation or exceedingly slow acquisition of $^1\text{H}$ from solvent. Most of the chemical shift differences between oxidized and reduced FeSOD are attributable to changes in paramagnetism, but significant differences are also observed in regions distant from Fe. Their small magnitude is consistent with FeSOD’s unique mechanism in which both oxidation states react with the same substrate. Nonetheless, such long-range effects hint at possible interplay between the active site and the rest of the protein. The effects of small competitively inhibitory anions on FeSOD’s HSQC indicate that interactions with anions in general affect SOD’s dimer interface, possibly by changing the relative orientations of the monomers, or by interacting near Phe118 in both oxidation states. This can explain the inhibition by anions observed by Bull and co-workers.$^{3,54}$ We have also identified a substrate binding site in Fe$^{2+}$SOD near Tyr34, and propose that this is the outer-sphere substrate binding site effective for the second half-reaction, reaction (1b).

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REFERENCES