Published in final edited form as:

Biochim Biophys Acta. 2010 February ; 1804(2): 275–284. doi:10.1016/j.bbapap.2009.11.009.

¹⁵N-NMR characterization of His residues in and around the active site of FeSOD†

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Summary

We have exploited ¹⁵N-NMR to observe histidine (His) side chains in and around the active site of Fe-containing superoxide dismutase (FeSOD). In the oxidized state, we observe all the non-ligand His side chains and in the reduced state we can account for all the signals in the imidazole spectral region in terms of the non-ligand His', paramagnetically displaced signals from two backbone amides, and the side chain of glutamine 69 (Gln69). We also observe signals from the His' that ligate Fe^{II}. These confirm that neither the Q69H nor the Q69E mutation strongly affects the Fe^{II} electronic structure, despite the 250 mV and > 660 mV increases in E_m they produce, respectively (Yikilmaz et al. (2006) *Biochem.* 45:1151–1161). In the Q69H mutant, we observe two new signals attributable to the His introduced into the active site in place of Gln69. One corresponds to a protonated N and the other is strongly paramagnetically shifted, to 500 ppm. The strong paramagnetic effects support the existence of an H-bond between His69 and the solvent molecule coordinated to Fe^{II}, as proposed based on crystallography. Based on previous information that His69 is neutral, we infer that the shifted N is not protonated. Therefore, we propose that this N represents a site of H-bond *acceptance* from coordinated solvent, representing a reversal of the polarity of this H-bond from that in WT (wild-type) FeSOD protein. We also present evidence that substrate analogs bind to Fe^{II}SOD outside the Fe^{II} coordination sphere, affecting Gln69 but without direct involvement of His30.

Keywords

N15-NMR; Fe-superoxide dismutase; paramagnetic; chemical shift; redox tuning; histidine

†Supported by the National Institutes of Health under GM R01 GM085302-01

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⁷We apologize to authors who may have published larger paramagnetic contributions to ¹⁵N chemical shifts in Fe^{II} systems, and request that they alert us to their publications.

Supplemental Information

Figure of ¹⁵N spectra of WT-Fe^{II}SOD His region at three temperatures: 10 °C, 25 °C and 40 °C. Curie Plot of the His-region ¹⁵N chemical shifts, table of chemical shifts and parameters of linear fits to a Curie temperature dependence, for each of WT-Fe^{II}SOD and Q69E-Fe^{II}SOD. Table of His Ns and their distances to Fe in Fe^{II}SOD.

Introduction

Readers of this special issue are already aware that superoxide dismutases (SODs) provide a crucial early line of defense against oxidative stress. Left unchecked, superoxide has a sufficiently long lifetime to diffuse substantial distances and even move between intracellular compartments [1]. It can then generate an array of different damaging daughter species including peroxide, hydroxyl radical, hypochlorous acid and peroxynitrite. Some of these are so short-lived that there is little chance of intercepting them. Instead, their formation is prevented by catalytic disproportionation of superoxide by SODs, in conjunction with scavenging of the product peroxide by catalases and peroxidases. Such is their importance, that SODs have evolved on at least three different occasions. Thus, three different enzymes bear this name: the Cu and Zn-containing SODs found in the cytoplasm of eukaryotes and extracellularly, the Ni-containing SODs found in several streptomycetes, and the family including Fe-containing SODs and Mn-containing SODs. MnSOD are found in the mitochondria of eukaryotes and some prokaryotes, whereas FeSOD is found in prokaryotes and in the chloroplasts of some plants [2]. FeSODs and MnSODs share substantial amino acid sequence homology, the same overall 3-dimensional structure for their monomers and identical Fe-binding residues, so far [3, 4].

This article focusses on the FeSOD from *Escherichia coli*, that has been a work-horse for structural and mechanistic studies [5–9], and whose active site resembles those of a large and diverse group of enzymes that activate dioxygen, called facial triad enzymes [10].¹ FeSOD's active site contains a single Fe ion coordinated by three histidines (His'), an aspartate (Asp[−]) and a molecule of solvent (Figure 1A). The coordinated solvent engages in a hydrogen bond (H-bond) with the Asp[−] ligand and another with the conserved active site glutamine 69 (Gln69) [9]. This H-bond plays a critical role in tuning the Fe site's reactivity (below).

FeSOD follows a ping-pong mechanism, in which superoxide reduces Fe^{III} upon binding to Fe in the first half reaction (eq. 1a, [6, 8]). The second half-reaction is believed to involve second-sphere binding of the next molecule of superoxide, which oxidizes Fe^{II} and departs as hydrogen peroxide (eq. 1b). Electron transfer has been shown to be coupled to proton transfer throughout the pH range of FeSOD's activity [8]. Moreover proton transfer to superoxide is critical for thermodynamic feasibility of the second half-reaction [11]. The coordinated solvent molecule is believed to be the redox-coupled proton acceptor and is likely the ultimate donor of one of protons acquired by peroxide [8, 12–14].

¹FeSOD's Fe is bound by three His' and an Asp[−] whereas the canonical facial triad enzymes employ two His' and either an Asp[−] or a Glu[−].

where L stands for the SOD protein ligands and the molecule of solvent coordinated to Fe is included in parentheses.

In the second half-reaction, superoxide is not believed to coordinate directly to Fe^{II} [15], but instead is thought to bind nearby in a site that is also close to the second-sphere residue tyrosine 34 (Tyr34, but see [16]). Crystal structures of FeSOD and MnSOD display a resolved solvent molecule H-bonded between the conserved residues Tyr34 and His30 [9, 17, 18]. In Fe^{II} SOD, this is 7.5–7.7 Å from Fe in the channel that links the active site to bulk solvent [9]. Temperature-jump studies indicated that superoxide binds to Fe^{III} SOD in two steps, coordinating Fe^{III} after passage through a prebinding state or site [5]. Additionally, substrate analogs that do not coordinate to Fe^{III} nonetheless competitively inhibit N_3^- binding [8]. Thus, the search continues for the site of substrate binding to the reduced enzyme, which may coincide with the prebinding site associated with the oxidized state. The fact that superoxide does not coordinate to Fe^{II} is likely critical to the nature of the chemistry that ensues. This is a key difference between FeSOD and the cytochrome p450s, that activate O_2 [19]. Nonetheless, the outer-sphere site where superoxide binds must be close enough for superoxide to accept an electron from Fe^{II} , and most likely a proton from the coordinated solvent molecule, possibly via Gln69 and Tyr34 [8, 13, 20–26]. Thus, we have sought means to test the possible role of His30 in substrate binding.

FeSOD's ability to both oxidize and reduce superoxide requires that its reduction midpoint potential (E_m) be intermediate between those of the two half reactions [27]. Because reduction of Fe is coupled to protonation of the coordinated solvent molecule, in this system [8, 12–14], H-bonds or other interactions by which the protein modulates the coordinated solvent's degree of protonation or bonding with Fe can have large effects on the E_m [28, 29]. In FeSOD, the conserved Gln at position 69 has been shown to contribute strongly to redox tuning via its H-bond to coordinated solvent [29–32]. Mutation of this Gln to His preserved 30% of activity and the structural integrity of the active site, but produced a 250 mV increase in E_m [33]. The crystal structure of the Q69H mutant indicates that the His69 replacing Gln69 also H-bonds with coordinated solvent (Figure 1A), but unlike Gln69, His69 is not constrained by other H-bonds [33]. Therefore we speculated that His69 would be free to act as either an H-bond donor or an H-bond acceptor, whichever is more favourable, and the 250 mV increase in E_m was attributed to loss of imposed redox tuning [31]. However the distance between the His69-N and the coordinated solvent O was sufficiently long (3.4 Å) that the significance of any H-bonding interaction was in doubt.

^{15}N NMR chemical shifts and ^1H -derived splittings can provide insight into the protonation state of His side chains, and their H-bonding status [34–36]. ^{15}N is also much less susceptible to paramagnetic relaxation than ^1H [37, 38]. ^1H resonances of ligands of Fe^{II} in FeSOD are as broad as 3000 Hz, producing severe overlap and thus limiting their utility as probes of active site electronics and structure [39]. However paramagnetic relaxation depends on the square of the gyromagnetic ratio, so the ten-fold lower gyromagnetic ratio of

^{15}N should make it a more useful probe of the ligand His side chains [40]. Markley's team has observed NMR signals from ^{15}N labelled backbone amides H-bonding to the S atoms coordinated to Fe in rubredoxin [41]. These were displaced some 400 ppm from their normal resonance frequencies and the effect was shown to be mediated by the H-bonds [42]. In WT-Fe^{II}SOD, we have previously detected the side chain amide N of Gln69, and found that it was shifted some 60 ppm from the normal (diamagnetic) position [43]. Thus, we now seek to observe an analogous signal from the His69 in the Q69H-FeSOD mutant, as a means of testing for the existence and possible significance of an H-bond between His69 and coordinated solvent.

In the following, we now report identification of ^{15}N -NMR resonances attributable to His side chains in Fe^{II}SOD. Despite paramagnetic effects, we were able to observe ^{15}N resonances of ligand histidines; the chemical shifts of these resonances are among the largest ever reported. Although we have not specifically assigned resonances to His30, none of the group of resonances that must include His30 respond to F^- addition to Fe^{II}SOD, indicating that His30 does not participate in binding this substrate analog to Fe^{II}SOD. Finally, we applied the method to Q69H-FeSOD, and observed a resonance unique to Q69H-FeSOD that is paramagnetically relaxed and shifted, consistent with promixity and through-H-bond coupling to Fe^{II}. His69 has been deduced to be neutral [31], so our observation of a second resonance attributable to His69 in the region of protonated imidazole Ns suggests that the N actually H-bonding with coordinated solvent is the one that is *not* protonated, and therefore that His69 *accepts* an H-bond from coordinated solvent.

Methods

Cloning, protein over-expression, purification and characterization for integrity were executed as before [33, 44, 45], except that bacteria were grown on M9 medium made with $^{15}\text{NH}_4\text{Cl}$. As previously reported, Q69H-FeSOD was isolated with only approximately 50% of the sites occupied by Fe, and 30% WT specific activity on a per-Fe basis [33]. Q69E-FeSOD was much more stable and bound Fe in more than 95% of the sites, but had less than 1 % of WT activity [33, 46]. WT-FeSOD contained Fe in 90% if active sites. Both WT- and Q69E-FeSOD tolerated elevated temperatures for days and retained their bound Fe over months at room temperature, but had to be discarded after several days at 50 °C. Spectra reported here were all collected at 40 °C or lower. Q69H-FeSOD released its bound Fe gradually on a time scale of a few weeks at 10 °C. Therefore multiple samples were made and each was only used for one week at most, at 10 °C, in a buffer chosen to optimize Q69H-FeSOD's stability.

Q69H-, Q69E- and WT-FeSOD were studied in a default buffer of 100 mM KBr, 100 mM pH 7.4 PIPES (piperazine- $\text{N,N}'$ -bis(2-ethanesulfonic acid)), except for the F^- -binding experiment, for which 10 mM MES pH 6.0 and 10 mM NaCl $\pm$ 100 mM KF was used (vs. a K_d of 40 mM [16]). All NMR samples were 10 % $^2\text{H}_2\text{O}$ by volume. For reduced-state studies, WT- and Q69H-FeSOD were degassed gently and maintained under an atmosphere of Ar. Reduction was accomplished by addition of 2 stoichiometric equivalents of dithionite in 50 mM KOH, after which samples were flame-sealed. Q69E-FeSOD is fully reduced as-isolated and required no treatment.

¹⁵N NMR spectra were collected at 60 MHz for ¹⁵N on a Varian INOVA spectrometer equipped with a 5 mm broad-band detection probe at 10 °C, or as specified otherwise. For typical spectra, a 1 second delay between scans was followed by a 30 ° excitation pulse (≈ 4 μs) and data acquisition for 100 ms with 10 kHz decoupling of ¹H, unless stated otherwise. Spectra collected for peak integration purposes employed relaxation delays of up to 3 s, and spectra devoted to observing paramagnetically shifted and relaxed resonances employed a 90 ° excitation pulse with relaxation delays as short as 200 ms and acquisition for as little as 40 ms. Typical processing involved application of 20 ms Gaussian line broadening and backward linear prediction to correct early data points prior to Fourier transformation, followed by baseline correction. Despite the use of samples that were approximately 1.5 mM in SOD dimers, typical spectra shown here were the result of averaging some 40,000 scans. Chemical shifts are quoted vs. neat liquid ammonia at 0 ppm based on an external standard of ¹⁵N urea in DMSO set to 77 ppm at 25 °C.

Results

Wild-type FeSOD

The ¹⁵N NMR spectra of proteins in general are well dispersed according to chemical type (Figure 2). The primary amines tend to be upfield near 33 ppm vs. ammonia; arginine guanidino N η and N ϵ resonate near 72 and 85 ppm respectively; backbone amides have chemical shifts as low as 105 ppm, particularly for glycine residues, and extend to roughly 130 ppm where the indole NHs of tryptophan side chains also resonate ². The side chain amides of glutamine and asparagine resonate near 112 ppm. Backbone imides of proline (Pro) are generally found between 130 and 140 ppm and are characterized by very sharp lines and long T₁ values due to their lack of bound ¹H and relative immobility. Well separated from the rest, the imidazole side chain Ns of His are dispersed in two almost non-overlapping spectral regions which are typically populated by H-bearing Ns between 160 and 190 ppm, and non-H-bearing Ns between 220 and 250 ppm [34–36]. Thus, ¹⁵N NMR allows us to observe His side chains with little interference from other functionalities, without the effort or expense of selective isotopic labelling. FeSOD contains only 7 His', of which three are ligands to Fe (Figure 1B).

The His region of oxidized Fe^{III}SOD's ¹⁵N-NMR spectrum reveals eight resonances (Figure 3, bottom). Even the most down-field Pro resonance at 143 ppm can readily be distinguished from His resonances based on the Pro imide N's much narrower line width than those of the His resonances, and the Pro's strong suppression under rapid inversion-recovery conditions (not shown). The eight imidazole resonances correspond exactly to the number expected from the four non-ligand His' in FeSOD (coloured amber in Figure 1B), indicating that we can observe not only His17 and 176 (more than 17 Å from either Fe of the dimer, supplemental table 3) but also His31 (8.0 and 8.6 Å from the nearest Fe for Nd₅ and Ne₂, respectively) and His 30 (6.2 and 6.7 Å from the nearest Fe for Nd₁ and Ne₂, respectively). Based on the three resonances between 260 and 220 ppm, there are three neutral and one cationic His. The His resonances have line widths that vary widely. This is not easily

²www.bmrb.wisc.edu/devise/histogram/html

explained by proximity to the Fe^{III} because there are two His more than 17 Å from the nearest Fe. These would be expected to produce four equally sharp lines in the absence of additional considerations (such as internal dynamics). Of the five H-bearing imidazole Ns, only the sharp one at 161 ppm has a sufficiently slowly-exchanging proton to produce splitting at 10 °C (not shown, but denoted by 'C' for 'coupled' in Figure 3).

Upon reduction, the strongly relaxing S=5/2 Fe^{III} is replaced by less relaxing S=2 Fe^{II}, and we anticipate that NMR spectroscopy will be able to detect nuclei that are closer to Fe [47]. However high-spin Fe^{II} is expected to have an anisotropic distribution of unpaired electron spin density because one d-orbital is populated by two electrons whereas the others are populated by only one each. Therefore, one expects larger dipolar paramagnetic contributions to chemical shift in Fe^{II}SOD [47]. Indeed, the His-region of the ¹⁵N spectrum now contains 11 resonances (Figure 3, middle spectrum).

The region between 156 and 165 ppm contains four overlapping resonances. Of these, a broad resonance near 161 ppm has been assigned to the side chain amide of Gln69 by selective labelling [43] (denoted 'Q' in Figure 3). Gln side chain amines normally resonate near 112 ppm, so Gln69 displays a 59 ppm paramagnetic contribution to chemical shift at 10 °C. Gln69's side chain N is close to Fe (5Å) and also coupled to it by a H-bond with the coordinated solvent molecule [9]. Hence we can also expect that resonances of backbone amides close to Fe, or coupled to it through bonds, could also appear in the His region as a result of paramagnetic contributions to chemical shift.

To identify chemical shifts reflecting paramagnetic contributions, we exploited the fact that the paramagnetic contributions to shift follow a Curie-type temperature dependence, decreasing linearly with the inverse of absolute temperature.³ A plot of chemical shift vs. 1/T reveals that three chemical shifts are strongly temperature-dependent (supplementary figure 2 and table 1). One of these is the resonance of Gln69. For the other two, extrapolation to 1/T= 0 (T tending to ∞) yields chemical shifts of 108 and 124 ppm, in the backbone amide region. Moreover, both display coupling to ¹H in non-decoupled spectra. Therefore, the resonances at 197 and 160 ppm are assigned to amides near Fe^{II} or linked to it by bonds and indicated by 'A' in figure 3. The remaining eight resonances account perfectly for the four non-ligand His'.

Of these eight resonances, two are within the sum of the two line-widths of positions of His resonances in oxidized Fe^{III}SOD, suggesting that they correspond to the same Ns in both oxidation states. We propose that they stem from one of the His' distant from Fe: His 17 or 176 (260 and 172 ppm, green verticals between bottom two spectra in Figure 3).

In addition to those of the non-ligand His', three resonances were observed near 1100 ppm (Figure 4). Their chemical shifts are strongly temperature dependent. These lines are also broader than the others and characterized by short T₁ values, indicating that they derive from the three His' that coordinate Fe^{II}. The most strongly-shifted of the three also has a T₁ only

³The observed dipolar paramagnetic contribution to chemical shift shrinks as the excess population with the ground-state electronic spin configuration. At temperatures tending to infinity, all spin states would be equally populated and their opposing contributions to dipolar paramagnetic chemical shift would cancel. Zero field splitting can contribute additional T-2 dependence

half those of the other two. We assign it to His26, based on previous work in which ^1H resonances assigned to His26 were found to be more strongly affected by paramagnetism than resonances of the other two ligand His', whose resonances were found to be similar [39]. It is simplest to assume that the coordinated Ns continue to elude us, and that the resonances near 1100 ppm correspond to the non-coordinated Ns.

Mutant Fe^{II}SODs

As a test of the identity of the resonance assigned to Gln69, the ^{15}N spectrum of Q69E-Fe^{II}SOD was compared with that of the WT. The feature at 161 ppm attributed to Gln69 was indeed absent (Figure 5). Two Q69E-Fe^{II}SOD resonances in the region of 150 – 260 ppm displayed strong Curie-type temperature dependencies with extrapolated chemical shifts near 110 ppm, in the amide region (supplemental figure 3 and table 2). Each is coupled to a slowly-exchanging ^1H . Therefore we attribute them to amide Ns. Both of these fall within a few ppm of the resonances we attribute to amides in WT-Fe^{II}SOD, so they likely reflect the same residues as in the WT (202 ppm in Q69E- and 197 ppm in WT-Fe^{II}SOD, 162 ppm in Q69E- and 160 ppm in WT-Fe^{II}SOD).

The spectrum of Q69H-Fe^{II}SOD revealed a much greater heterogeneity of integrated peak areas than was observed for WT- or Q69E-Fe^{II}SOD. The H-coupled Q69H-FeSOD resonance closest in chemical shift to the WT amide resonance at 197 ppm is one of the low-amplitude peaks. Similarly, the three Q69H resonances that are coupled to an H and close to the other WT paramagnetically-shifted amide are also weak. Paramagnetically-shifted amides would only be evident for the SOD monomers that contain Fe^{II}, so Q69H's retention of Fe in only some half the sites can explain the weakness of paramagnetically-shifted resonances.

Conversely, the six strongest resonances of Q69H include both of the resonances that in WT-FeSOD were insensitive to the oxidation state of Fe, in addition to the resonances at 237 and 189 ppm that have clear counterparts within 2 ppm in the spectra of all three proteins. We reason that these four strong resonances stem from His17 and 176, which are far from Fe and residue 69.

The last two of the strong Q69H- resonances are closest to WT-Fe^{II}SOD resonances at 240 and 166 ppm that respond to mutation of residue 69. The resonance near 166 ppm responds very little to Fe oxidation state but the resonance near 240 ppm in the Q69H spectrum displays a broad base suggestive of heterogeneity. This is readily explained by the fractional occupancy the Fe site. Since His30 is closer to Fe but His31 is closer to residue 69, we propose that the resonances at 240 and 166 ppm in WT-FeSOD could stem from His31.

Thus, the six resonances that are relatively strong in Q69H-Fe^{II}SOD are tentatively attributed to three His residues that are less sensitive to the Fe content of the active site and therefore represent simply the amount of protein. Similarly, we propose that the weak resonances correspond to His side chains in the active site and close to Fe: His30 and His69 (in Q69H-FeSOD). This reasoning also indicates that the two non-ligand WT-Fe^{II}SOD His resonances that are not among Q69H-Fe^{II}SOD's strong six must stem from His30. These are the resonances at 163 and 158 ppm, suggesting that His30 is cationic in Fe^{II}SOD. This was

not anticipated, given that in MnSOD, His30 can be replaced by Asn and Ala with relatively minor cost to activity and stability [48, 49]. We will seek a more definitive assignment of His 30 by site directed mutagenesis.

His30 in Fe-complete sites could produce one pair of signals and His30 in Fe-free sites could produce another pair, for four possible signals, in Q69H-Fe^{II}SOD. We would also expect to see a pair of signals for each of the ligand His' in Fe-free sites, as well as two pairs of signal from His69. However we do not see so many weak signals, and nor do we see the two strong sharp signals we would expect if the His side chains of Fe-free active sites became freely mobile and unstructured. Thus NMR signals from Fe-free sites are not apparent in our spectra, perhaps due to conformational heterogeneity and/or intermediate time-scale dynamics in these incompletely constrained active sites.

The His spectral region provides only one candidate resonance for His69 of Q69H-Fe^{II}SOD, at 150 ppm. Based on this chemical shift, this N bears an H, consistent with coupling observed in the non-decoupled spectrum (Figure 5). However, based on the large (59 ppm) paramagnetic contribution to the chemical shift displayed by Gln69's side chain N, we can anticipate a comparably large effect on the N of His69 that is closest to Fe (5.1 Å [33]). Such a shift would place this resonance between 220 and 310 ppm for a comparable H-bond, but possibly further a-field for a stronger H-bond. Upon searching down-field, we found a resonance that is unique to Q69H, despite the strong parallels between the spectra of Q69H-, Q69E- and WT-Fe^{II}SOD in other spectral regions (besides 150 ppm). Figure 6 displays the spectral region beyond 300 ppm, that is normally devoid of signals in diamagnetic proteins. In addition to the three signals we attribute to ligand His' near 1100 ppm, Q69H-Fe^{II}SOD alone displays a well-behaved resonance at 500 ppm that is broader and has a shorter T₁ than unshifted His resonances. Therefore, we attribute this signal to the His69 introduced into this protein alone.⁴

Substrate analog binding

Upon addition of F⁻ to WT-Fe^{II}SOD most resonances do not respond at all. However there is a slight shift in the resonance near 197 ppm (attributed to an amide, above), and a substantial movement of the Gln69 resonance from 161 ppm to 168 ppm. Since we have signals from all the non-ligand His', and none of them shifts significantly, we find no evidence for direct participation of His30's side chain in binding the substrate analog F⁻.

Discussion

The ¹⁵N-NMR spectra we present permit collective assignments only, but we are able to observe signals even from ligand His'. The fact that their chemical shifts do not change much upon mutation of residue 69 demonstrates that the covalency of their bonds to Fe^{II} is very similar, as is the anisotropy of Fe^{II}'s unpaired electron spin density distribution. Thus, the mutations have little effect on the electronic structure of the Fe^{II}. This finding agrees

⁴We note that at pH 9.6, Q69H-Fe^{II}SOD lacked this resonance, possibly as a result of ionization of nearby Tyr34. However the significance of this observation is unclear as two of the strong His signals were also lost, rather than shifted, raising the possibility of damage to the protein (not shown).

with those of extensive magnetic circular dichroism studies [32, 50], retention of active site integrity based on X-ray crystallography [33] and the relatively small changes observed among the paramagnetically shifted ^1H resonances [31].

Resonances from His69 and H-bonding with coordinated solvent

There is a strongly paramagnetically shifted resonance unique to the Q69H-Fe^{II}SOD mutant. This resonance has a linewidth and chemical shift intermediate between those of the conventional diamagnetic His N signals and those of the ligands. Thus it is very likely a signal from His69. One might argue that the other resonance unique to Q69H-Fe^{II}SOD, at 150 ppm, represents a non-His signal present in the His spectral range due to paramagnetic chemical shift contributions. Unfortunately, it was not possible to test the temperature dependence of the chemical shift of this resonance, due to the instability of Q69H-FeSOD. However the close correspondence between His chemical shifts observed for WT- and Q69E-FeSOD, including for the paramagnetically-shifted amides, and the retention of that correspondence for the strong resonances in Q69H-Fe^{II}SOD, makes it unlikely that a residue not subject to large shifts in the first two should become so in the third. These arguments favour attribution of the Q69H-Fe^{II}SOD-specific signals at 500 ppm and 150 ppm to His69, rather than other resonances experiencing altered paramagnetic contributions to their chemical shifts in the Q69H mutant.

The strong paramagnetic shift of the 500 ppm signal, amounting to a paramagnetic contribution of 240 ppm at least, is comparable to the paramagnetic contributions observed for backbone amide Ns hydrogen bonded to ligand S atoms in rubredoxin [51]. This supports the crystallographic suggestion that His69 engages in an H-bond with coordinated H₂O [33]. His69 is indicated to be neutral, based on the fact that titrations of nearby Tyr34 yield a pK_A similar to that observed for the WT protein containing (neutral) Gln [31]. Thus His69 should have one non-protonated N and one protonated N. The 150 ppm resonance is most reasonably attributed to a protonated imidazole N, in turn suggesting that the strongly shifted N is not protonated, and therefore that His69 *accepts* an H-bond from coordinated H₂O rather than donating one. This constitutes a reversal of the H-bond between residue 69 and coordinated solvent, and can explain Q69H-FeSOD's 250 mV higher-than-WT E_m [31, 33].

Histidine 30

A pair of resonances has been identified that are not paramagnetically shifted but are sensitive to mutation of Gln69 to His and loss of Fe, at 163 and 158 ppm. These are tentatively attributed to His30, with the only viable alternative being His31, because these are the only two non-ligand His' close to the active site. His30 has shorter distances to Fe and appears linked to the coordinated solvent by a chain of hydrogen bonds, explaining the weakness of these resonances in terms of partial Fe occupancy. Although His31 is packed among protein residues including residue 69 not far away, the packing of the residues that surround it and its two H-bonds (with the carbonyl O of Val70 and the indole NH of Trp77) are essentially unaffected by the mutation, based on comparison of the crystal structures (coordinates 1ISA and 1ZA5 [9, 33]), indicating that it should not be as sensitive to Fe. Moreover, although His30 is substantially solvent-exposed, and retains an apparent H-bond

with Tyr163, its interaction with Tyr34 is altered by the mutation [33].⁵ Thus, assignment to His30 is preferred. The chemical shifts of the two resonances in question indicate that the His is cationic, which is difficult to envision in either of the above two environments. A definitive assignment is premature until a His30 mutant has been studied by ¹⁵N NMR.

Substrate binding to reduced Fe^{II}SOD

FeSOD's ping-pong mechanism requires that one molecule of substrate bind to the oxidized enzyme and then that another molecule of substrate bind to the reduced enzyme. For oxidized Fe^{III}SOD, substrate is understood to coordinate directly to Fe^{III} [13, 45], however debate continues as to the location of substrate binding to Fe^{II}SOD, outside the coordination-sphere site. F⁻ is a competitive inhibitor that binds to reduced Fe^{II}SOD [8], and may provide clues as to where substrate binds [16]. F⁻ causes some paramagnetically shifted ¹H resonances to move in a way similar to their response to deprotonation of Tyr34, indicating an outer-sphere event [16]. Tyr34 in turn appears to H-bond with a crystallographic water that also derives an H-bond from His30 in some crystal structures of Fe- or MnSODs [9, 17, 18]. Mutation of His30 in MnSOD has been shown to lower MnSOD activity by over two orders of magnitude for the H30V mutant [52], with a smaller effect resulting from more polar substitutions [48, 49]. This was attributed to disruption of H-bonding, conformational change and occlusion of space required for substrate access and binding [48, 49, 52]. In an effort to clarify the issue, we have sought to test His30's possible significance to substrate binding via the effect of addition of F⁻. Although we do not have a definitive assignment for His30, the fact that none of the diamagnetic His resonances respond to F⁻ indicates that His30 does not participate in outer-sphere substrate binding. Our results at this point are more consistent with a role for His30 in proton transfer or maintenance of structure, including structure of water around the active site.

In their thoughtful computational study, Abreu *et al.* find that substrate binds to Fe or Mn end-on and in MnSOD the distal end H-bonds with Tyr34 and the Gln equivalent to Gln69 [22]. The computational studies indicated that Gln69's slightly greater distance from Fe in FeSOD than in MnSOD prevents Gln69 from H-bonding to Fe^{III}-bound substrate. However spectroscopic studies argue that substrate analogs do not bind directly to Fe^{II} [15]. In the reduced state, it may suffice for them to bind at the other end, to amino acid binding partners. Precedent is provided by the oxidized state of MnSOD, for which Whittaker and Brunold have documented an equilibrium between substrate coordination to Mn^{III} vs. second-sphere binding involving Tyr34 [53, 54]. When Fe is the catalytic metal ion, there is chemical reason not to create peroxide coordinated to Fe^{III}, as this is known to precipitate enzyme inactivation [55]. Thus a second-sphere binding site may serve to balance the need to bring substrate sufficiently close to Fe^{II} to undergo efficient reduction, the requirement that substrate be in contact with the active site proton network, and the need for substrate to not get trapped by Fe⁶. Moreover Abreu *et al.* argue that substrate participates actively in

⁵In the WT-Fe^{II}SOD the distance between the phenolic O and His30's Nd is 5.0 Å, but it is 3.6 Å in the Q69H mutant, allowing for a more direct connection with the active site. However the very different pHs used for crystallization make this difference difficult to interpret (pH 4.5 in the WT vs. pH 8 for Q69H)

⁶Reduction of superoxide is most unfavourable unless it is protonated, and Fe oxidation is also coupled to proton release from the coordinated solvent.

proton transfer to coordinated OH^- in the first half reaction, and find that the active site can accommodate superoxide near both Tyr34 and Gln69 [22]. Thus, we propose that F^- may bind near Gln69 in $\text{Fe}^{\text{II}}\text{SOD}$, despite not coordinating to Fe^{II} .

Our observation that Gln69 responds to addition of the substrate analog F^- supports a proposal that is consistent with the above three mechanistic constraints. The chemical shifts of the ligand His' do not change much upon F^- binding (1151, 1119 and 1030 ppm in the presence of F^- vs. 1156, 1130, 1061 ppm in its absence), indicating that Fe^{II} does not go from 5-coordinate to 6-coordinate, or release a His ligand. Asp^- is too strong a ligand for F^- to displace it and replacement of the coordinated solvent by F^- would have a very large effect on Gln69's chemical shift and line width, since coordinated solvent mediates Gln69's interaction with Fe^{II} . Thus we conclude that F^- does not coordinate to Fe^{II} corroborating published work [15]. Nonetheless our spectra do show a 7 ppm response from the side chain amide of Gln69, suggesting that F^- interacts with it. We also find that Tyr34 responds to F^- binding [16]. Thus, Gln69 (and Tyr34) appear to provide a stronger binding motif than does Fe^{II} : they out-compete it. Consistent with the small effect on chemical shift, binding is indeed weak ($K_d \approx 40 \text{ mM}$ [16]), as F^- is a poor size-homologue of superoxide. However our results suggest that Gln69 may contribute to the site of substrate binding to $\text{Fe}^{\text{II}}\text{SOD}$.

Ligand N chemical shifts

Literature values for ^{15}N chemical shifts of ligands to Fe^{II} are rare, and our report of those of the three His ligands in $\text{Fe}^{\text{II}}\text{SOD}$ therefore provides a valuable benchmark for computational and synthetic model studies, as well as studies of other non-heme mononuclear Fe proteins. ^{15}N coordinated to Fe^{II} has been observed by SS-NMR for nitrosoarenes coordinated axially to horse heart Fe^{II} myoglobin and human Fe^{II} hemoglobin, at 562 and 580 ppm, respectively [56]. However in this system Fe^{II} is low-spin and possible paramagnetic effects due to excited spin states were deemed less significant than the electronic consequences of bond formation on the electronic structure of the nitrosoarene N (the free nitrosoarene had an ^{15}N chemical shift near 840 ppm). A high-spin Fe^{III} complex has been investigated by ^{15}N solid-state NMR [57]. Although not coordinated to Fe^{III} , the observed ^{15}N had a chemical shift of 970 ppm as opposed to the 180 ppm observed in the absence of paramagnetism. In this case the H atom of an imino group was engaged in an H-bond to a coordinated O in an arrangement similar to that of our Gln69 or His69, however the Fe was $S=5/2 \text{ Fe}^{\text{III}}$ instead of our $S=2 \text{ Fe}^{\text{II}}$, preventing direct comparison of this published 790 ppm paramagnetic contribution to ours.

Despite the magnitude of our paramagnetic contribution to chemical shift, we nonetheless interpret our ligand His signals as stemming from the non-coordinated N atoms, because only three signals are observed and the coordinated Ns are expected to be the more strongly broadened and shifted set, and therefore less-easily observed. At chemical shifts as large as 1150 ppm, these signals therefore incorporate paramagnetic contributions as large as 950 ppm, since the non-coordinated Ns bear protons and thus have diamagnetic chemical shifts no greater than 200 ppm. We are not aware of other chemical shifts or paramagnetic contributions as large, for ^{15}N ligands to high-spin Fe^{II} .⁷

Conclusions

We have exploited the chemical shift dispersion and insensitivity to paramagnetic relaxation of ^{15}N NMR, to identify resonances from all the His side chains in FeSOD. Eight resonances are collectively identified as those of His 17, 176, 30 and 31 and proposals are made for specific assignments. None of these responds strongly to F^- binding, arguing against a role for His30 in substrate binding to Fe^{II} SOD. Gln69's side chain amide N does respond to F^- , suggesting that binding of this substrate analog, and thus possibly substrate, could involve Gln69. We have observed three strongly shifted and broadened resonances that we assign to non-coordinated N atoms of ligand His'. Their chemical shifts between 1000 and 1200 ppm are the largest of which we are aware in Fe^{II} systems. Finally, we have observed a resonance attributable to the His introduced in place of Gln69 in Q69H- Fe^{II} SOD. Its chemical shift of 500 ppm argues for the presence of a favourable H-bond between His69 and coordinated solvent. This is consistent with our explanation for the elevated E_m of this mutant in terms of modulation of the energies associated with proton transfer coupled to electron transfer [31].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by GM R01 GM085302-01. The NMR spectrometer was purchased by the Research Challenge Trust Fund of Kentucky.

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Figure 1A

Figure 1B

Figure 1.

Diagram of the active site of FeSOD (left) and ribbon diagram of a FeSOD monomer in the context of the dimer (right). The active site is shown to include the ligands to Fe as well as His30, Tyr34 and Gln69 (counter clockwise from the left), based on the crystallographic coordinates of Lah *et al.*[9]. The residues introduced by mutation of Gln69 to His and Glu are shown as well, with the replacement residues shown as placed by overlays of each mutant active site with the WT site. The His69 of Q69H-FeSOD is depicted with green C and the Glu69 of Q69E-Fe²⁺SOD is depicted with yellow C atoms. The ribbon diagram of FeSOD shows the ligand histidines in CPK, non-ligand His' in amber and the His introduced in the case of the Q69H mutant in green, for one monomer of the dimer. The other monomer is between the viewer and the page, and is shown as a partially transparent thin ribbon structure. Active site overlays optimized the superposition of all side-chain atoms of the amino acid residues 26, 30, 34, 73, 76 122, 156, 158, 160 and 161, but did not take into account residue 69, the Fe or the coordinated solvent. Overlays were performed using swisspdbviewer [58], the coordinates 1ZA5 and 2BKB of Yikilmaz *et al.*[33] and the coordinates 1ISA of Lah *et al.* [9]. Figures were generated using Molscript [59] and Raster 3D [60].

Figure 2.

Dispersion of different protein functionalities over different regions of the ^{15}N spectrum. In order to avoid invoking the ^{15}N -nOe, the spectrum was collected without ^1H decoupling. Data were acquired at 10 °C over 0.25 s and 2 s recovery delay was allowed prior to the next 60 ° excitation pulse. An 80 ms Gaussian line broadening function was applied.

Figure 3.

^{15}N spectra of WT FeSOD, comparing the oxidized state (bottom) with the reduced state (top two). For the reduced state, comparison of the top spectrum collected without ^1H decoupling, and the middle spectrum collected with ^1H decoupling, identifies N sites with a slowly-exchanging ^1H bound, as split in the top spectrum (orange vertical lines). A blue brace identifies a region including four overlapping resonances in WT-Fe^{II}SOD, 'A' identifies resonances attributed to backbone amides, 'Q' identifies the broad resonance of Gln69 and green vertical lines identify resonances that appear common to the reduced and oxidized states. All spectra were collected at 10 °C. A 45° excitation pulse and 100 ms acquisition were used with 2 s relaxation delays between scans.

Figure 4.

Chemical shifts of strongly-shifted and relaxed resonances of WT- (blue) and Q69E0Fe^{II}SOD (red) as functions of 1000*1/T. (Q69H-Fe^{II}SOD did not tolerate temperatures above 10°C for the long intervals needed to collect ¹⁵N spectra.) Data were collected at each of 10, 25 and 40 °C. 80,000 scans using a 30 ° excitation pulse (to cover a broad spectral width), 60 ms of acquisition and a 1 s relaxation delay between scans. 5 ms Gaussian line broadening was applied.

Figure 5. Comparison of the imidazole-region of the ^{15}N spectrum of WT-Fe^{II}SOD with those of Q69E-Fe^{II}SOD (top two spectra) and Q69H-Fe^{II}SOD (lower two spectra). A 90° excitation pulse and 250 ms acquisition were used with 5 s relaxation delays between scans.

Figure 6. Comparison of the down-field region of ^{15}N spectra of WT-, Q69E- and Q69H- Fe^{II} SOD. A 90° excitation pulse and 250 ms acquisition were used with 5 s relaxation delays between scans. 5 ms Gaussian line broadening was applied.

Figure 7.

Effect of addition of the substrate analog F^- to WT-Fe^{II}SOD. WT-Fe^{II}SOD was in 10 mM morpholinoethanesulfonate (MES) buffer at pH 6.0 with 10 mM NaCl with (top) or without (bottom) addition of 100 mM KF. The K_d for F^- binding to Fe²⁺SOD is 40 mM (F^- inhibits FeSOD with a K_I of 19 mM but binds to oxidized Fe³⁺SOD with a K_d of 7 mM). A 30° excitation pulse and 60 ms acquisition were used with 1 s relaxation delays between scans. The control spectrum (no F^-) displays two signals that are attributed to damaged protein in this older sample, near 175 and 215 ppm. These are not present in fresher material, see for example Figure 3.

Table 1Proposed resonance assignments and parallels between WT- and Q69E-Fe^{II}SOD.

Chemical shift at 10 °C (ppm)	slope vs. 1000/T	¹ H splitting	assignment (tentative)	corresponding resonance in Q69E (ppm)	slope vs. 1000/T
1156	343		Ligand	1158	340
1130	328		Ligand	1073	295
1061	310		Ligand	1023	286
258	-3		(His17 or 176)	259	-3
240	-6		His31	244	-6
237	0		(His176 or 17)	238	6
197	21 +		Amide	202	27
189	0		(His176 or 17)	191	-9
172	0		(His17 or 176)	175	-15
166	-3		(His31)	170	-12
163	-3		(His30)	167	0
160	15 +		Amide	162	15
161	29 +		Gln69		
158	-3	?	(His30)	160	-3
142	3		Pro	144	3