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^{15}N-NMR characterization of His residues in and around the active site of FeSOD

Anne-Frances Miller [a,b,*], Emine Yikilmaz [a,b], Surekha Vathyam [b,1]

[a] Dept. Chemistry, University of Kentucky, Lexington, KY 40506-0055, USA
[b] Dept. Chemistry, The Johns Hopkins University, Baltimore, MD 40508, USA

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ABSTRACT

We have exploited ^{15}N-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. 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.

1. 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 three-dimensional structure for their monomers and identical Fe-binding residues, so far [3,4].

This article focuses 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[2] [10]. FeSOD's active site contains a single Fe ion coordinated by three histidines (His′), an aspartate (Asp$^-$) and a molecule of solvent (Fig. 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

* Corresponding author. Dept. Chemistry, University of Kentucky, Rose Street, Lexington, KY 40506-0055, USA. Tel.: +1 859 257 9349; fax: +1 850 323 1069.
E-mail address: afm@uky.edu (A.-F. Miller).
[1] Current address: National Institutes of Health, Office of Technology Transfer, 6011 Executive Blvd. St. 325, Rockville, MD 20852-3804, USA.

[2] FeSOD'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$^-$.

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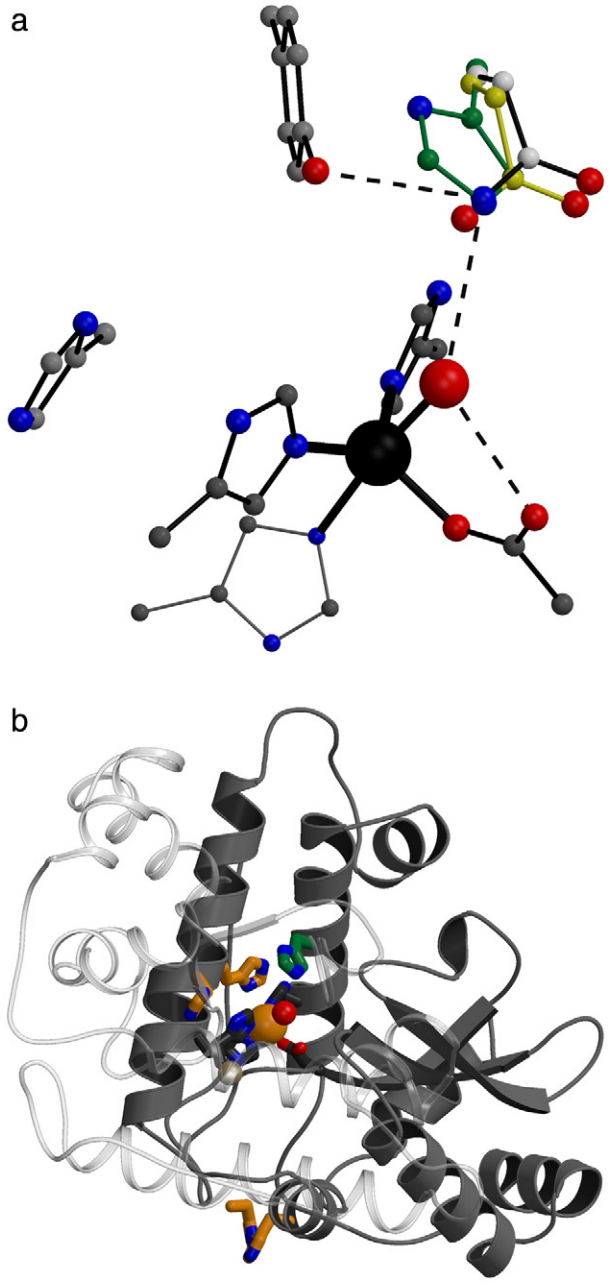

Fig. 1. Diagram of the active site of FeSOD (left) and ribbon diagram of a FeSOD monomer in the context of the dimer (right). The active site is shown to include the ligands to Fe as well as His30, Tyr34 and Gln69 (counter clockwise from the left), based on the crystallographic coordinates of Lah et 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-Fe^{2+}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].

proton acceptor and is likely the ultimate donor of one of protons acquired by peroxide [8,12–14].

$$O_2^{\cdot -} + H^+ + LFe^{3+}(OH^-) \rightarrow O_2 + LFe^{2+}(OH_2) \quad (1a)$$

$$O_2^{\cdot -} + H^+ + LFe^{2+}(OH_2) \rightarrow H_2O_2 + LFe^{3+}(OH^-) \quad (1b)$$

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 (Fig. 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 favorable, 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 10-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 labeled 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.