Fe superoxide dismutase

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Fe superoxide dismutase

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**FUNCTIONAL CLASS**

Enzyme; E.C. 1.15.1.1; mononuclear non-heme; non-sulfur Fe enzyme.

Non-sulfur Fe enzyme known as superoxide dismutase, SOD or SD. Catalyzes disproportionation of $\text{O}_2^-$:

$$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2.$$ 

**OCCURRENCE**

FeSOD is found in bacteria, especially the more primitive ones, the chloroplasts of plants, \(^1\,^2\) a few protists \(^3\) and possibly eukaryotes. \(^4\,^5\) The homologous MnSODs are found in bacteria and mitochondria, and are believed to protect DNA from endogenous oxidative stress, whereas FeSOD may serve as a housekeeping enzyme and provide

**3D Structure**  
Cartoon of the dimer structure of Fe\(^{3+}\)SOD from *E. coli*, showing the Fe\(^{3+}\) as a large black sphere and the heavy atoms of the ligands from C\(_\alpha\) out in ball and stick format. Based on the coordinates of Lah et al., I1SB, \(^44\) and produced using **MOLSCRIPT**. \(^37\)
resistance to environmental oxidative stress, by virtue of its periplasmic location.6 Throughout this article, ‘SOD’ refers collectively to the closely related MnSODs and FeSODs, but not the unrelated Cu, ZnSODs or NiSODs.

BIOLGICAL FUNCTION

With catalases and peroxidases, SOD provides protection against oxidative damage caused by the chemical progeny of $\text{O}_2^-; \text{H}_2\text{O}_2$ and OH-$^\cdot$.7–10 The regulatory role of NO and $\text{O}_2^-\cdot$’s rapid reaction with NO raise the possibility that SOD’s activity may also have regulatory ramifications.

AMINO ACID SEQUENCE INFORMATION

FeSOD, MnSOD and Cu, Zn–SOD have been studied as an example of parallel enzyme evolution.1 The amino sequences of FeSODs and MnSODs are highly conserved, especially with respect to residues in and around the active site. Well over two hundred SOD amino acid sequences have been reported as of March 2000 with the number increasing every week. An up to date list is best obtained via a BLAST search, which can be performed on-line at http://www.ncbi.nlm.nih.gov:80/BLAST/. Because the amino acid sequence between residues 143 and 157 is ‘low in complexity’ this may be excluded as a criterion for homology by performing searches with the ‘filter on’.

Representative sequences are those of:
- Escherichia coli, accession number gi.640114.11
- Bordetella pertussis, gi.586007.12
- Nicotiana plumbaginifolia chloroplast, gi.170234.13
- Helicobacter pylori, gi.4218197.14
- Plasmodium falciparum, gi.4139318.15
- Plectonema boryanum gi.1711435.16

PROTEIN PRODUCTION, PURIFICATION AND MOLECULAR CHARACTERIZATION

E. coli FeSOD is relatively easy to purify owing to its high stability and orange color. The purification protocol of Slykhuis and Fee17 works very well, though with a little fine-tuning. After cell lysis in 0.1 M KCl and pH 7.4 phosphate buffer, DNA is removed by precipitation with streptomycin sulfate or 30 mM MnCl$_2$, and proteins less stable than SOD are removed by denaturation at 60 °C for 3 min and centrifugation. Further substantial purification is achieved by means of a 50% (NH$_4$)$_2$SO$_4$ cut. At this point, FeSOD is by far the majority species present if it was expressed well. Pure FeSOD is obtained following anion exchange chromatography on DE52 at pH 7.4 and, if necessary, a flow-through column of CM52 at pH 5.5.

METAL CONTENT AND COFACTORs

Atomic absorption spectroscopy, as well as the X-ray crystal structures, reveal that each SOD monomer contains one metal ion and no other cofactors. Good preparations of FeSOD contain one Fe per monomer but very often when FeSOD is purified from bacteria grown in rich or ill-defined medium SOD is found to contain a mixture of metal ions including most importantly Mn$^{2+}$ and Zn$^{2+}$.

ACTIVITY TEST

By far the most common assay is the original one in which the reduction of cytochrome $c$ by $\text{O}_2^-\cdot$ is monitored spectroscopically and the activity of SOD is assessed based on its ability to interfere with cytochrome $c$ reduction. Xanthine oxidase is used as a source of nM $\text{O}_2^\cdot$·.18 A variety of other assays exist19,20 and are useful under different circumstances including in native electrophoresis gels.21 Stopped-flow22 or pulse radiolytic assays23 yield the kinetic constants $k_{\text{cat}}$ and $K_M$.

SPECTROSCOPY

Fe$^{3+}$SOD has been studied by UV/vis spectrophotometry and the broad overlapping bands peaking at 330 nm assigned to ligand-to-metal charge transfer transitions.17,24,25 The EPR spectrum has been described in terms of $D = -1.7$ cm$^{-1}$ and $E/D = 0.24$.26–28 Mössbauer spectroscopy29 and EXAFS30,31 have also been applied to both the oxidation states, and the reduced state was studied by MCD.32 NMR spectroscopy was used to observe the active site of Fe$^{2+}$SOD from Methanobacterium thermoautotrophicum33 and E. coli,34,35 in which the ligands’ resonances have been assigned.36 Anion binding has been studied by solvent proton magnetic resonance dispersion.37 The results of these studies have provided invaluable insights into the mechanism of FeSOD, as discussed below.

THE X-RAY CRYSTALLOGRAPHIC STRUCTURE AND METAL SITE OF FeSOD

Crystallization of FeSOD

FeSOD has most commonly been crystallized from the medium of pH 4.5–4.8 containing 40–55% (NH$_4$)$_2$SO$_4$ or 35% methyl pentanediol and 20 mM CaCl$_2$38–41 or 50 mM phosphate buffer of pH 6.1 with 2.15 M (NH$_4$)$_2$SO$_4$.42 FeSODs from more exotic organisms have been crystallized at higher pHs, including a pH 7.5 100 mM Tris and 23% PEG 6000 for Mycobacterium
**Fe-superoxide dismutase**

Figure 1  Ribbon diagram of a monomer of *E. coli* Fe$^{3+}$SOD showing the Fe$^{3+}$ as a large black sphere and the heavy atoms of the ligands from C$_n$ out in ball and stick format. The orientation is the same as that of the monomer on the left side of the 3D Structure. Large alphanumerics are used to label the different helices (a1, a2, a3…) and β-strands (b1, b2, b3) according to Lah *et al.*$^{45}$ Residue numbers are indicated in smaller numbers. Based on the coordinates of Fe$^{3+}$SOD published by Lah *et al.* (1ISB)$^{45}$ and produced using *MOLSCRIPT.$^{57}$

*tuberculosis,$^{43}$* and pH 8.5 100 mM Tris 8% PEG 8000 for *Sulfolobus solfataricus.$^{44}$

**Overall description of the structure**

FeSODs and MnSODs occur as dimers or tetramers of a ~22 kDa monomer whose fold is highly conserved (3D Structure and Figure 1).$^{38,39,43–49}$ The monomer is composed of two domains, each of which contributes two ligands to the single active site metal ion. The N-terminal domain is made up of two long helices separated by a shorter and a more variable one (residues 1–80; amino acid numbering of FeSOD from *E. coli* is used throughout, except when referring to a MnSOD, in which case the numbering of *E. coli* MnSOD is used). The first
Fe-superoxide dismutase

Figure 2  Coordination of Fe³⁺ in Fe³⁺SOD at low pH as per the 1SB coordinates of Lah et al. Metal ion-to-ligand distances in Å are as follows and the crystal structure boasts 1.85 Å resolution at R = 0.18: Fe³⁺-His26 N₃ = 2.16 Å, Fe³⁺-His73 N₃ = 2.05, Fe³⁺-Asp156 O₃ = 1.91, Fe³⁺-His160 N₃ = 2.07. Fe³⁺-O of OH⁻ = 1.96. For Fe²⁺SOD the resolution is 1.80 Å at R = 0.19 and the analogous distances from Fe²⁺ are 2.18, 2.04, 1.94, 2.12, 2.04 Å, respectively. H atoms and backbone C', O and N atoms are omitted for clarity. Produced using MOLSCRIPT.³⁷

Helix contains a conserved kink which orients the universally conserved Tyr34, His30 and ligand His26 into the active site. Ligand His73 derives from the second long helix of the first domain.⁴⁵ A linker of ≈10 variable amino acids connects the two domains and the C-terminal domain (residues 89–192), an open α/β structure, consists of a three-stranded β-sheet flanked on both sides by a total of four α-helices. The C-terminal domain supplies ligands Asp156 and His160 from the C-terminus of the β-sheet and the loop that follows. The fifth ligand to Fe is a coordinated solvent molecule believed to be OH⁻ in the oxidized (Fe³⁺) state and H₂O in the reduced (Fe²⁺) state (Figure 2).⁴⁹ The interface between the domains is predominantly hydrophobic.⁴⁵ The interface between the subunits is highly conserved and generates symmetry-related funnels that provide substrate access to the active sites.⁴⁵

Each monomer’s metal ion and ligands are all inaccessible to the bulk solvent, sequestered behind Trp77, Tyr34 and His30 at the base of the funnel out to the bulk solvent.⁴⁵ The residues participating in the second coordination sphere are derived from both domains and even include residues from the other monomer of the dimer (Figure 3). Indeed, the funnel through which the substrate is believed to access each active site is lined with residues from both monomers.⁴⁵

Thus, SOD displays a theme common among metalloenzymes, of metal binding at the interface between domains or subunits. This device may allow protein domains to fold around individual hydrophobic cores before coordinating the metal ion with ligands distant in the amino acid sequence. The metal ion can then be sequestered between domains to obtain the low dielectric environment needed for tight binding and activity, without requiring a single hydrophobic core to swallow a metal ion.

The metal ion coordination is approximately trigonally bipyramidal with His26 and coordinated solvent as axial ligands (Figure 2). Coordinated solvent is supported by an extensive conserved hydrogen bond network including Gln69, Tyr34, Trp122, Asn72, Asp156 and (via a solvent) His30 and Tyr163B from the other subunit (Figure 3).⁴⁵,⁵⁰ There is a conserved Arg nearby⁵¹ and the active site is surrounded by a shell of highly conserved predominantly aromatic amino acids. These may serve to protect the protein from radical inactivation by virtue of their relatively long-lived free radical states, that may be stable long enough to be rereduced in the next redox cycle of the
metal ion instead of undergoing irreversible oxidative chemistry.

**FUNCTIONAL ASPECTS**

**Mechanism and inhibition**

Fe-SOD from *E. coli* disproportinates $\text{O}_2^{-}$ with saturation kinetics characterized by a turnover number of $k_{\text{cat}} = 2.6 \times 10^4$ s$^{-1}$ and a $K_M$ of 80 $\mu$M for $\text{O}_2^{-}$ at pH 8.4 and 25 $^\circ$C, consistent with the observed second-order rate constant $k_{\text{cat}}/K_M = 3 \times 10^8$ M$^{-1}$ s$^{-1}$. Pioneering work demonstrated that at modest $\text{O}_2^{-}$ concentrations disproportionation occurs in two sequential steps (see Equations (1) and (2))

$$\text{O}_2^{-} + \text{SOD(M}^{2+}) \rightarrow \text{O}_2 + \text{SOD(M}^{2+}) \quad (1)$$

$$\text{O}_2^{-} + \text{SOD(M}^{2+}) + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{SOD(M}^{3+}) \quad (2)$$

where SOD signifies the enzyme and M indicates the bound iron or manganese ion, which cycles between its +3 and +2 oxidation states. The rate-limiting step is believed to be dissociation of $\text{H}_2\text{O}_2$ and to involve $\text{H}^+$ transfer, as it is accelerated by weak acids.

This is only one of several manifestations of the importance of protons to redox catalysis.

FeSOD is competitively inhibited by $\text{N}_3^{-}$, which coordinates directly to $\text{Fe}^{3+}$ resulting in hexacoordinate $\text{Fe}^{3+}$. This suggests that the substrate binds directly to $\text{Fe}^{3+}$ and that substrate oxidation occurs by an innner-sphere mechanism (but see references 17, 55). $\text{F}^{-}$ also coordinates to $\text{Fe}^{3+}$ as does $\text{OH}^{-}$. Two $\text{F}^{-}$ ions can bind to $\text{Fe}^{3+}$, consistent with one replacing the coordinated solvent. Additional inhibitors do not coordinate to $\text{Fe}^{3+}$ but do compete with $\text{N}_3^{-}$.

The $K_{DB}$ of $\text{N}_3^{-}$ and $\text{F}^{-}$ are both much lower than their $K_{DB}$ suggesting much weaker interactions with the reduced rather than the oxidized state. By contrast, the non-coordinating inhibitors $\text{ClO}_4^{-}$ and $\text{SCN}^{-}$ have $K_{DB}$ and $K_{DB}$ that are larger and comparable. Thus, an outer-sphere binding site is inferred for the reduced state that may be the same as the prebinding site inferred for the oxidized state.

FeSOD is inactivated by $\text{H}_2\text{O}_2$ in a reaction that involves an initial stoichiometric reduction of Fe followed by...
by Fe release and a chemical modification of active site residues including Trp.\textsuperscript{56–59} conserved among SODs and is expected to have a pK near the pK of 9 that affects the catalytic activity.\textsuperscript{29,45,49,61–64}

**Protons involved in catalysis**

Two protons participate directly in SOD’s reaction, in Equation (2). It is likely that the first proton is derived from the coordinated solvent, as the latter is expected to have a much lower pK when Fe is oxidized than when it is reduced.\textsuperscript{49,60} The second proton was assumed to be derived from Tyr34 because this residue is universally

**pKs affecting the oxidized state**

SOD activity decreases at increasing pH with a pK of \textit{\sim}9 (at 25 °C). This is ascribed in part to the onset of competitive inhibition caused by OH\textsuperscript{−} binding to Fe\textsuperscript{3+} for the following reasons. The pK affects \textit{K}_{d} but not \textit{k}_{cat}, implying a greater significance to substrate binding than to the rate-limiting chemical steps, and is associated with a

**Figure 4**  Signatures of the pK of Tyr34 in Fe\textsuperscript{3+}SOD. The nuclear magnetic resonance chemical shifts of virtually every active site resonance titrate with a pK of 8.5 in WT Fe\textsuperscript{3+}SOD, but not in Y34F Fe\textsuperscript{3+}SOD.\textsuperscript{35} The pH dependence of the N\textsubscript{31} proton of His73 is shown in WT (open circles) and Y34F (filled circles) Fe\textsuperscript{3+}SOD. The same pK affects the solvent exchange rate of an active site hydrogen bond network proton (the N\textsubscript{31} proton of His160, which is hydrogen bonded to Glu159),\textsuperscript{45} making this proton inaccessible to OH\textsuperscript{−}. Log(base-catalyzed exchange) is expected to increase with pH linearly with a slope of 1.\textsuperscript{98} This behavior is indeed observed in Y34F Fe\textsuperscript{3+}SOD. However, ionization of Tyr34 at pH 8.5 interrupts this increase in WT Fe\textsuperscript{3+}SOD.
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A decrease in the affinity of Fe\(^{3+}\)SOD for azide.\(^{52}\) The latter indicates that either a proton required for substrate analog binding is lost at pH 9 or that substrate analog binding occurs in competition with OH\(^-\) binding. Since the pK coincides with a change in the EPR signal of Fe\(^{3+}\) suggesting a change in the coordination sphere,\(^{24}\) and is associated with expansion of the coordination sphere based on EXAFS spectroscopy at a low temperature,\(^{40}\) it is assigned to the coordination of OH\(^-\). However, the active site geometry may not be the same at a low temperature as at room temperature.\(^{17,55}\)

The pK affecting the reduced state

Fe\(^{2+}\)SOD was predicted to also have a pK near 9 based on elegant kinetic studies and the fact that a single proton is taken up on reduction both above and below the oxidized state pK.\(^{52}\) Anaerobic NMR pH titrations directly detected this pK of 8.5 ± 0.07\(^{35}\) and assigned it to Tyr34 based on its complete absence from FeSOD in which Tyr34 had been mutated to Phe, Y34F FeSOD (Figure 4).\(^{65}\)

Given Tyr34’s universal conservation among SODs and its predicted role as a proton donor to substrate, it was a big surprise that Y34F SOD retains at least 40% of the normal wild-type (WT) activity in the standard assay.\(^{50,65–67}\) However proton exchange measurements demonstrated that upon ionization, Tyr34 bars OH\(^-\) from the active site. This implies that Tyr34 \(-\) repels other anions too,\(^{35}\) consistent with the increase in K\(_M\) above Tyr34’s pK.\(^{52}\) Thus, Tyr34 \(-\) shuts down SOD at high pH. Arguably this would happen anyway when the concentration of OH\(^-\) became sufficient to outcompete substrate for its binding site. However, Tyr34 is shown to help protect FeSOD from inactivation by HO\(_2\).\(^{50,67}\) possibly by denying HO\(_2\)\(^-\) access to the active site Fe\(^{2+}\) when the pH rises sufficiently for H\(_2\)O\(_2\) to dissociate significantly.

Substrate analog binding to Fe\(^{2+}\)SOD via Tyr34

Tyr34 has also been shown to participate in substrate binding and protonation in Fe\(^{2+}\)SOD. In contrast to the inner-sphere reaction implied for the oxidized state, MCD studies at low temperatures indicated no change in the coordination number for the reduced state in the presence of 500 mM K\(_N\) or concentrated NaF or NaOCl.\(^{32}\) NMR spectroscopy indicates that F\(^-\) binds to Fe\(^{2+}\)SOD as an outer-sphere ligand,\(^{68}\) implying an outer-sphere mechanism for substrate reduction. The low-pH K\(_D\) is 40 mM but the binding affinity decreases at high pH with a pK of 8.5.\(^{69}\) Thus, F\(^-\) binding either requires the phenolic proton of Tyr34 or is prevented by Tyr34.\(^{-}\) Because Y34F Fe\(^{2+}\)SOD binds F\(^-\) in a pH independent manner, we conclude that Tyr34 \(^-\) inhibits F\(^-\) (and substrate) binding.\(^{69}\)

The phenolic H of neutral Tyr34 nonetheless does appear to contribute a little to F\(^-\) binding at low pH, as the K\(_D\) of Y34F Fe\(^{2+}\)SOD is higher than that of WT FeSOD (10 and 2 mM, respectively) and the same is true for Fe\(^{2+}\)SOD (K\(_D\) = 70 mM for Y34F and 40 mM for WT).\(^{59}\) By contrast, the fact that Y34F Fe\(^{2+}\)SOD’s K\(_D\) for N\(_3\), is 20 times smaller than that of WT probably reflects the interference between the three-atom substrate analog and the OH of Tyr34.\(^{66}\) Crystal structures of FeSOD complexed with N\(_3\) indicate that N\(_3\) pushes Tyr34 out of its native position.\(^{45}\) Thus, Tyr34 exploits both steric and electrostatic mechanisms to enforce binding specificity for small anions.

Tyr34 as a possible proton shuttle

We have located the F\(^-\) binding site near residues 35–40 by high-resolution NMR spectroscopy,\(^{58}\) although Tyr34 itself could not be observed in these experiments because of paramagnetic relaxation of its resonances. In addition, F\(^-\) binding raises the pK of Tyr34, suggesting that F\(^-\) hydrogen bonds with it or binds near it.\(^{69}\)

The crystal structures of FeSOD and MnSOD contain a solvent molecule in a position consistent with our proposal, 3.3 Å from Tyr34’s O and 3.5 Å from the N\(_3\) of His30.\(^{45,70}\) His30 is absolutely conserved in FeSODs and MnSODs and the mutation of His30 in human MnSOD decreases k\(_{cat}\) and can increase K\(_M\) (but see reference 72). If F\(^-\) were to associate with Fe\(^{2+}\)SOD in the place of this solvent molecule, it would be 7.5 Å from Fe\(^{2+}\). However, this distance should be regarded as a maximum, and electron transfer could be facilitated by aromatic residues His30 and Tyr34 which are 4.4 and 5.2 Å from the ligands His160 and His73, respectively, and appear to stack parallel with them (Figure 5). Thus, Tyr34 may begin transferring a proton to substrate as part of substrate binding. This would have the desirable effect of favoring substrate reduction and could progress to full proton transfer in the course of electron transfer to substrate. At the same time, Tyr34’s proton would be replaced by proton transfer from the coordinated solvent, which becomes increasingly acidic as Fe is oxidized. This second part of the transfer could occur naturally via the hydrogen bond network between Tyr34 and the coordinated solvent.\(^{45,70}\)

The high activity of Y34F FeSOD in the standard assay argues that either the above proton relay is not necessary or that a similarly effective mechanism for protonating substrate and deprotonating the coordinated solvent exists in Y34F SOD. Indeed, the Y34F mutation leaves more space in the active site, results in significantly faster base-catalyzed proton exchange in and out of the active site at high pH,\(^{45}\) and also allows greater substrate analog access to the metal ion.\(^{66,67,69}\) Thus, at the low substrate concentrations of the standard assay, proton transfer may
be able to keep up with substrate binding, without Tyr34. By contrast, stopped flow kinetics resolves $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ and demonstrates that $k_{\text{cat}}$ decreases by an order of magnitude in human Y34F-MnSOD.\textsuperscript{50}

Thus, Tyr34 functions as a selective barrier to the active site that passes protons and bars $\text{HO}_2^-$. Others have proposed that Tyr34 mediates proton transfer from the bulk solvent to the substrate in the active site,\textsuperscript{50} but we propose that it shuttles protons between the bulk and coordinated solvent or between the coordinated solvent and substrate, in conjunction with outer-sphere electron transfer.

**Redox tuning: a model explaining SOD’s metal ion specificity**

In addition to substrate binding and proton transfer, electron transfer is central to redox catalysis. This is inextricably linked to the identity of the metal ion, a property of the enzyme that is so basic that it is usually taken for granted. The metal ion determines what $E_{\text{ox}}$ are possible, and the $E_{\text{ox}}$ places a fundamental thermodynamic limit on the chemistry the enzyme can perform.

Some SODs are active in the standard assay with either Fe or Mn bound, and are called ‘cambalistic’.\textsuperscript{73–75}

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**Figure 5** A proposed location of outer-sphere substrate binding to Fe$^{2+}$SOD and a possible mechanism of proton transfer from coordinated solvent via Tyr34, initiated upon substrate binding and progressing in conjunction with Fe$^{2+}$(H$_2$O) oxidation to Fe$^{3+}$(OH$^-$). Dashed lines indicate hydrogen bonds involving coordinated solvent or the crystallographic solvent molecule whose position may be indicative of an outer sphere substrate binding site. Heavy dashed lines indicate a possible proton transfer relay linking coordinated solvent and outer sphere bound substrate. Coordinates of Fe$^{2+}$ SOD were used (1HSA)\textsuperscript{45} and the figure was produced using MOLSCRIPT.\textsuperscript{97}
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Table 1  Reduction midpoint potentials of analogous high-spin Fe and Mn complexes in mV vs. NHE

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Fe complex</th>
<th>Mn complex</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(H₂O)₆</td>
<td>770</td>
<td>1510</td>
<td>740</td>
</tr>
<tr>
<td>EDTA</td>
<td>96</td>
<td>825</td>
<td>730</td>
</tr>
<tr>
<td>(Fe)SOD</td>
<td>220⁹</td>
<td>&gt;900b</td>
<td>&gt;670</td>
</tr>
<tr>
<td>(Mn)SOD</td>
<td>−240⁹</td>
<td>290b</td>
<td>540</td>
</tr>
</tbody>
</table>

a  Vance and Miller.82  
b  Vance, unpublished.

However, this is not the rule. It has been known for more than 20 years that FeSOD protein ([Fe]SOD hereafter) can be reconstituted with Mn (resulting in Mn(Fe)SOD), and Fe can be incorporated into (Mn)SOD protein to make Fe(Mn)SOD. Although both metal ions can perform SOD chemistry and both proteins obviously can support it, the majority of metal-exchanged SODs are not active in the standard assay.76-79  Yamakura showed that Fe⁶⁺(Mn)SOD displays a pK two pH units below that of native Fe⁹⁺SOD and that Fe(Mn)SOD acquires a low but significant activity below this pK.⁸⁰,⁸¹  Whittaker and Whittaker found that the mutation of Tyr34 to Phe raised the pH onset of activity and proposed that different ligand basicities in the (Fe)SOD and (Mn)SOD proteins are responsible for the inactivity of Fe(Mn)SOD.⁸²  We have demonstrated that Fe(Mn)SOD and Mn(Fe)SOD have strongly shifted the reduction midpoint potentials (Eₘₙ) that can simply and fully account for their inactivities.⁸³

Since SODs must both oxidize and reduce O₂⁻⁻, their Eₘₙs should be half way between the Eₘₙs of these two half reactions, or approximately 360 mV vs. NHE, for optimal turnover.⁸⁴  Indeed, this appears to be the case, based on the published Eₘₙs of FeSOD, MnSOD and Cu, ZnSOD, which range from 190–420 mV.⁸³,⁸⁴  However, octahedral high spin Mn tends to have a much higher 3+2+/Eₘₙ than octahedral high spin Fe (Table 1). Thus, (Mn)SOD must depress the Eₘₙ of Mn much more than (Fe)SOD depresses that of Fe. Given the structural similarity of Fe and Mn, and the apparent similarities of the (Fe)SOD and (Mn)SOD proteins, we proposed that the differences in Eₘₙ tuning

![Figure 6](image_url)  A simple model that can explain the inactivities of Fe(Mn)SOD and Mn(Fe)SOD on the basis of Eₘₙs that are too low (or too high) to support O₂⁻⁻ oxidation (or reduction).⁸³  Since high-spin Fe complexes have lower Eₘₙs than analogous high-spin Mn complexes, yet the Eₘₙs of FeSOD and MnSOD are comparable, the MnSOD protein, (Mn)SOD, must depress the Eₘₙ of Mn³⁺²⁻ more than (Fe)SOD protein depresses the Eₘₙ of Fe⁹⁺²⁻. If this difference persists in metal-exchanged SODs then Fe (the lower Eₘₙ metal ion) bound in (Mn)SOD protein, Fe(Mn)SOD, will be subjected to greater Eₘₙ depression, and display a very low Eₘₙ. Conversely, Mn will display an unnaturally high Eₘₙ in Mn(Fe)SOD. The reference compounds chosen are the hexaquo complexes of Mn³⁺²⁻ and Fe³⁺²⁻. The Eₘₙs obtained for E. coli FeSOD, MnSOD and Fe(Mn)SOD are indicated as well as a lower limit for the Eₘₙ of Mn(Fe)SOD.⁸³,⁸⁹
Figure 7  Overlay of the active sites of Fe$^{3+}$/MnSOD (grey Ca) and Fe$^{3+}$/SOD (white Ca) based on the A-chain coordinates of Edwards et al. (1MMD) and the A chain coordinates of Lah et al. (11SB). Coordinates were superimposed in Midas plus based on the Fe$^{3+}$, the coordinating O of Asp and the coordinating Ns of the His. The rms difference between atomic coordinates was 0.2 Å. The figure was produced using Molscript.\textsuperscript{77}

 persist in the metal-exchanged SODs. Thus our model predicted that the $E_m$ of Fe(Mn)SOD would be much lower than those of FeSOD or MnSOD, and that the $E_m$ of Mn(Fe)SOD would be much higher (Figure 6). Since the potentials of Fe and Mn are different by a large amount (Table 1), we predicted that the $E_m$ of Fe(Mn)SOD would be below that required for it to oxidize $O_2$\textsuperscript{2-}, but that if there were nothing else wrong with the active site, then Fe(Mn)SOD should still be able to reduce $O_2$\textsuperscript{2-}. Similarly, Mn(Fe)SOD could fail to turn over due to inability to reduce $O_2$\textsuperscript{2-} because of an $E_m$ higher than that of $O_2$\textsuperscript{2-}/H$\textsubscript{2}$O\textsubscript{2}.\textsuperscript{82}

Proof of the model

The $E_m$ of \textit{E. coli} FeSOD is 220 mV vs. NHE based on both ascending and descending titrations and two different mediators\textsuperscript{82} but a significantly lower value is obtained using a mediator cocktail. We have also evaluated the $E_m$ of \textit{E. coli} MnSOD to be 290 mV vs. NHE.\textsuperscript{85} However, the $E_m$ of Fe bound to (Mn)SOD is $-240$ mV, almost half a V lower than Fe's $E_m$ in (Fe)SOD\textsuperscript{82} and Mn(Fe)SOD's $E_m$ is more than 900 mV, much higher than that of MnSOD.\textsuperscript{85} We have shown that metal-substituted SODs retain the ability to bind substrate analogs and that their active sites are similar to those of the native enzymes, ruling out gross disruption as the cause of inactivity.\textsuperscript{86} Moreover, Fe(Mn)-SOD retains ability to reduce substrate and therefore both electron and proton transfer activity.\textsuperscript{82} Our model is consistent with Fe(Mn)SOD's acquisition of activity at low pH\textsuperscript{67,80,81} since protonation of an active site group is expected to raise the $E_m$. Thus, the catalytic inactivities of Fe(Mn)SOD and Mn(Fe)SOD can be simply explained by their very low and very high $E_m$s, respectively.\textsuperscript{82}

Mechanisms of $E_m$ tuning

The two SOD proteins' dramatically different $E_m$ tuning indicates that despite their similar appearance in crystal structures, the two proteins are very different indeed with respect to their interactions with the metal ion. However, differences between the active sites of FeSOD and MnSOD are overshadowed by the similarities in the crystal structures (Figure 7).\textsuperscript{39,45} In both, the same ligands coordinate the metal ion in virtually superimposable geometries. The second sphere contains few notable differences.\textsuperscript{87,88} FeSODs have a Tyr at position 76 which is occupied by Phe in MnSODs, but mutation of Tyr to Phe in \textit{S. solfataricus} FeSOD did not increase the Mn-supported activity.\textsuperscript{89} Mutation of the Glu that bridges the dimer interface was found to confer higher Fe binding on MnSOD, but no Fe-supported activity.\textsuperscript{90} Even the
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computed electrostatic potentials of *E. coli* FeSOD and *Thermus thermophilus* MnSOD do not differ substantially at the active site (M Gunner, personal communication) Thus, the two SOD proteins appear to use the same tools to effect different redox tuning Subtle biophysical differences have been reported between *E. coli* FeSOD and MnSOD but it is not known whether these differences are general.\(^7\) The major difference is that the Gln conserved at residue number 69 in FeSODs is absent from MnSODs and replaced by a Gln conserved at position 146 instead.\(^8\) This Gln side chain is believed to hydrogen bond with the same residues in both SODs.\(^45,88\)

The appearance of similarity may be deceptive and largely due to the relatively low resolution possible in crystal structures of proteins, compared to the sensitivity of metal ion electronics to relatively small perturbations in placement, orientation and protonation of ligands. Many of the SOD crystal structures published were obtained using protein not fully metallated or containing a mixture of metal ions. In these, the finer details of the active site reflect the average of several different species and cannot be interpreted in terms of aspects by which the individual species might differ, including subtle positional and hydrogen bonding differences. Indeed, spectroscopic studies directly addressing the metal ion have revealed significant differences between Fe coordinated to (Fe)SOD and (Mn)SOD.\(^86\)

FeSOD and Fe(Mn)SOD are significantly different with respect to the effects of substrate analog binding and protonation or OH\(^-\) binding equilibria, both of which could be related to electron transfer.\(^86\) A pK near 8.4 is associated with a dramatic change in Fe\(^{3+}\)-(Mn)SOD active site geometry from weakly rhombic to a much more axial structure.\(^86\) This is completely unlike the effect of increasing pK on Fe\(^{3+}\)SOD, which is to cause the close-to-rhombic EPR signal to approach the rhombic limit.\(^24\)

Edwards et al. reported observing a non-native active site geometry in the crystal structure of Fe\(^{3+}\)-(Mn)SOD.\(^48\) However, their crystallization pH of 8.5 was significantly higher than the pHSs used for the reference structures of FeSOD\(^49,45,48\) and moreover roughly coincides with the pK of 8.4 identified by EPR with a coordination geometry change.\(^86\) Since the other published Fe\(^{3+}\)SOD structures represent the active site below its pK (where known) and the Fe\(^{3+}\)-(Mn)SOD structure represents the active site at or above its pK, it is not surprising that the sites in the Fe\(^{3+}\)-(Mn)SOD structure display a second coordinated solvent molecule but that none of the Fe\(^{3+}\)SOD sites do. The Edwards result indicates that the pK of Fe\(^{3+}\)-(Mn)SOD corresponds to the coordination of a second OH\(^-\), like the pK of Fe\(^{3+}\)SOD.\(^30\)

The very different EPR signatures associated with N\(^-\) and OH\(^-\) binding to Fe\(^{3+}\)SOD vs. Fe\(^{3+}\)-(Mn)SOD\(^67,86\) underline the different behaviors of Fe\(^{3+}\) in these superficially similar sites, and may be related to the different substrate analog binding modes observed crystallographically for N\(^-\) in Fe\(^{3+}\)SOD and Mn\(^{3+}\)SOD.\(^45\) Thus the large \(E_m\) differences are accompanied by large spectroscopic and substrate analog binding mode differences that are not easily explained based on the available crystal structures.

**The position of the active site Gln differs in Fe\(^{2+}\)SOD and Fe\(^{2+}\)(Mn)SOD**

The degree to which the ligands are protonated can exert a very strong effect on the metal ion \(E_m\). In particular, the ionizable proton of coordinated solvent greatly stabilizes the electronic orbitals of the coordinated O and therefore has a very large effect on its influence on the metal ion \(E_m\). Based on computations, the \(E_m\) of Mn could vary by 1.5 V depending on whether the coordinated solvent is protonated or not.\(^52\) Thus, differences in the degree to which the coordinated solvent is protonated, for example by different hydrogen bonding, could explain the half V difference between the \(E_m\) of metal ions bound to (Fe)SOD vs. (Mn)SOD.

Gln69 (and Asp156) hydrogen bond directly to coordinated solvent. Asp156 is common to FeSODs and MnSODs but the Gln comes from different directions, with slightly different orientations and positions relative to the metal ion.\(^45,88\) The side chain N is significantly closer to the metal ion in Fe(Mn)SOD and MnSOD than it is in FeSOD, and interacts much more strongly with the Fe\(^{2+}\) of Fe\(^{2+}\)(Mn)SOD than that of Fe\(^{2+}\)SOD.\(^54\) Thus, a strong contact between the Gln and the Fe correlates with a much lower \(E_m\). The same correlation recurs in human (Mn)SOD, in which Q143N MnSOD has a much higher-than-native \(E_m\), and weaker-than-native hydrogen bonding between the side chain amide of Asn and Mn.\(^93\) Thus, strong interaction between the side chain amide and the metal ion depresses \(E_m\) and destabilizes additional electron density on the metal ion. It could do this by stabilizing the coordinated OH\(^-\).\(^54\)

**Coordinated solvent as a device for \(E_m\) tuning**

Since the coordinated solvent is believed to be OH\(^-\) in the oxidized state of both SODs, but to take up a proton upon reduction, the acid dissociation constants of the coordinated solvent in the oxidized and reduced states (\(K_{red}\) and \(K_{ox}\)), contribute to the \(E_m\), observed at a given pH (see Equation (3))

\[
E_m = E_{AH} + \frac{RT}{F} \ln \frac{K_{red} + [H^+]}{K_{ox} + [H^+]} \tag{3}
\]

where \(E_{AH}\) is the potential relating the protonated oxidized state to the protonated reduced state. \(K_{red}\) is relatively unimportant since \(K_{red} \ll [H^+]\),\(^35,86\) but if \(K_{ox}\) differs between FeSOD and Fe(Mn)SOD, it will contribute to the
$E_{m}$ tuning difference. Thus, stabilization of OH\(^-\) could lower $E_{m}$ via $K_{eq}$. An implausibly large decrease in $pK_{eq}$ of 8 pH units would be required to fully account for the 0.5 V lower $E_{m}$ observed in Fe(Mn)SOD than in FeSOD. However, modulation of pKs by 5 pH units is well known in proteins and indicates that this mechanism could be an important contributor to differential redox tuning.

Since proteins achieve exquisite and dramatic control over residue protonation states and pKs, coordinated solvent molecules and hydrogen bonds between them and protein residues offer a potentially general and powerful mechanism by which proteins may tune the $E_{m,S}$ of bound metal ions. Thus, coordinated solvents may not only serve to hold open labile coordination sites and supply the protons whose transfer accompanies electron transfer, but may also serve a general role as adapters between protein and metal ion by which the protein's control over proton density can be translated into control over metal ions' $E_{m,S}$, and thus reactivity.

>7% WT FeSOD activity.$^{54}$ This activity is well within the range observed for cambialistic SODs. Consistent with the greater Mn-supported than Fe-supported activity, the active site remains spectroscopically much more similar to that of (Mn)SOD than that of (Fe)SOD with either Fe or Mn bound.$^{54}$ That mutagenesis of the active site Gln does not completely convert a MnSOD to a FeSOD (or vice versa) is not surprising,$^{55}$ considering that Gln is only one of the several amino acids that interacts with the metal ion. Moreover, it is the side chain of Gln that is involved in tuning metal ion reactivity, and this is positioned only remotely by the peptide backbone, and much more precisely by packing and hydrogen bond interactions with residues that retain their MnSOD positions and identities. Precise positioning of chemical functionalities may account for many of the chemical differences observed when a metal ion is bound to the two different proteins, and be as important as the more often-cited conserved differences between the identities of the active site amino acids.

**Mutation of the active site Gln**

Several groups have prepared mutants of the active site Gln.$^{54,55,56}$ Mutagenesis to the Glu enhanced Mn-binding over Fe-binding but did not confer Mn-supported activity.$^{54}$ In a more conservative strategy, Hiraoka \textit{et al.} mutated the Gln characteristic of FeSOD to Gly and Ala141 to the Gln characteristic of MnSOD, in a cambialistic SOD. The mutant displayed increased the Mn-supported activity and decreased the Fe-supported activity but remained cambialistic.$^{55}$ The complementary mutant has also been prepared (Figure 8). \textit{E. coli} G77Q,Q146A-(Mn)SOD lacking the Gln characteristic of MnSOD and containing the Gln characteristic of FeSOD instead retains 70% of WT Mn-supported activity in the standard assay. However, in contrast to WT Fe(Mn)SOD which has no detectable Fe-supported activity in the standard assay, Fe-G77Q,Q146A-(Mn)SOD displays

**Relating proton transfer to redox tuning**

Coupling between proton transfer and electron transfer is an important mechanism of biological energy conservation. It is also a virtually ubiquitous feature of biological redox catalysis, and is explicit in SOD. The ionizable proton of the coordinated solvent and the protons in the hydrogen bond network not only tune the $E_{m}$ of the metal ion, they also participate in the reaction. Thus, SOD proteins may bring about a balance between the two half reactions of superoxide disproportionation by modulating the pKs of the two protons involved. Since (lower potential) Fe has a natural tendency to reduce substrates whereas (higher potential) Mn has a natural tendency to oxidize them, (Fe)SOD protein might promote substrate oxidation at the expense of reduction by favoring proton uptake by coordinated solvent. This is essentially the opposite of what happens in O\(_2\) carrier proteins where Fe$^{2+}$.
coordinates O₂ to reversibly form a [Fe³⁺O₂⁻] state that is stabilized by hydrogen bonding to O₂⁻. FeSOD may provide a proton to coordinated OH⁻ instead, and thereby favor electron transfer from O₂⁻ to Fe³⁺, towards coordinated solvent (Figure 9). (Mn)SOD might promote substrate reduction by transferring a proton to the non-coordinating O of O₂⁻ simultaneously with electron transfer (the pK of O₂⁻ is only 4.5) and by stabilizing coordinated solvent as OH⁻ instead of protonating it. Different hydrogen bonding to substrate is consistent with the different modes of substrate analog binding observed in FeSOD vs. Fe(Mn)SOD or MnSOD. Indeed, this alternative use of active site protons to draw electron transfer onto and off of substrate is equivalent to the different stabilization of coordinated H₂O/OH⁻ invoked earlier to explain some of the difference in Eₘₛ and constitutes an effective reversal of the active site proton flow in one protein relative to the other.

CONCLUDING REMARKS

In FeSOD as in many other redox-active enzymes, substrate binding, proton transfer and electron transfer are inextricably intertwined. Although FeSOD’s overall reaction is readily catalyzed by many simple inorganic systems, FeSOD achieves the specificity characteristic of enzymes via the protein’s participation in and control over substrate binding and protons. Our measurements of Kᵥₐₛ and pKs provide a necessary thermodynamic framework for understanding FeSOD’s mechanism while spectroscopic studies permit interpretation in terms of conformational changes and individual residues, complementing the wealth of mechanistic, crystallographic and mutagenesis-derived information available for this enzyme.

The apparent implausibility of Fe as an agent of protection against oxidative stress is reconciled by an outer-sphere mechanism for the second half reaction and exclusion of HO₂⁻ from the active site Fe²⁺ by ionized Tyr34⁻. We propose that a conserved crystallographic solvent approximately represents the outer sphere substrate binding site and that Tyr34 serves to shuttle a proton from the coordinated solvent to the substrate in conjugation with its reduction in MnSOD. Gln also occupies a pivotal position in FeSOD’s active site, linking at least four other amino acids to the coordinated solvent. We propose that the differences between the strength of hydrogen bonding to the coordinated solvent in the (FeSOD) and (Mn)SOD proteins goes a long way toward producing the very large difference between the Eₘₛ's achieved by these two proteins. This in turn is sufficient to explain the two proteins’ intriguing metal ion specificities. Since coordinated solvent is not only crucial to Eₘₛ tuning but also proposed to be the source of one of the protons required to generate the product, its pKs are crucial to activity. Protein control over the degree to which coordinated solvent is protonated, via hydrogen bonding and other protein-determined interactions, may provide a general mechanism for protein determination of metal ion Eₘₛ and reactivities.

By virtue of the very large range of Eₘₛ observed within a conserved overall structural context, the FeSODs and MnSODs provide an excellent system for understanding redox tuning. The possibility of manipulating the Eₘₛ at will by amino acid and metal ion substitution opens the way for designing metal ion sites to catalyze desired chemistry.
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