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Review

Activation of superoxide dismutases: Putting the metal to the pedal

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Abstract

Superoxide dismutases (SOD) are important anti-oxidant enzymes that guard against superoxide toxicity. Various SOD enzymes have been characterized that employ either a copper, manganese, iron or nickel co-factor to carry out the disproportionation of superoxide. This review focuses on the copper and manganese forms, with particular emphasis on how the metal is inserted in vivo into the active site of SOD. Copper and manganese SODs diverge greatly in sequence and also in the metal insertion process. The intracellular copper SODs of eukaryotes (SOD1) can obtain copper post-translationally, by way of interactions with the CCS copper chaperone. CCS also oxidizes an intrasubunit disulfide in SOD1. Adventitious oxidation of the disulfide can lead to gross misfolding of immature forms of SOD1, particularly with SOD1 mutants linked to amyotrophic lateral sclerosis. In the case of mitochondrial MnSOD of eukaryotes (SOD2), metal insertion cannot occur post-translationally, but requires new synthesis and mitochondrial import of the SOD2 polypeptide. SOD2 can also bind iron in vivo, but is inactive with iron. Such metal ion mis-incorporation with SOD2 can become prevalent upon disruption of mitochondrial metal homeostasis. Accurate and regulated metallation of copper and manganese SOD molecules is vital to cell survival in an oxygenated environment.

Keywords: Copper; Manganese; Iron; Mitochondria; ALS; Superoxide dismutase; SOD; CCS; Copper chaperone; Posttranslational modification; Disulfide isomerase; SOD1; SOD2; EC-SOD

1. Introduction

The superoxide dismutases (SODs) are ubiquitous components of cellular antioxidant systems. As described by McCord and Fridovich over 36 years ago, these proteins protect redox sensitive cellular machinery from damage by catalyzing the disproportionation of superoxide anion to oxygen and hydrogen peroxide [1]. Several diverse families of SODs have been widely studied and disruptions or accentuations of their function are associated to varying degrees with several diseases in man such as arteriosclerosis [2], diabetes mellitus [3,4] and Down syndrome [5]. The strongest connection between SODs and human disease is found for the copper and zinc dependent forms, mutations in which can cause the neurodegenerative disease amyotrophic lateral sclerosis (ALS) [6]. The activity of each of the superoxide dismutase families relies upon a specific redox active metal ion, and depending on the SOD molecules, this could either be a manganese, iron, copper or nickel ion. This brief review will focus on recent insights into the question of how the correct metal gets into the copper- or manganese-dependent families of these important antioxidant proteins.

All known SODs require a redox active transition metal in the active site in order to accomplish the catalytic breakdown of superoxide anion. A generic mechanism for the metalloenzyme-dependent dismutation steps is below.

$$\begin{array}{c} M^{oxidized}\text{–SOD} + O_2^- \rightleftarrows M^{reduced}\text{–SOD} + O_2 \\ M^{reduced}\text{–SOD} + O_2^- + 2H^+ \rightleftarrows M^{oxidized}\text{–SOD} + H_2O_2 \\ \hline 2O_2^- + 2H^+ \rightleftarrows O_2 + H_2O_2 \end{array}$$

The metal cofactors catalyze both a one-electron oxidation (see first step) and a one-electron reduction of (second step) separate superoxide anions to give the overall disproportionation reaction. These reactions typically require no external source of redox equivalents and are thus self-contained

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components of the antioxidant machinery. This allows SODs to function in a variety of intracellular and extracellular environments. Other antioxidant enzymes are known to consume superoxide, such as the prokaryotic superoxide reductases (SORs). In contrast to the SODs, these enzymes require an external source of electrons which are typically supplied by accessory redox proteins, such as a ferrodoxin [7]. Under some biochemical conditions the SOD proteins can bypass the dismutation cycle and use external redox equivalents. For instance, catalytic SOR or superoxide oxidase (SOO) activities have been reported for some SOD proteins; however, a physiological role for such reaction pathways is not known [8].

Superoxide dismutases are typically soluble secreted or cytosolic proteins but are also found in a number of subcellular compartments such as the cell envelope of gram-negative bacteria or the mitochondria of eukaryotic cells, as well as the extracellular milieu. While there is no similarity in sequence or structure between Cu/ZnSOD and Mn/FeSOD families, these metalloproteins do share some intriguing properties. For instance, both are quite stable relative to most proteins found in mesophilic organisms. Members of these families of enzymes are found across the continuum of life: examples are known in prokaryotes, archea and eukaryotes. The Cu/ZnSODs and MnSOD, which are the focus of this review, are the only forms found in yeast and mammals. Excellent overviews of the prokaryotic nickel and iron SODs can be found elsewhere [9,10].

1.1. SOD nomenclature

Before discussing the activation of the mammalian and yeast versions of these proteins, it is useful to narrow down the diverse SOD nomenclature otherwise observed in the literature (Table 1). First the names for highly similar orthologs of the various metal containing SOD molecules can vary greatly among different species. For example, the manganese containing SOD in E. coli is known as sodA, in *C. elegans* as Sod-2 and Sod-3, in *C. sapidus* as mtMnSOD and in bakers yeast and mammals as SOD2. Furthermore, SOD3 refers to extracellular Cn/ZnSOD in mammals, but to intracellular MnSOD in *C. albicans*. To avoid such nomenclature confusion in this review, we employ the designations Cu/ZnSOD, MnSOD and FeSOD for all organisms other than human and yeast, where the SOD1 (intracellular Cu/Zn) and SOD2 (mitochondria MnSOD) designations will be used.

2. Copper, zinc superoxide dismutase

2.1. Sub cellular localization of intracellular Cu/ZnSOD of eukaryotes

SOD1 in mammals and *S. cerevisiae* is mainly localized in cytosol with a smaller fraction in the intermembrane space of mitochondria [21–25]. It has also been reported in nuclei, lysosomes and peroxisomes using immunocytochemical methods [26]. While superoxide is produced in cytosol by several enzymes such as xanthine oxidase [27], respiratory components in mitochondria are thought to be a major source of O_2^- generation [28]. In fact, in mammals, more than 95% of consumed daily oxygen is reduced to water in the respiratory chain, and 1–2% of it has been estimated to be converted to O_2^- by proteins in the electron transport chain in mitochondria. Most of mitochondrial O_2^- is disproportionated by high levels of Mn-superoxide dismutase (1.1×10^{-5} M) [29] in the mitochondrial matrix. While the presence of SOD1 in the mitochondrial matrix is a controversial issue [30] its localization in the inter-membrane space (IMS) is now well established [21,23–25,31]. Interestingly, in gram-negative bacteria, Cu/ZnSOD is localized in a compartment that is distantly related to the IMS, namely the periplasmic space [32–34]. Cu/ZnSOD is important for survival of prokaryotes in relatively late/stationary phase and contributes to the ability to grow aerobically [34]. The periplasmic sources of O_2^- have not yet been characterized but it is likely that Cu/ZnSOD protects components of this compartment against both endogenous and exogenous sources of O_2^-, for instance those arising from host pathogen responses.

Mammalian SOD1 is highly expressed in the liver and kidney [35] and is also abundant in motor neurons [36]. Interestingly, xanthine oxidase (which can serve as a source of superoxide) is highly expressed in the liver. Knockout studies indicate that elimination of the SOD1 gene in rodents is associated with

Table 1
Nomenclature for select SOD molecules across various species

Redox metal co-factor	Name	Species	Localization	Reference
Manganese	sodA	*E. coli*	Intracellular	[11]
	Sod-2 and Sod-3	*C. elegans*	Presumed mitochondrial	[12]
	CytMnSOD	*C. sapidus*	Cytosolic	[13]
	MtMnSOD	*C. sapidus*	Mitochondria	[13]
	SOD3	*C. albicans*	Cytosolic	[14]
	SOD2	*S. cerevisiae* Mammals	Mitochondrial matrix	[15]
Iron	sodB	*E. coli*	Intracellular	[16]
Nickel	NiSOD	*S. seoulensis*	Intracellular	[9]
Copper	Sod-1 and Sod-5	*C. elegans*	Presumed cytosolic	[17,18]
	sodC	*E. coli*	Periplasmic	[19]
	SOD1	*S. cerevisiae* Mammals	Cytoplasm and mitochondrial IMS	[1]
	SOD3	Mammals	Extracellular	[20]

alcohol-induced liver injury [37,38], hepatocarcinogenesis [39], infertility in females [40], age-related hearing loss via cochlear hair cell degeneration [41], vulnerability to motor neuron loss after axonal injury [42], and decrease in life span [39]. Also, in humans, the level of glycated SOD1 is increased in the erythrocytes of patients with diabetes mellitus [43]. Given that glycation in SOD1 may cause fragmentation of the enzyme [4], loss of SOD1 activity may lead physiological problems in diabetic patients [3]. The most widely studied connection between SOD1 and human diseases involves the late onset neurodegenerative disease ALS. Over one hundred mutations in the human gene SOD1 are now known to lead to some of the inherited forms of ALS, and several outstanding reviews cover the physiology and biochemical mechanisms that are under evaluation [44–47].

2.2. SOD1 chemistry and mechanism

The structure and mechanism of this remarkable family of proteins have been reviewed comprehensively elsewhere [48]. SOD1 is quite robust with respect to physical or chemical denaturation; enzymatic activity is observed in the presence of stringent denaturants such as 10 M urea or 4% SDS, and activity in standard buffers is observed at 80 °C [49]. While thermal (non-enzymatic) dismutation of O_2^- to O_2 and H_2O_2 is somewhat fast under typical conditions (5×10^5 M^{-1} s^{-1}), SOD1-catalyzed dismutation is accelerated by four orders of magnitude and approaches the diffusion-controlled limit (1.6×10^9 M^{-1} s^{-1}). It is important to note that the rate of disproportionation by these enzymes is very similar to that of buffer solutions containing transition metal salts. This observation led to a significant amount of debate concerning the actual function of SOD. Recent in vitro and in vivo studies strongly support a model for the inorganic physiology of the cytosol in which intracellular free zinc and copper ion concentrations are vanishingly small under normal aerobic growth [50–54]. This is consistent with a selection processes favoring organisms that elaborate a means of localizing transition metal catalyst for superoxide dismutation to different parts of the cell, depending upon the origin and nature of the oxidative stress. Free iron, on the other hand, has been proposed to be more available [55].

The sequence and structure of Cu/ZnSOD is highly conserved from prokaryotes to eukaryotes [56]. This protein associates to form a dimer with a dissociation constant of $\sim1.0\times10^{-10}$ M^{-1} [57], and each subunit has an immunoglobulin-like fold that provides an active site with one copper and one zinc ion. One remarkable feature is the intra-subunit disulfide bond, which is stable and observed in most if not all Cu/ZnSOD structures published to date. In the native state of human SOD1 two of the four cysteines, namely Cys 57 and Cys 146 are oxidized to form a disulfide that stabilizes Loop IV, which plays an important role in SOD1 dimerization. The copper and zinc ions bind to the protein in two similar, but chemically distinct environments. In this structure, a Cu^{2+} ion is shown bound by four histidine nitrogen atoms (His 46, 48, 63, 120), forming a distorted plane, and water binds as a fifth ligand to complete a square pyramidal structure. A bridging role is shown for His 63, which also binds to the adjacent Zn^{2+} ions through the other imidazole nitrogen, which must be deprotonated in order to bind both metals simultaneously. Interestingly, reduction of the Cu^{2+} to Cu^{1+} leads to a significant change in the coordination number and geometry. Regardless of copper oxidation state, the zinc ion binds to His 63 as well as His 71, His 80, and Asp 83 in a distorted tetrahedral arrangement. Binding of Zn^{2+} ion is not essential for the dismutation reaction but confers higher thermal stability [58]. Similar structures are observed for the bacterial forms of CuSOD, with a few notable exceptions: a stable monomeric form is observed for the *E. coli* protein [59], a heme binding site is observed in *H. ducreyi* [60], and no clear zinc binding site is known for the *M. tuberculosis* protein [61].

2.3. Insertion of copper is facilitated by the CCS metallochaperone

It has long been known that the apo-form of SOD1 can be generated in vitro and then reconstituted with the native copper and zinc ions or with other metals [48]. Most cells do not leave the insertion of copper to diffusion reactions and employ an accessory protein known as the copper chaperone for SOD1 (CCS) to facilitate this process. CCS docks with and transfers the metal ion to the disulfide-reduced apo SOD1 [53,62] as seen in Fig. 1 and discussed below. Accounts of the discovery and structure of CCS can be found in several recent reviews [63–66]. The mechanism shown in Fig. 1 reveals that CCS activates its target SOD1 by a very different mechanism than that used by the copper chaperone Atx1, where Cu^+ is readily transferred to the partner domains in the presence or absence of oxygen. It is now clear that the CCS copper chaperone employs a more complex oxygen- and disulfide-dependent mechanism. Before describing the copper loading process, we examine the generally overlooked intrasubunit disulfide of SOD1.

2.4. Role of the disulfide in SOD1 structure and activity

An intrasubunit disulfide is observed in all structurally characterized forms of native SOD1 published to date. It appears that SOD1 is the most abundant disulfide-containing protein in the eukaryotic cytosol [58]. Given the reducing character of the cytosol, most pairs of solvent exposed Cys residues would not be expected to form a persistent disulfide. The reduction potential for the disulfide of yeast SOD1 ($E^0=-0.23$ V) [58] is similar to the resting or average reduction potential for the eukaryotic cytosol [67]. This potential is also similar to that observed for the protein disulfide isomerases (PDI) such as thioredoxin, where the active site pairs of cysteines cycle between the reduced and oxidized state (see [58] and references therein for comparisons). Thus the robustness of this disulfide in the eukaryotic cytosol (or in the presence of reducing buffers in vitro) is remarkable. The physiological stability of the disulfide most likely originates from kinetic as opposed to thermodynamic factors [58]. The disulfide reduced form of the yeast SOD1 protein can accumulate in the cell under anaerobic conditions or in the absences of CCS [58,68]. This immature state of the protein is

important to the physiology of SOD1: only the disulfide reduced and apo form of the polypeptide can be imported into the IMS of the mitochondria [21]. The presence of SOD1 in this compartment has received considerable attention as a potential site for neuronal damage by mutant forms of SOD1 that give rise to ALS [23,69–73] (Fig. 2).

Recent in vitro studies have shown that reduction of the conserved disulfide bond in human SOD1 lowers the barrier to oligomerization and predisposes the protein to formation of incorrect disulfide cross-links [58] and higher order aggregation in the presence of oxidants [74]. Reduction of the disulfide also contributes to significant global destabilization of protein structure [74–76]. One or more of these characteristics is significantly exacerbated in most ALS-related SOD1 mutations mentioned above.

Several recent studies have shown that the disulfide is essential for SOD activity and provide initial insights into how the copper loaded form of CCS might catalyze the oxidation of SOD to its disulfide form [45,58,68,75]. Overall, our mechanism for the maturation of SOD1 involves four sequential steps as shown in Fig. 1. The mechanism places the CCS/SOD1 complex observed in the cocrystal structure of a mutant SOD1 bound to CCS [77] on the physiological pathway for SOD activation [58].

The disulfide may play several structural and functional roles, but one key attribute is that changes in the disulfide status alters the oligomerization of SOD1. The reduced protein favors the monomeric state over the dimeric state at equilibrium. In fact, when no metals are bound to the disulfide reduced state human SOD1, no dimeric form is observed. Recent in vivo studies have shown that the copper chaperone CCS controls formation of this disulfide in an O_2 responsive step [68]. The oxidizing equivalents are employed in the disulfide formation, first leading to a heterodimeric disulfide linking Domain III of CCS with Cys 146 of SOD1. In a subsequent step, the intermolecular disulfide undergoes an exchange reaction in which SOD1 Cys 56 replaces the Cys of CCS-Domain III to form the Cys56–146 SOD1 intramolecular disulfide characteristic of the native enzyme. At this point, it is not known whether copper transfer into the active site precedes or is concurrent with disulfide formation.

This oxygen-responsive disulfide process is catalyzed by the copper form of CCS and appears to be part of a physiological cycle that regulates the amount of active, copper loaded SOD1protein [58]. It also modulates the half-life of the apo reduced protein, which is thermally unstable relative to the more mature states [74]. While the immature form of the WT SOD1 is folded at physiological temperatures and shows little propensity for aggregation, many of mutant forms that are known to cause ALS are destabilized, and several are completely unfolded at 25 °C. Not all ALS mutations lead to thermal destabilization [78]; however, all of the mutant proteins studied to date show a great propensity to aggregate. When the disulfide-reduced forms aggregate, the oligomeric forms that result are exceedingly sensitive to adventitious oxidation by even mild oxidants, such as oxidized glutathione [74]. Interestingly mild oxidation of the ALS mutant forms of disulfide-reduced SOD1 (but not WT) lead to insoluble species which when solubilized with SDS and loaded onto a nonreducing gel appear as a ladder of higher order disulfide crosslinked SOD1 species in vitro [74]. Recently these same phenomena have been observed in the spinal cord of transgenic mouse models for ALS [45,79,80]. Ladders of oxidized SOD1 from monomers to hexamers are clearly

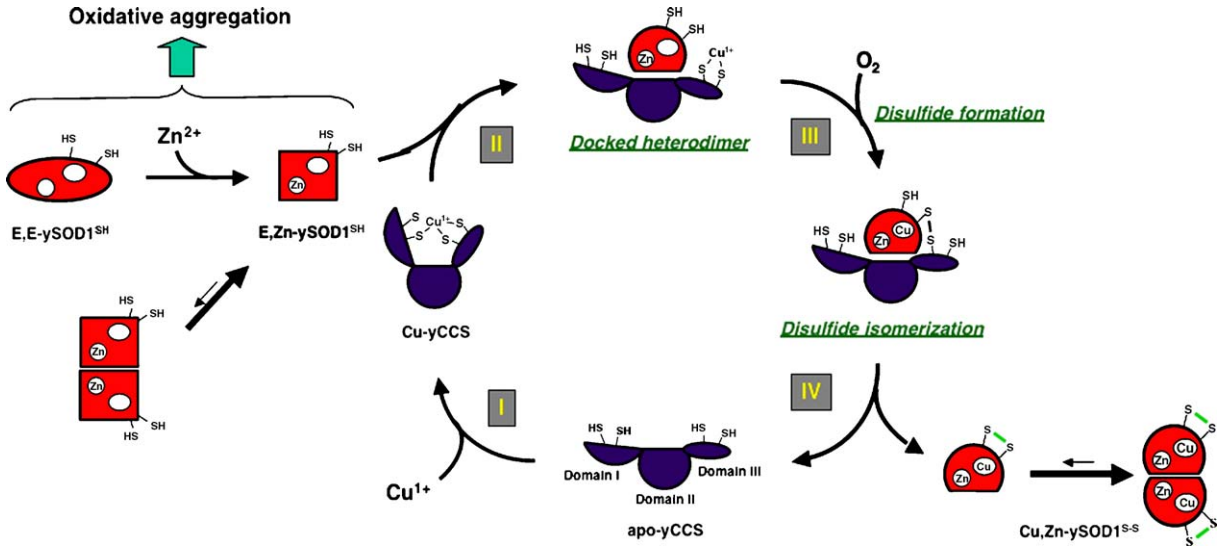

Fig. 1. Proposed mechanism of copper insertion into SOD1 by its metallochaperone, CCS. The copper chaperone acquires copper through unknown routes and then docks with a disulfide reduced form of SOD1 (steps I and II). This complex is inert to further reaction unless exposed to oxygen or superoxide (step III), at which point a disulfide-linked heterodimeric intermediate forms. An analogous complex containing mutant SOD1 has been trapped and structurally characterized. In the case of the WT protein, this complex undergoes disulfide isomerization to an intramolecular disulfide in SOD1 (step IV). Copper is transferred at some point after introduction of oxygen and the mature monomer is proposed to be released from CCS. The left side of the image depicts several immature states of the protein in which the essential disulfide bond has not yet formed. If the conserved Cys are oxidized to form incorrect disulfide linkages, this can lead to SOD crosslinking and aggregation.

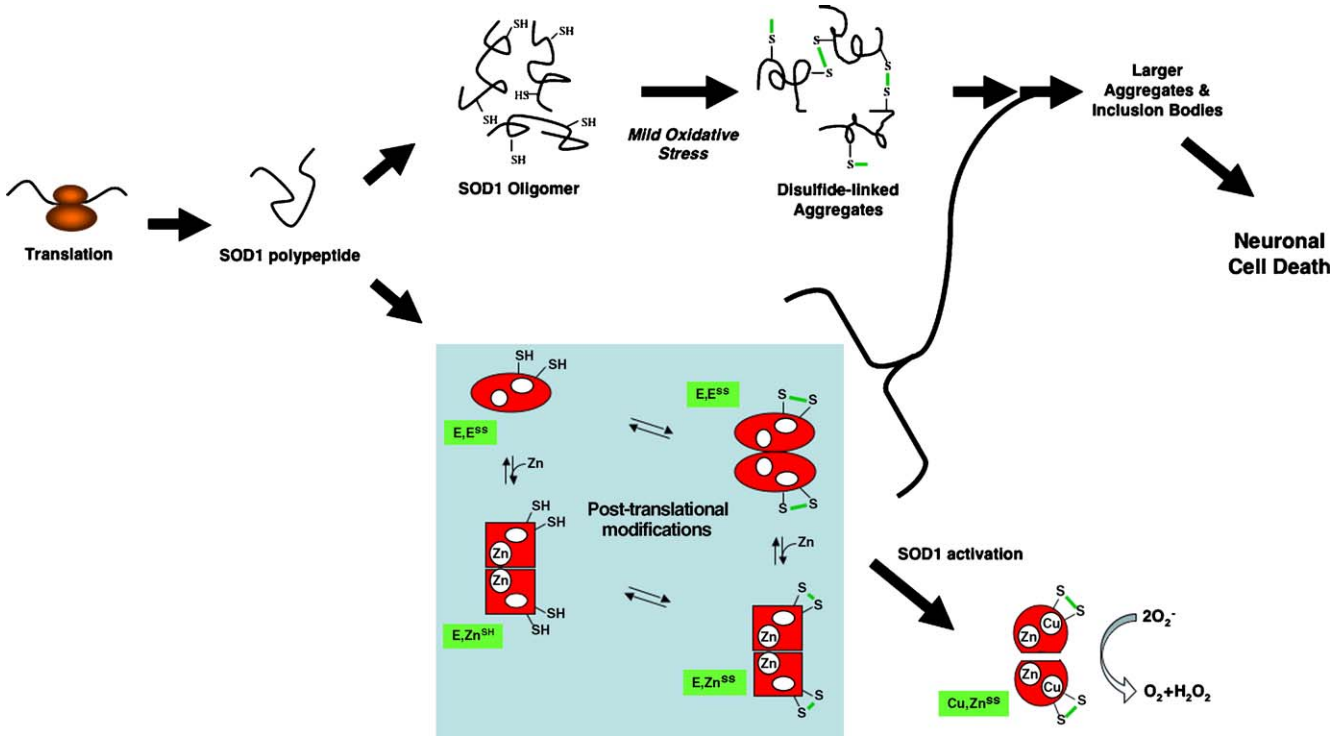

Fig. 2. Oxidative aggregation model for ALS-linked mutations in SOD1: competition between folding and disulfide crosslinking pathways. The lower pathway traces the maturation of the WT SOD1, while the upper pathway depicts reactions of ALS-causing mutant SOD1. The disulfide-reduced apo-SOD1 molecules emerge from the ribosome and undergo folding and modification processes. In the case of the WT protein, the polypeptide folds and once zinc is acquired equilibrates between monomer and dimer forms. Both zinc binding and intra-molecular disulfide formation lend significant additional thermodynamic stability to the dimer (see green box), however the enzyme is not active in the absence of copper. In contrast to the WT protein, the apo and reduced states of many ALS mutant SODs are unfolded or misfolded at physiological temperature. The mutants studied to date are also predisposed to oligomerization when they are in the E,E-hSOD1^{SH} state. In the presence of oxidants, these oligomeric forms of SOD1 are susceptible to formation of disulfide-linked multimers. In this model, these oxidized multimers ultimately nucleate the formation of larger insoluble aggregates that may contain folded or more mature forms of the protein such as those shown in the blue box. Formation of these oxidatively crosslinked aggregates are proposed to cause neuronal cell death. Under normal conditions the cellular machinery can degrade aggregates stabilized by non-covalent interactions. If, however, these oligomers experience even mild oxidative stress, they under go covalent crosslink formation which stabilizes the aggregates and leads to precipitation. Once the rate of insoluble aggregate formation outpaces the rate of degradation by the cellular quality control machinery, the cell or compartment may incur damage. Thus, any stress events that increase the expression of the most immature forms of the mutant SOD1 protein (see first branchpoint above) or otherwise increases its concentration (for instance by reverse of the lower path) may stimulate the rate of formation of robust aggregates. This model for SOD1-linked ALS thus involves two key steps: (a) oligomerization of misfolded reduced forms of the protein, (b) oxidation of the oligomers to form covalently linked insoluble aggregates that initiate cell death. Recent observations provide evidence for oxidative crosslinked aggregates in mitochondria of afflicted spinal cord neurons.

apparent in the insoluble fractions of neuronal extracts for animals expressing several ALS mutant proteins, but no such ladders are observed in mice that overexpress WT SOD1 [69]. These studies support the proposal that the build up of disulfide-reduced SOD1, either by oxidative-stress induced expression of new SOD1 pools or reduction of existing pools of the native protein may lead to intermolecular disulfide adducts that seed nucleation of insoluble aggregates that can cause neuronal toxicity[58]. Other studies of the occurrence of detergent-resistant non-native dimers also reported evidence of a significant amount of the disulfide reduced SOD1 in spinal cord of mouse models and ALS patients [81]. While these observations provide strong support for a role of the SOD1 disulfide in ALS, there are still many mechanistic alternatives. It is becoming apparent that this copper chaperone does far more than deliver copper: it has both sulfhydryl oxidase and protein disulfide isomerase activities that appear to allow for higher order types of physiological regulation in response to oxidative stress.

2.5. CCS independent pathways for copper-loading of SOD1

In addition to CCS, metazoan SOD1 can acquire copper by a CCS-independent pathway. Cu/Zn SOD from humans, mouse and *C. elegans* have been shown to retain activity in cells devoid of CCS, and CCS-independent activity has been observed with both yeast and mammalian expression systems [18,82–85]. Although the copper donor in this case has not been identified, CCS-independent activation of SOD1 can be discerned from CCS by two criteria. First, CCS-independent activation has a strict dependence on reduced glutathione or a low redox potential of the cell [18,85]. By comparison, CCS is reactive over a range of intracellular GSH concentrations. Secondly, the CCS-independent pathway is sensitive to certain structural perturbations in Cu/Zn SOD1 structure. Specifically, prolines at SOD1 positions 142 and 144 (corresponding to *S. cerevisiae* and human SOD1) prohibit CCS-independent activation, but have no effect on CCS [18,85]. *S. cerevisiae*

SOD1 naturally contains these prolines and is totally dependent on CCS for acquiring copper in vivo. Yet the prevalence of such prolines is rare among eukaryotes, and it is predicted that the vast majority of SOD1 molecules can acquire copper by both pathways [85]. It is noteworthy that these prolines occur near the intrasubunit disulfide bond of SOD1 [75]. The Cu/Zn SOD of *C. elegans* is unique in that it appears refractory to CCS and is totally reliant on CCS-independent activation. In accordance with this, there are no known CCS-encoding loci in the genome of *C. elegans* [18]. Why different organisms have evolved to employ different methods of copper acquisition is unclear but may reflect unique lifestyle requirements for copper and oxygen. Such evolutionary adaptations to optimize metal co-factor capture, utilization and localization is probably not restricted to copper and SOD1, but may be expanded to include many aspects of metallophysiology across evolution.

2.6. Extracellular superoxide dismutase (EC-SOD)

A distinct superoxide dismutase activity is observed in the circulatory system of many mammals. This activity arises from a secreted copper and zinc containing enzyme encoded by the human SOD3 gene that is related to the dimeric Cu,Zn SOD family described above. EC-SOD is typically made in vascular smooth muscle cells and secreted into the extracellular environment where it binds to extracellular matrix and endothelial surface components [20]. Its postulated functions include prevention of superoxide-dependent inactivation of endothelial cell enzymes and products including nitric oxide.

The central core of EC-SOD polypeptide is homologous to SOD1 but possesses extensions at the N- and C-termini. Unlike SOD1, it forms stable tetramers with interchain disulfides that stabilize the quaternary structure [86]. The copper-loading pathways for EC-SOD are also quite different from those observed for SOD1. The secreted EC-SOD protein appears to be loaded with copper in CCS-independent intracellular steps that occur within the secretory compartments. The latter compartments utilize the Atox1 copper chaperone pathway [20,87]. Atox1, also known as HAH1, is the human homologue of the first example of a copper chaperone, namely Atx1. This small protein is a diffusable copper binding protein which docks specifically with the cytosolic domain of the Menkes disease protein, an integral membrane P-type ATPase proteins which transport Cu^{+1} across vesicular membranes into secretory compartments in both yeast and mammalian cells [88]. Several reviews of these mechanisms can be found elsewhere [51,64,66,89].

3. Manganese superoxide dismutase

3.1. The well conserved family of manganese and iron containing SODs

The family of MnSOD or FeSOD enzymes first discovered by Fridovich [11,16] has been well conserved throughout evolution. Across various phyla of archae, eubacteria and eukaryotes, the family of Mn/Fe SODs is comprised of dimers or tetramers of ≈21 kDa subunits with considerable sequence homology and well conserved protein folds (reviewed in [10]). Within each subunit, a single manganese or iron atom bound at the active site serves to catalyze the disproportionation of superoxide to oxygen and hydrogen peroxide. Regardless of whether the cofactor is manganese or iron, the metal is buried well within the protein interior and co-ordinated in a trigonal bipyramidal geometry to three histidines, one aspartate and a solvent water molecule.

Based on 261 aligned sequences and 12 X-ray structures, two phenetic trees of Mn/Fe SODs have been constructed, and a clear distinction has been made for dimeric and tetrameric SODs (for details see [10]). Dimeric forms of MnSOD or FeSOD are typical of bacteria, and in many organisms (e.g., E. coli) both metal forms are expressed in the same cell. A limited number of prokaryotes, especially hyperthermophiles, express tetrameric Mn- or FeSOD enzymes [10]. Eukaryotes in general harbor only MnSOD as a tetramer; FeSOD is commonly absent from eukaryotes.

3.2. Promiscuity in metal binding of Fe and MnSODs

Based on the close homology of manganese and iron SODs, one might expect facile metal co-factor substitution. Indeed, Whittaker has shown that the MnSOD of E. coli binds iron with affinities equal to that of manganese [90]. Metal mis-incorporation with bacterial MnSODs has also been observed in vivo. MnSOD of E. coli is expressed as a mixture of manganese and iron bound forms [91], and iron binding to MnSOD increases during anaerobic conditions and when extracellular iron is abundant [92,93]. The tetrameric MnSOD from *Thermophilus* also binds both manganese and iron when expressed in E. coli [92], and recombinant FeSOD from *A. ambivalens* can associate with cobalt, nickel or manganese depending on the extracellular environment of the *E. coli* host [94]. It has been proposed that metal selectivity in the bacterial Fe/Mn SOD molecules is determined by the differential bioavailability of manganese versus iron in the cell [90].

Although bacterial Fe/Mn SOD molecules can bind non-native metals with high affinity, they are very specific with regard to metal-catalyzed reactivity. MnSOD molecules are inactive with iron at the active site, and the same is true for Mn-loaded FeSOD. This would seem surprising based on the virtually identical metal binding sites for Mn and FeSODs [92]. However, slight differences in the outer sphere of the metal site may be critical [92] which in turn affect redox potential of the catalytic site [95,96]. SOD enzymes are efficient at disproportionating superoxide simply because the redox potential of the metal site (+200 to +400 mV) lies between that of oxygen/superoxide (−160 mV) and superoxide/hydrogen peroxide (+890 mV). In MnSOD, the active site environment has been fine-tuned to accommodate manganese, and iron binding lowers the redox potential to a level that cannot complete the disproportionation reaction [95,96].

It is worth mentioning that the metal ion promiscuity of Fe/Mn SODs is highly irregular among metalloproteins. To our knowledge, this represents the only documented case of metal ion misincorporation in a metalloenzyme expressed phys-

iologically in its native host. Mistakes in metal co-factor insertion typically inactivate enzymes and can have a detrimental effect on the cell. In the case of bacterial MnSOD, it is possible that metal ion misincorporation serves in a regulatory mode to down regulate enzyme activity under conditions of low need, e.g., under anaerobic conditions.

While metal ion misincorporation seems wide spread with bacterial MnSOD, the MnSOD of eukaryotic mitochondria (SOD2) appears highly metal specific. Until recently, there have been no reports of iron association with eukaryotic SOD2. SOD2 is the sole SOD of the mitochondrial matrix and loss of mitochondrial SOD2 is known to have severe consequences on organism survival [97–102]. Eukaryotes have therefore evolved with methods for largely preventing iron incorporation into SOD2. Yet as described below, when cellular iron or manganese homeostasis is disrupted, changes in metal ion bioavailability can lead to iron insertion in eukaryotic SOD2 as well. The mechanism by which eukaryotic SOD2 acquires its manganese co-factor and the effects of iron homeostasis on SOD2 activity will be the subject of the remaining discussion.

3.3. Manganese insertion into SOD2 requires mitochondrial import of the polypeptide

In eukaryotes, the manganese form of SOD generally resides in the matrix of the mitochondria. Rare exceptions include the cytosolic MnSODs of the fungi *C. albicans* [14] and crustaceans [13]. The mechanism by which these cytosolic enzymes acquire their manganese co-factor is unknown. In this review, we shall highlight recent advances made in understanding the manganese activation steps for the mitochondrial SOD2.

In all known cases of SOD2, the polypeptide is encoded by a nuclear gene and is imported into the mitochondrial matrix. SOD2 is synthesized as a precursor polypeptide containing, at its N-terminus, a pre-sequence for mitochondrial targeting that is subsequently cleaved in mitochondria. We engineered a mutant form of *S. cerevisiae* SOD2 that lacks this mitochondrial pre-sequence. [The yeast protein is denoted as Sod2p.] The modified Sod2p polypeptide accumulated in the cytosol rather than mitochondria, and was enzymatically inactive. Lack of activity reflected manganese deficiency in the enzyme, because activity was restored upon culturing yeast cells in medium containing high manganese [103]. These studies demonstrated that Sod2p requires a mitochondrial localization to efficiently acquire manganese.

Is manganese inserted into a pre-existing pool of apo SOD2? As described in the accompanying section of this chapter, the Cu/Zn containing SOD1 of eukaryotes can exist as an apo enzyme that is readily activated by copper without new protein synthesis [68,104,105]. However, the same is not true for manganese SOD2. Using *S. cerevisiae* Sod2p as a model, we noted that metal activation requires protein translation and that manganese insertion only occurs with newly synthesized Sod2p molecules that are freshly imported into mitochondria [103]. The ribosomes for SOD2 translation are juxtaposed on the outer membrane of mitochondria [106,107], which allows for tight coupling of translation and mitochondrial import of SOD2 [103]. Our studies are consistent with a model in which the mitochondrial import process facilitates manganese insertion into SOD2 (see Fig. 3).

Why would mitochondrial import be necessary for co-factor insertion? In studies of the highly homologous bacterial MnSOD, metal insertion requires a temperature-dependent switch in SOD structure. At ambient temperatures, MnSOD molecules largely exist as conformers where metal access to the active site is blocked by steric and electrostatic constraints [90,108]. However, a specific switch from this “closed” to an “open” conformation allows manganese entry. Such a switch in conformation can be induced in the test tube by elevations in temperature [90,108,109]. Yet in living cells, other forces for disrupting SOD structure must be at play. What better way to disrupt protein structure than to pass it through a biological membrane? During co-translational import of SOD2 into mitochondria, the polypeptide may remain sufficiently unfolded to facilitate manganese insertion (as in Fig. 3). Alternatively, an accessory factor may contain SOD2 in the unfolded state during import to allow rapid manganese insertion prior to SOD2 folding [103]. Overall, the mitochondrial import process provides an excellent evolutionary advantage to overcoming the physical barriers associated with manganese binding to SOD2.

3.4. Membrane transporters that facilitate manganese insertion into SOD2

Through yeast molecular genetics, we have identified two membrane transporters that are needed for manganese trafficking to SOD2. These include the Smf2p manganese transporter and Mtm1p, a member of the mitochondrial carrier family of transporters.

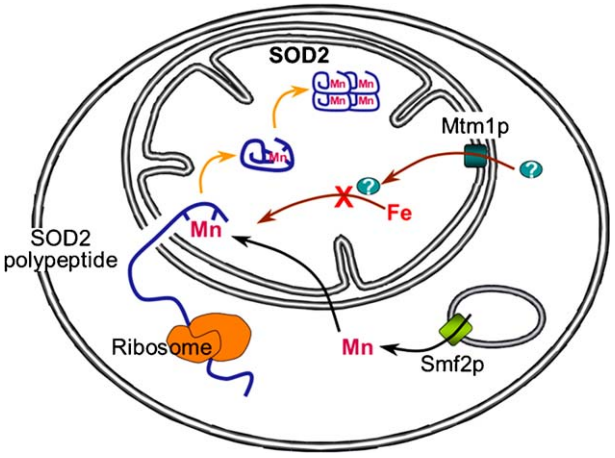

Fig. 3. Role for mitochondrial import in metallation of SOD2. Shown is a model by which the translation, mitochondrial import and metal insertion of SOD2 are coupled. As the polypeptide enters mitochondria, SOD2 remains sufficiently unfolded to allow manganese insertion. Following manganese binding and complete entry into the mitochondrial matrix, the polypeptide can assemble into the quaternary enzyme. The mitochondrial carrier transporter Mtm1p helps prevent iron from interacting with SOD2. The substrate for transport by Mtm1p is unknown. Mitochondrial manganese is derived from intracellular vesicles harboring the Nramp manganese transporter, Smf2p.

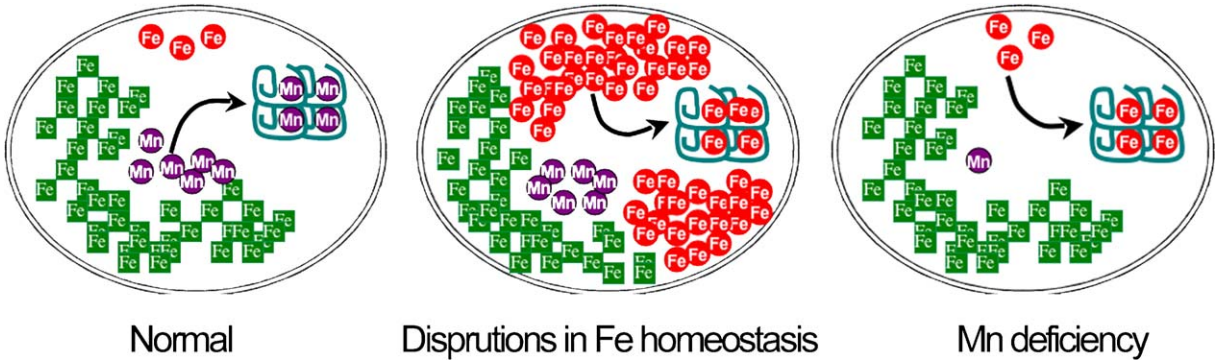

Fig. 4. Metal binding to SOD2 is determined by the differential bioavailability of iron versus manganese. Shown is a model for how changes in mitochondrial iron and manganese homeostasis can change metal specificity in SOD2. Under normal conditions, mitochondrial iron is in vast excess over total mitochondrial manganese (by 1–2 orders of magnitude), but this iron largely exists in a "SOD2-inert" state (green boxes) that is unavailable to SOD2. The small fraction of "SOD2-reactive" iron (red circles) cannot compete well with manganese (violet circles) for binding to SOD2. However, with disruptions in iron homeostasis caused by *mtm1* mutations or by specific defects in Fe–S cluster assembly, SOD2-reactive iron levels substantially rise, and this iron competes well with manganese for binding to SOD2. Under manganese deficiency conditions, the small level of SOD2-reactive iron gains access to the active site of SOD2.

3.4.1. SMF2

Smf2p is one of two Nramp metal transporters that function in manganese uptake and trafficking in *S. cerevisiae*. In *smf2*Δ null yeast mutants, mitochondrial manganese levels are very lo h elevated, but non-toxic concentrations of manganese (e.g., 5 μM $MnCl_2$ added to the growth medium that has no inhibitory effect on yeast cell growth) [110].

The effect of *smf2* mutations on manganese is not unique to mitochondria. The cytosol of *smf2*Δ mutants is also manganese-deficient, and manganese-dependent sugar transferases in the secretory pathway are inactive in *smf2* mutants [110]. This cell-wide deficiency in manganese suggests that Smf2p is important for cell surface uptake of manganese. However, to date there is no evidence that Smf2p resides at the plasma membrane, only at an intracellular vesicular localization of unknown origin [110]. The effect of *smf2*Δ mutations on cell surface uptake of manganese is either indirect or involves a small fraction of plasma membrane Smf2p that escapes detection.

In *smf2* mutants, the Sod2 polypeptide stably accumulates in the mitochondria in a manganese-deficient form. This pool of "apo" Sod2p cannot be activated by manganese, due to the aforementioned requirement for new protein synthesis in manganese insertion [103]. But is this enzyme really apo with respect to metal content or just manganese deficient? In our recent studies, we find that the Sod2p of *smf2* mutants is in fact bound to iron [111]. Most likely, mitochondrial SOD2 never accumulates in the fully apo form, but binds to either manganese or iron depending on which metal is more bioavailable.

3.4.2. MTM1

Mtm1p is a member of the mitochondrial carrier family (MCF) of transporters. MCF transporters (≈30–40 distinct forms) all reside in the mitochondrial inner membrane and function to exchange solutes between the mitochondria and cytosol [112]. We identified *S. cerevisiae MTM1* as a gene which when deleted, resulted in Sod2p inactivation due to manganese deficiency in the enzyme [113].

We surmised that Mtm1p might serve as the *m*anganese *t*ransporter for the *m*itochondria *or* a *m*anganese *t*rafficking factor for *M*nSOD, hence the name Mtm1p. As with *smf2* mutants, Sod2p activity in *mtm1*Δ cells could be restored by supplementing the growth medium with manganese. However, unlike *smf2* mutants, the manganese levels required to restore Sod2p activity in *mtm1* mutants was exceedingly high, approaching cytotoxic quantities [113]. As such, Sod2p inactivation in *mtm1* mutants was not likely to result from a simple deficiency in mitochondrial manganese. In fact, our analysis revealed that mitochondrial manganese was somewhat higher than normal in *mtm1* mutants [113]. Yet this manganese cannot bind to Sod2p.

Mtm1p is clearly not the manganese transporter for the mitochondria and to date, the substrate for transport by Mtm1p remains elusive. In any case, Mtm1p plays a critical role in mitochondrial metal homeostasis and as described below, the Mtm1p effects on bioavailability of manganese versus iron greatly impacts on Sod2p activity.

3.5. Mistakes in metal ion incorporation in eukaryotic SOD2

As mentioned above, bacterial forms of MnSOD readily associate with iron in vivo, however a similar mis-incorporation of iron has not been reported for eukaryotic SOD2. Based on the strong homology between bacterial and eukaryotic MnSOD enzymes, one might expect SOD2 to bind iron with affinities similar to that of manganese. In addition, the levels of total mitochondrial iron exceed manganese by 1 to 2 orders of magnitude [114]. How then does SOD2 manage to avoid interactions with iron? In the following, we provide evidence that iron can indeed associate with SOD2, particularly when mitochondrial iron homeostasis is disrupted.

The first clue of iron association with SOD2 was obtained during analysis of *mtm1* yeast mutants defective in Sod2p activity. These mutants were seen to hyperaccumulate iron in both the mitochondria and cytosol [113]. Such iron accumulation without exposure to high environmental iron is reminiscent

of several *isc*-like mutants of *S. cerevisiae*, or mutants defective in Fe–S cluster biosynthesis (reviewed in this issue by R. Lill). Although we have no evidence to date that Mtm1p functions in Fe–S cluster synthesis [111], Mtm1p nevertheless is playing a critical role in mitochondrial iron homeostasis.

The elevation in mitochondrial iron of *mtm1* mutants ranges from 2 to 5 fold depending on the yeast strain or growth conditions, but regardless of this variation in iron levels, the mitochondrial iron of *mtm1* mutants is toxic to Sod2p. Sod2p activity of *mtm1* mutants is rescued by depleting mitochondrial iron through either iron chelator treatment or by mutating the Aft1p transcription factor [115] for iron uptake in yeast. Moreover, mitochondrial fractionation and ICP analysis demonstrates that in *mtm1* mutants, iron binds to Sod2p, not manganese [111]. As with bacterial MnSOD, iron binding to eukaryotic SOD2 irreversibly inactivates the SOD enzyme.

Iron inactivation of mitochondrial Sod2p is not unique to *mtm1* mutants and was also seen with certain *isc* mutants defective in mitochondrial Fe–S cluster synthesis. By comparison, treatment of wild type yeast with high environmental iron did not inactivate Sod2p in spite of elevated iron levels [111]. Overall, these studies have led us to conclude that mitochondrial iron exists in at least two states. Normally the bulk of mitochondrial iron in wild type cells exists in a "SOD2-inert" form that is inaccessible to SOD2. Mitochondrial manganese is more bioavailable and as a result, SOD2 is assembled in the manganese-bound, enzymatically active state. However, certain disruptions in mitochondrial iron homeostasis can lead to a change in iron bioavailability and a "SOD2-reactive" form of iron accumulates (Fig. 4). This iron pool is so accessible to SOD2 that it takes extremely high elevations in mitochondrial manganese to out-compete iron for binding to SOD2 [111]. It is noteworthy that a small pool of "SOD2-reactive" iron also exists under normal iron homeostasis conditions. In wild type yeast cells, a minor fraction of iron can be seen associating with Sod2p. And as described above, iron readily associates with yeast Sod2p when mitochondrial manganese is low, as in *smf2* mutants [111] (Fig. 4). Currently the precise nature of SOD2-reactive versus SOD2-inert iron is unknown but may reflect changes in oxidation state or ligand complexes of the metal. It is unlikely that free ionic manganese and iron serve as the source of metal for SOD2, but rather the metals exist as available organic or inorganic complexes.

4. Outlook

The copper and zinc dependent SODs play important, but not necessarily essential, roles in protecting components of the bacterial cell envelope or one of the many compartments of eukaryotic cells. In addition, there is accumulating evidence that copper dependent SODs play roles in signal transduction pathways. For instance, the EC-SOD enzyme can modulate the half-life of endothelial-derived nitric oxide in regions of the vasculature where superoxide levels would otherwise inactivate this important signal molecule by forming peroxynitrite [116]. The products of superoxide dismutation, i.e. hydrogen peroxide and oxygen, may also play direct signaling roles in the intracellular milieu as well [117]. With these more complex SOD functions in mind, it is not surprising that numerous posttranslational mechanisms for regulating SOD activity in response to physiological signals are beginning to emerge [68]. Intriguingly, metal insertion, proteolytic processing and disulfide formation are important posttranslational modifications that alter SOD1 and/or EC-SOD activity. The factors such as CCS that are involved in SOD1 modifications may be employed in different physiological contexts, depending upon the degree of oxidant stress, nutritional copper availability [118] or the local signaling requirements of specialized cells. It will be interesting to see how these and other metal-specific posttranslational modification pathways play a part in metabolic, neurodegenerative and vascular diseases.

In contrast to the copper-requiring SODs, the manganese containing SOD2 of the mitochondria plays an *essential* role in oxidative stress protection. Complete loss of the enzyme results in neonatal lethality in mice [102] and is also critical for growth and viability of other eukaryotic organisms [98,99]. Even haploinsufficiency of SOD2 can be detrimental [97,119]. As such, the precise assembly of SOD2 into a mature tetrameric, manganese-containing enzyme is critical for aerobic survival. Mammalian candidate homologs to Smf2p [120] and Mtm1p [113] have been identified, and these may play an important role in ensuring proper activation of mammalian SOD2 as well. While many key findings have emerged from studies in *S. cerevisiae*, the picture is far from complete. We still do not understand how manganese enters the mitochondria or how mitochondrial iron is largely maintained in a bio-unavailable state with regard to SOD2. The substrate for transport by Mtm1p is still elusive, and current studies are aimed at understanding how disruptions in mitochondrial iron homeostasis by *mtm1* mutations increases iron bioavailability to Sod2p. These studies on the promiscuous metal binding by SOD2 can have important implications with regard to human disorders of oxidative stress and iron overload. A number of iron overload diseases have been associated with severe oxidative damage [121,122]. It is generally believed that high iron causes oxidative stress mainly through iron catalyzed Fenton chemistry. However, our recent analyses of SOD2 suggest a new component to iron toxicity: inactivation of an important mitochondrial anti-oxidant enzyme. Future studies of iron overload disorders should consider effects on SOD2 as a potential etiologic agent.

Finally, the studies on the various metal containing SODs raise questions about the evolution of SOD catalysts. It is quite possible that environmental metal ion availability and cell physiology have played important roles in determining the nature of the specific metal ion and SOD-type used in scavenging toxic superoxide anion.

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