Published in final edited form as:

Biochim Biophys Acta. 2010 February ; 1804(2): 298. doi:10.1016/j.bbapap.2009.08.014.

Metal Uptake by Manganese Superoxide Dismutase

James W. Whittaker^{a,*}

^a Department of Science and Engineering School of Medicine Oregon Health and Science University
20000 N.W. Walker Road Beaverton, OR 97006-8921

Abstract

Manganese superoxide dismutase is an important antioxidant defense metalloenzyme that protects cells from damage by the toxic oxygen metabolite, superoxide free radical, formed as an unavoidable by-product of aerobic metabolism. Many years of research have gone into understanding how the metal cofactor interacts with small molecules in its catalytic role. In contrast, very little is presently known about how the protein acquires its metal cofactor, an important step in the maturation of the protein and one that is absolutely required for its biological function. Recent work is beginning to provide insight into the mechanisms of metal delivery to manganese superoxide dismutase *in vivo* and *in vitro*.

Keywords

apoprotein; metal binding; metallation; activation; metalloenzyme; metalloprotein;
metallochaperone; free radical; antioxidant

1. Introduction

Metalloproteins are abundant in biology [1] and may actually comprise the majority of the proteome of living cells [2,3], performing a wide range of essential functions including DNA biosynthesis [4-6], core energy metabolism [7-9], transcriptional regulation [10,11], stress response [12] and detoxification [13]. Yet, in spite of their importance, virtually nothing is known about how most metalloproteins and metalloenzymes acquire their metal cofactor, a critical step in their maturation and activation. For many of these proteins, investigation of the metal binding mechanism is just beginning. This review surveys recent progress towards understanding the mechanism of metal binding for the essential antioxidant metalloenzyme, manganese superoxide dismutase (MnSOD) [14-17], that protects cells against the toxic effects of superoxide free radical.

1.1 Superoxide biochemistry

Oxygen-based respiration has evolved as an extraordinarily efficient mode of energy metabolism, but the efficiency comes at a price, as oxygen's aggressive reactivity results in the formation of a variety of reactive oxygen species (ROS) within the cell [14,18]. These toxic

© 2009 Elsevier B.V. All rights reserved.

* Corresponding author. Department of Science and Engineering, School of Medicine, Oregon Health and Science University, 20000 N.W. Walker Road, Beaverton, OR 97006-8921 USA. Tel.: +1 503-748-1065; fax: +1 503-748-1464. jim@ebs.ogi.edu (J.W. Whittaker).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

by-products of cellular metabolism, including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO\bullet$), are formed by one-, two- and three-electron reduction of O_2 , respectively. The cellular reactions of superoxide include destruction of Fe-S clusters in metalloproteins and mutagenesis (Fig. 1). In more general terms, ROS are capable of breaking into the bonding of proteins, lipids and nucleic acids, altering their biological activity and creating genetic mutations.

Reduced organic cofactors (e.g., $FADH_2$, $NADH$, hydroquinones), metal ions, respiratory chain components, and redox-active metabolites (e.g., thiols) are all susceptible to spontaneous auto-oxidation by dioxygen, forming superoxide. The main sources of superoxide in living cells are thought to be components of the electron transport chain (especially Complex I and Complex III) and the TCA cycle [21-24]. As a free radical, superoxide can initiate radical chain processes in the cell, react with nitric oxide to form peroxynitrite, and add to unsaturated lipids to form peroxides. Iron-sulfur clusters are especially vulnerable to attack by ROS, which destroys the cluster and releases free Fe^{2+} , propagating the damage [25,26]. Because of this special sensitivity of Fe-S clusters, disruption of metabolic pathways that depend on Fe-S metalloenzymes is a major consequence of oxidative stress. The cumulative effects of ROS and oxidative stress are genetic lesions resulting from DNA damage and a wide range of metabolic problems, the basis for “oxidative aging” theories [27-29].

1.2 Superoxide dismutases

The superoxide (hydroperoxyl) radical formed by one-electron reduction of dioxygen is a weak acid ($pK_a = 4.88$) that ionizes to its conjugate base at physiological pH, forming a radical anion [30]. While spontaneous dismutation of hydroperoxyl free radical (protonated superoxide) is fast (diffusion controlled) at low pH, the bimolecular reaction is very slow at physiological pH as a result of the electrostatic repulsion of ionized superoxide radical anions:

In cells this dismutation reaction is instead catalyzed by a family of enzymes known collectively as superoxide dismutases (SODs) [31-42]. SODs serve as the front-line defense against ROS in living cells, catalyzing the redox disproportionation of superoxide into hydrogen peroxide and molecular oxygen.

All known SODs are metalloenzymes containing a redox-active metal cofactor (Cu, Ni, Fe, or Mn) in their active site. During turnover, the active site metal ion undergoes cyclic oxidation and reduction, storing an electron and avoiding the electrostatic repulsion barrier that prevents direct reaction of the two superoxide anions:

The importance of this reaction for aerobic life may be gauged by the observation that superoxide dismutases have evolved independently at least three times during evolution, reflected in three distinct families of enzymes sharing a common catalytic function but based on divergent structures. Cu,Zn-SOD is based on an immunoglobulin (beta-sandwich) fold, and binds a pair of metal ions (Cu and Zn) in a binuclear complex bridged by a bidentate histidine imidazolate side chain [33]. NiSOD, which mainly occurs in actinobacteria (including

Streptomyces sp.) and cyanobacteria, is based on an up-and-down four-helix bundle architecture and binds a single Ni ion in each subunit [35]. Mn and Fe SODs share a common structure, with an all-alpha-helical N-terminal domain and an alpha/beta C-terminal domain, binding the catalytic metal center in the interior of the protein [37,39,42]. In many cases, organisms contain multiple forms of SOD, with different patterns of expression and distinct sites of subcellular localization. For example, *Escherichia coli* contains a constitutive FeSOD and a MnSOD induced by oxidative stress, as well as a periplasmic Cu,Zn-SOD [12,32]. Eukaryotic (yeast, human) cells typically contain a cytoplasmic Cu,Zn-SOD, and a MnSOD that is localized in the mitochondrial matrix [40,41].

The essential role played by SOD in living organisms is also reflected in the severe consequences of deletion of the SOD gene. *E. coli* Δ sodA mutants lacking MnSOD exhibit a growth defect under aerobic conditions [20,43]. This phenotype is exacerbated in SOD-null strains produced by deletion or disruption of both *sodA* (encoding MnSOD) and *sodB* (encoding the FeSOD), which exhibit enhanced susceptibility to spontaneous and induced mutagenesis. Eukaryotic cells (including yeast) are particularly sensitive to loss of MnSOD [44]. Δ sod2 knock-out strains lacking mitochondrial MnSOD exhibit impaired respiratory metabolism and decreased viability [45-48]. Conversely, over-expression of MnSOD in eukaryotes leads to an increased lifespan (at least for fruit flies and mice) [49,50].

Acquisition of the metal cofactor is clearly an essential step in the maturation of SOD metalloenzymes, required for expression of their catalytic function. While many aspects of the catalytic mechanisms of these enzymes are well-established, the mechanisms involved in delivery of the metal cofactors are still poorly understood.

2. Pathways for metal delivery in living cells

Cells have evolved complex mechanisms for regulating the availability of metal ions that are required for life, trafficking them across cell membranes and among subcellular compartments, and delivering them to target sites within the cell. Delivery of the correct metal ion to its cognate acceptor site is clearly essential for biological function, and two basic paradigms for metal delivery to protein binding sites have emerged from recent studies [51-53] (Fig. 2): (1) chaperone-dependent metal binding (generally restricted to toxic metal ions, e.g. Cu, Ni, and Zn [54]; or complex clusters, including Fe-S clusters and Mo-cofactors [55]); and (2) chaperone-independent metal binding. These metal homeostasis mechanisms involve a large number of components, and the details of the trafficking pathways have been elucidated in only a few cases. The basic principles are illustrated by the established pathways for copper delivery to Cu,Zn-SOD (Fig. 3A) [56-63].

2.1 Chaperone-dependent metal binding

Extracellular copper is imported by metal-specific uptake pumps in the cell membrane that concentrate the metal ion from the environment. Because copper is a heavy metal, it is toxic to cells and intracellular trafficking of the metal ion involves a family of metallochaperones (including the chaperone for Cu,Zn-SOD, CCS [56,60,61]), which sequester the toxic metal ion. Metallochaperones [52,53,56] are proteins that sequester and traffick toxic metal ions in the cell interior, preventing deleterious oxidation-reduction and complexation side reactions. The chaperone complex may buffer the free metal ion to very low (zeptomolar, 10^{-21} M) [64] levels for on-demand delivery to specific target proteins to complete their maturation, although recent studies suggest that the non-proteinaceous pool of “free” copper inside cells may actually be higher than originally estimated [65,66]. The best-characterized examples of metallochaperones are found in copper biochemistry [56-58], where at least three distinct chaperone families have been discovered, involved in Fe uptake [59], activation of Cu,Zn-SOD [60,61] and maturation of cytochrome *c* oxidase [62]. The yeast Cu chaperone for Cu,Zn-SOD

(CCS), identified by genetic screening methods, is needed for efficient maturation of Cu,Zn-SOD *in vivo* and is believed to form a heterodimer with the precursor SOD apo-protein in the process of metal insertion [63], as illustrated in Fig. 2B. This example illustrates some of the key features of chaperone-dependent metal binding: a requirement for protein-protein interactions that can be detected in genetic screens, and an association with toxic heavy metals. The tendency of heavy metal ions to form toxic complexes places special biochemical constraints on these species, including strict buffering and chaperone escorts within the cell. Zinc, like copper, is a heavy metal, and the intracellular concentration of Zn is buffered by forming complexes with metallothionein [67], which may also function as a metallochaperone, delivering Zn to some target proteins [68].

Are there chaperone systems for all essential metal ions? Probably not. Many metal ions appear to be present as free aquo ions as weak complexes with metabolites (including amino acids and anions) that mobilize the ions inside the cell. The ability of many divalent metal ions (Mg^{2+} , Mn^{2+}) to functionally substitute for each other *in vivo* appears to provide evidence for the presence of pools of “free” metal ions in the cell.

2.2 Chaperone-independent metal binding

While chaperone-dependent pathways have been established for copper delivery, other metal ions follow distinct pathways within the cell. These processes all involve metal transporters in cell membranes that recognize specific metal ions (including Cu, Zn, Ni, Mn, and Fe) and deliver them to the cytosol (Fig. 3A&B). From there, intracellular trafficking depends to a large extent on the toxicity of the free metal ion. Some metal ions (including alkali and alkali earth cations: Na, K, Mg, Ca) which are nonredox-active and therefore relatively innocuous *in vivo*, are simply accumulated as free ions in the cell. The metal ions are then available for direct, chaperone-independent binding to apo-metalloproteins.

Chaperone-independent metal binding (Fig. 2A) in fact appears to be the more general scheme for metal delivery. In this case, the free metal ion is available inside the cell at levels comparable to the dissociation constant for the protein complex. For example, Ca^{2+} and Mg^{2+} ions present at μM - mM concentrations in the cell bind rapidly and reversibly to their cognate proteins, controlled mainly by the availability of the metal ion [69,70]. Other proteins also appear to follow this plan, and Fe binding by the transport protein transferrin has been especially well-studied *in vitro* [71-75]. The metal binding site of transferrin lies on a buried protein domain interface, with metal binding and release being conformationally gated as the domains open, Pac-Man-style [74]. The conformational transition in transferrin is sensitive to pH: at low pH (characteristic of the vacuolar lumen, where the Fe is released following cellular uptake of serum transferrin) a di-lysine motif on the surface of the protein becomes protonated, triggering domain opening and mobilizing the metal ion [75]. However, both chaperone-dependent and – independent pathways may operate for a given metal ion. In eukaryotic cells, a protein has been identified that appears to function as a metallochaperone delivering Fe to the storage protein ferritin [76].

The alternative pathways for metal delivery can be distinguished by several characteristics, including metal selectivity and the dependence on protein-protein interactions. Unlike chaperone-dependent metal delivery systems, there is typically no evidence from genetic suppression screening and mutagenesis for involvement of other gene products in the chaperone-independent process. Thus, the *in vitro* reaction is fundamentally the same as that which occurs *in vivo*.

3. Metal binding by Manganese Superoxide dismutase

At present very little is known about how MnSOD acquires its essential cofactor inside the cell. Studies on *E. coli* MnSOD show that metal binding *in vivo* exhibits only a modest selectivity, and Mn, Fe, and mixed (Mn,Fe) forms of the protein are all present [77-79]. In spite of this relatively promiscuous metal binding behavior, only the Mn form is catalytically active, reflecting a strict catalytic specificity for the metal ion. This stark contrast between low selectivity in metal binding but strict specificity for catalysis is an ironic paradox of MnSOD function [17].

In principle, the metal may bind at any stage of protein biosynthesis. As the nascent polypeptide emerges from the ribosome and begins to acquire secondary structure, elements of the metal binding motif may form weak complexes with metal ions (Fig. 4A). Alternatively, metal binding may only occur after the protein has fully folded and the metal binding site is completely formed, but before elements of quaternary structure have formed through subunit assembly (Fig. 4B). Finally, metal may bind to the mature, fully folded, dimeric product (Fig. 4C).

In vivo metal activation of yeast mitochondrial MnSOD has been most extensively studied [80-83]. Eukaryotic mitochondrial MnSODs are nuclear-encoded and synthesized on ribosomes in the cytoplasm, fused with an N-terminal mitochondrial-targeting signal peptide [40,41]. Metal binding occurs in the mitochondrial matrix after the protein is imported via translocation machines [84] in the mitochondrial outer (TOM) and inner (TIM) membranes (Fig. 3A). Translocation is coupled to cleavage of the signal peptide to produce the mature polypeptide in the matrix space. Mtm1p, a protein of the mitochondrial carrier family, has been described as a potential chaperone for delivery of Mn to the SOD apoprotein, since its deletion prevents MnSOD activation in the mitochondria [83]. However, it appears that this defect may be an indirect result of disruption of mitochondrial Fe homeostasis leading to misincorporation of Fe for Mn in MnSOD [85,86]. Mtm1 deletion thus creates a model for diseases of Fe homeostasis, including Friedreich's ataxia, that result in mitochondrial Fe overload. Inactivation of MnSOD by incorporation of the "wrong" metal ion in these disorders elevates oxidative stress, illustrating the close relation between Fe and Mn metabolism in the cell and the importance of SOD metal binding processes in both health and disease.

3.1 Thermally triggered metal binding

In work on thermophilic and hyperthermophilic SODs, recombinant enzymes from *Thermus thermophilus* ($T_{\text{opt}} = 65^{\circ}\text{C}$) and *Pyrobaculum aerophilum* ($T_{\text{opt}} = 100^{\circ}\text{C}$) were unexpectedly found to be expressed as metal-free apoprotein, lacking any metal cofactor, when produced in a mesophilic host (*E. coli*) [87,88]. This apo-MnSOD did not bind Mn when incubated at room temperature, but could be activated by insertion of the metal ion when heated to higher temperatures, approaching the physiological growth temperature of the source organism. Mn binding was detected by end-point activity assay after heating for a fixed time in the presence of Mn salts. While the reduced metal ion (Mn^{2+}) is thermodynamically more stable in aqueous solution and is also kinetically more labile than Mn^{3+} , the protein-bound metal ion spontaneously oxidizes in the presence of air *via* the reverse of Equation 1. The "thermally triggered" metal uptake exhibited by these thermophilic proteins demonstrates that a degree of thermal excitation of the protein is required for the metal to bind, indicating that dynamical features of the protein structure are involved in the process.

The mesophilic *E. coli* MnSOD exhibits similar metal uptake behavior [89]. For many years, the *E. coli* MnSOD apoprotein, prepared by chemical denaturation, metal extraction and renaturation, was assumed to have an adventitious metal ion bound in its active site, since attempts to activate it by incubation with Mn^{2+} were unsuccessful unless the metal ion was

present during the denaturation/renaturation process [77,90]. A reexamination of the Mn activation of *E. coli* apo-MnSOD has shown that, like its thermophilic homologs, the *E. coli* protein requires thermal excitation to allow the metal to bind, although the triggering temperature is much lower for the mesophilic protein [89].

3.2 Calorimetric studies of metal binding equilibria

Calorimetric methods have been used to quantitatively define the thermodynamic equilibria associated with protein stability and metal binding affinity in MnSOD complexes [89]. Differential scanning calorimetry (DSC) [91,92], yields thermochemical parameters (unfolding midpoint temperature, T_m , and enthalpy, ΔH) that can be interpreted in terms of the free energy of unfolding (Fig. 5). Comparing results for apo-MnSOD and its Mn^{2+} and Mn^{3+} complexes gives indirect estimates of the stability of the metal complexes, based on the fact that metal binding affinity is reflected in the global stabilization of the folded protein (a simple thermodynamic box argument). The analysis makes use of Brandt's theory [93-95] for coupled equilibria to evaluate the metal binding constants (Eqn. 4):

$$K_{Mn^{2+}}(T_{Mn}) = \frac{\exp \left[\frac{-\Delta H_{Apo}}{R} \left(\frac{1}{T_{Mn}} - \frac{1}{T_{Apo}} \right) + \frac{\Delta C_p}{R} \left(\ln \frac{T_{Mn}}{T_{Apo}} + \frac{T_{Apo}}{T_{Mn}} - 1 \right) \right]}{[Mn^{2+}]_{T_{Mn}}} \quad (4)$$

Here, $K_{Mn^{2+}}(T_{Mn})$ is the equilibrium binding affinity for formation of the protein- Mn^{2+} complex at the transition temperature for unfolding (Mn^{2+})₂-MnSOD, ΔH_{Apo} is the transition enthalpy for apo-MnSOD, T_{Apo} is the transition temperature for unfolding of apo-MnSOD, T_{Mn} is the transition temperature for unfolding (Mn^{2+})₂-MnSOD, ΔC_p is the heat capacity change for unfolding apo-MnSOD, and $[Mn^{2+}]_{T_{Mn}}$ is the concentration of free metal ion at T_{Mn} . Using this approach, the binding affinities for Mn^{2+} and Mn^{3+} complexes have been estimated: $K_{Mn^{2+}} = 3 \times 10^9 \text{ M}^{-1}$; $K_{Mn^{3+}} = 5 \times 10^{17} \text{ M}^{-1}$ [89].

The metal binding affinities found for these complexes are surprisingly low, given the essentially irreversible character reported for the metal binding reaction. For example, Mn stays bound to MnSOD during extensive dialysis against chelators (e.g., EDTA) that should be able to extract a relatively weakly bound metal ion. This apparent paradox is resolved by recognizing that both binding and release of the metal ion by MnSOD is *kinetically*, rather than *thermodynamically*, controlled, and that the barrier to metal release is related to the stability of some elements of protein structure.

The thermodynamic box picture (Fig. 5B) also gives insight into the catalytic specificity of MnSOD for Mn over Fe. The redox potential of the metal couple is perturbed from the free solution value by the relative stabilities of the protein complexes in the two oxidation states (Eqn. 5):

$$\Delta \Delta G = -RT \ln (K_{red}/K_{ox}) = -nF\Delta E \quad (5)$$

For the Mn-substituted protein, the relatively tight binding of the Mn^{3+} complex relative to the Mn^{2+} complex reflects an 0.7 V lowering of the $E_{Mn^{2+/3+}}$ redox potential to a value that is within the range of SOD activity ($E^\circ = 0.1\text{-}0.3 \text{ V}$, limited by superoxide reduction and oxidation redox couples). In the Fe-substituted protein, the relative affinities for Fe^{2+} and Fe^{3+} complexes results in a similar (0.8 V) lowering of the redox potential, but in that case the reference potential ($E_{Fe^{2+/3+}}$) is smaller, shifting the redox potential for Fe_2 -MnSOD to a value that is too low to support a superoxide dismutation cycle (Eqns. 2&3).

The structural perspective provides a complementary view of the metal specificity of MnSOD (Fig. 6). X-ray crystal structures of Fe-substituted *E. coli* MnSOD and native Mn-containing enzyme show that the metal binding sites are nearly identical in the two complexes [96]. However, the Fe center (Fig. 6, Right) is distorted from the trigonal bipyramidal coordination geometry of the Mn site (Fig. 6, Left) towards a tetragonal complex by formation of a solvent adduct, assigned as exogenous hydroxide (OH). Formation of a ferric hydroxide complex in the active site has been described as “rusting” of the catalytic cofactor following misincorporation of Fe for Mn [17], which physically blocks the substrate access channel and stabilizes the oxidized metal center.

3.3 Continuous fluorimetric metal binding assay

Analysis of the apo-MnSOD thermally triggered metal uptake behavior has been dramatically improved over the original end-point determination used in earlier studies by the development of a continuous, fluorimetric assay for metal binding [97]. This technique is based on the efficient quenching of intrinsic protein tryptophan luminescence by transition metal ions, particularly Co^{2+} (Fig. 7). Non-radiative energy transfer from the excited state of the amino acid chromophores to the ligand-field manifold of the bound metal ion quenches the fluorescence intensity of the metal complex by approximately 60%, a signal for metal binding that can be monitored continuously, in real time during the metal binding reaction.

Using this approach, it has been possible for the first time to investigate the metal uptake kinetics of apo-MnSOD in detail. The metal binding reaction turns out to be biphasic, revealing an unexpected kinetic complexity (Fig. 7, Bottom). By systematically varying conditions, it has been possible to show that the amplitudes of the two phases are temperature-dependent (Fig. 8, Top), tracing out a sigmoidal transition profile. At sufficiently low temperature ($T < 35^\circ\text{C}$ at pH 7) virtually no metal uptake is observed, consistent with earlier *in vitro* reconstitution experiments. These results appear to reflect an intrinsic equilibrium behavior of the protein sample.

The observation of two distinct kinetic phases indicates the presence of at least two species in the sample, interconverting in a dynamic equilibrium. The fast kinetic phase represents a fraction of the protein that is capable of rapidly binding metal ion from solution (the “open” state), while the slow phase reflects relatively slow conversion of the low-temperature stable “closed” state of the protein to the “open” state (Fig. 9). The A_0/A_{TOT} ratio maps out the transition region for the temperature-dependent equilibrium between “closed” and “open” states, similar to a melting curve for unfolding of DNA. The sharpness of the transition reflects the cooperativity of the process, and a Van't Hoff analysis allows the data to be interpreted in terms of thermodynamic parameters.

The pH-dependence of the metal uptake kinetics is as striking as their temperature-dependence (Fig. 8, Bottom). When the metal uptake assay is performed in buffers spanning a range of pH values (6.5 – 8.5) at constant temperature (37°C), the open fraction (A_0/A_{TOT}) increases from < 0.2 to > 0.8 , reflecting extensive conversion to the “open” form of the protein at higher pH. The data can be fit to a modified Henderson-Hasselbalch equation for a pH-dependent equilibrium (Eqn 6), including a Hill coefficient (n) to model titration of interacting sites [98]:

$$(A_0/A_{\text{TOT}}) = \left(10^{n(\text{pH}-\text{pK}_a)}\right) / \left(1 + 10^{n(\text{pH}-\text{pK}_a)}\right) \quad (6)$$

This analysis yields an apparent pK_a of 7.8 for the pH-dependent process, and a Hill coefficient $n = 0.7$. The magnitude of the Hill coefficient suggests that interaction with a second site

titrating over this pH range gives rise to a modest anti-cooperativity for the deprotonation event governing the metal uptake kinetics.

The rate constant associated with the slow phase (k_{slow}) is also pH dependent. The pH sensitivity of the kinetics arises from a hydrogen ion ($[H^+]$) term in the underlying empirical rate law, leading to expressions that allow proton ionization steps to be detected through the pH dependence of the rate constant (Eqns 7&8) [99]:

$$k=c[H^+]^m \quad (7)$$

$$\log k=\log c - m\text{pH} \quad (8)$$

Eqn 8 describes a log-linear pH-rate relation, with a slope ($-m$) whose sign (plus or minus) indicates whether the transition state involves proton loss or gain, respectively, and whose magnitude may be interpreted as the number of protons actively involved in the transition state for the rate process. In the present case, the data exhibits a clear a log-linear rate profile with a slope ($-m = +1.1$) reflecting ionization of a single proton in the closed→open transition associated with the slow phase kinetics (Fig. 8, Bottom, Insert)

These experimental results are consistent with kinetically gated metal binding by apo-MnSOD, where the rate limiting step in the kinetics is a transition in the protein rather than insertion of the metal ion. On the basis of these results, two models for the gating transition have been proposed: (1) separation of N- and C-terminal domains, allowing access to the metal binding site on the domain interface, or (2) dissociation of the subunits, exposing the binding site on the subunit interface.

3.4 Conformationally gated metal uptake

These two models have been tested by systematically introducing covalent disulfide cross-links into the protein structure by mutagenesis [100], restricting motion at specific molecular interfaces [101] and measuring the effects on metal binding using the fluorimetric metal uptake assay [97].

Target sites for mutagenesis were selected by inspecting the structure of *E. coli* MnSOD (PDB ID 1VEW) [39], importing C β coordinates into a spreadsheet program and constructing a distance matrix. Estimates of geometric parameters (e.g., C β distances) that optimal for disulfide bond formation [102] derived from structural studies on protein disulfides were used to identify pairs of residues having C β separation between 3.5 and 5.0 Å. These residues were further evaluated by visual inspection of the structure with the Cys mutation modeled *in silico* (Fig. 10). Three sites on the subunit interface (S126, E170 and Y174) were identified as targets based on this analysis. In each case, the residue was predicted to form a relatively unstrained disulfide bond with the corresponding residue in the opposite subunit. S126C and Y174C disulfides constrain the subunit interface at opposite faces of the protein, while the E170C disulfide secures the dimeric structure at the middle of the interface. Iodosobenzoate [103] was used to convert the thiol-containing proteins isolated from *E. coli* into the corresponding disulfide form, followed by metal extraction and renaturation to yield the disulfide cross-linked apoprotein for metal uptake measurements.

Two additional pairs of Cys variants were also prepared, constraining the domain interface at opposite ends of the molecule. One variant (H17C/N190C) was predicted to prevent domain separation at the N-terminal side of helix-1. The best candidates for covalently constraining

the domain interface at the opposite side of the molecule were a pair of residues (R72, D147) forming a salt-bridge “clasp” feature between N- and C-terminal domains in the WT protein. The C β atoms in the (R72C/D147C) variant are predicted to be too far apart (7.2 Å, corresponding to a minimum predicted S-S distance of 4.7 Å) to permit unstrained disulfide bond formation. However, a bifunctional thiol reagent (dibromobimane, dBm) [104] was used to convert the R72C/D147C dithiol to the bridged product. Each of these disulfide constructs is designed to specifically constrain either the subunit (S126C, E170C, Y174C) or domain (R72C/D147C/dBm, H17C/N190C) interfaces. Surprisingly all of these disulfide variants were able to bind Co²⁺. Thus, neither subunit dissociation nor domain separation appears to be required for metal uptake to occur.

Other experiments have provided evidence identifying the structural elements responsible for the “conformational gating” behavior. The pH profile for the metal uptake reaction (Fig. 8, Bottom) implies that the gating process involves ionization of a single proton associated with a group having an apparent pK_a of 7.8, consistent with a Glu-His salt bridge [100]. Repeating the pH profile for a series of active site mutational variants showed that all were perturbed in terms of temperature dependence and that all except the H171A variant retained the pH sensitivity of the WT protein. Similarly, the E170C disulfide variant lacked this pH sensitivity, suggesting that the E170-H171 salt bridge that is a conserved feature of the protein structure plays an important role in the metal uptake process. It is interesting to note that the E170-H171 salt bridge plays an important role in the stability of the metallated dimer. Substitution of Ala for Glu (E170A) by directed mutagenesis leads to extensive dissociation of the metal-containing protein [105].

Based on these studies, a detailed model for the conformational gating transition that controls metal uptake in apo-MnSOD has been proposed (Fig. 11). At the lower temperature and pH range, a salt bridge between E170 and H171 stabilizes the “closed” form of the protein. This form closely resembles the metallated holoprotein, except for the absence of metal ion in the binding site. As the temperature and/or pH is raised, the salt bridge is disrupted, allowing E170 to reorient, forming a new salt bridge complex with R181 and Y173 within the same subunit. This rearrangement occurs within a channel that transects the subunit interface and is a conserved feature of SOD protein structure, although previously no function was known for the channel. The alternative conformation of E170 in turn allows H171 to rotate, forming new hydrogen bond with the E170 carboxylate in the “open gate” conformation. Reorientation of both E170 and H171 is predicted to open a pathway into the buried binding site allowing access to metal ions in solution, accounting for the creation of a metal entry channel in the gating transition (Fig. 11).

4. Conclusions

Manganese delivery to apo-MnSOD is an essential step required for the functional maturation of the enzyme and expression of its biological activity. Recent studies on metal activation both *in vitro* and *in vivo* have provided new insight into the basic features of this process, revealing a complex interdependence of metal trafficking pathways and subtle features of protein structure. A detailed mechanism has been proposed for post-translational metal uptake by the dimeric *E. coli* apo-MnSOD, involving activation of a conformational gating transition for metal binding. The structural basis for conformational gating, its role in biological metal uptake mechanisms, and relevance to metal binding by tetrameric apo-MnSOD remain to be investigated.

Acknowledgments

This work has been supported by the National Institutes of Health (GM 042680 to J. W. W.) and the Oregon Health and Science University Medical Research Foundation.

References

1. Bertini, I.; Gray, H.B.; Stiefel, E.I.; Valentine, J.S., editors. *Biological Inorganic Chemistry: Structure and Reactivity*. University Science Press; Sausalito: 2007.
2. Bertini I, Rosato A. From genes to metalloproteins: A bioinformatic approach. *Eur. J. Inorg. Chem* 2007;2007:2546–2555.
3. Degtyarenko, K. Metalloproteins. In: Dunn, M.J.; Jorde, L.B.; Little, P.F.R.; Subramaniam, S., editors. *Encyclopedia of Genetics, Genomics, Proteomics and Bioinformatics, Part 3: Bioinformatics*. John Wiley and Sons; New York: 2005.
4. Eklund H, Uhlin U, Färnegårdh M, Logan DT, Nordlund P. Structure and function of the radical enzyme ribonucleotide reductase. *Prog. Biophys. Mol. Biol* 2001;77:177–268. [PubMed: 11796141]
5. Stubbe J, van der Donk W. *Chem. Rev* 1998;98:2661–2662. [PubMed: 11848974]
6. Frey PA. Radical mechanisms of enzymatic catalysis. *Annu. Rev. Biochem* 2001;70:121–148. [PubMed: 11395404]
7. Schatz G. The magic garden. *Annu. Rev. Biochem* 2007;76:673–678. [PubMed: 17313357]
8. Rees D. Great metallocusters in enzymology. *Annu. Rev. Biochem* 2002;71:221–246. [PubMed: 12045096]
9. Brudvig GW. Water oxidation chemistry of photosystem II. *Philos. Trans. R. Soc. Lond. B Biol. Sci* 2008;363:1211–1218. [PubMed: 17954436]
10. Giedroc DP, Arunkumar AI. Metal Sensor Proteins: Nature's metalloregulated allosteric switches. *Dalton Trans* 2007:3107–3120. [PubMed: 17637984]
11. Hantke K. Iron and metal regulation in bacteria. *Curr. Opin. Microbiol* 2001;4:172–177. [PubMed: 11282473]
12. Touati D. Iron and oxidative stress in bacteria. *Arch. Biochem. Biophys* 2000;373:1–6. [PubMed: 10620317]
13. Cobbett C, Goldsbrough P. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol* 2002;53:159–182. [PubMed: 12221971]
14. Fridovich I. Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem* 1995;64:97–112. [PubMed: 7574505]
15. Robinson BH. The role of manganese superoxide dismutase in health and disease. *J. Inherit. Metab. Dis* 1998;21:598–603. [PubMed: 9728339]
16. Whittaker JW. Manganese superoxide dismutase. *Met. Ions Biol. Syst* 2000;37:587–611. [PubMed: 10693146]
17. Whittaker JW. The irony of manganese superoxide dismutase. *Biochem. Soc. Trans* 2003;31:1318–1321. [PubMed: 14641053]
18. McCord JM. Oxygen-derived free radicals. *New Horiz* 1993;1:70–76. [PubMed: 7922395]
19. Archibald FA. Oxygen toxicity and the health and survival of eukaryote cells: A new piece is added to the puzzle. *Proc. Natl. Acad. Sci. USA* 2002;100:10141–10143. [PubMed: 12939409]
20. Carliz A, Touati D. Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? *EMBO J* 1986;5:623–630. [PubMed: 3011417]
21. Kussmaul L, Hirst J. The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *Proc Natl. Acad. Sci. USA* 2006;103:7607–7612. [PubMed: 16682634]
22. Esterházy D, King MS, Yakovlev G, Hirst J. Production of reactive oxygen species by complex I (NADH:ubiquinone oxidoreductase) from *Escherichia coli* and comparison to the enzyme from mitochondria. *Biochemistry* 2008;47:3964–3971. [PubMed: 18307315]
23. Muller FL, Liu Y, Van Remmen H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J. Biol. Chem* 2004;279:49064–49073. [PubMed: 15317809]

24. Messner KR, Imlay JA. Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase. *J. Biol. Chem* 2002;277:42563–42571. [PubMed: 12200425]
25. Gardner PR, Fridovich I. *J. Biol. Chem* 1991;266:19328–19333. [PubMed: 1655783]
26. Keyer K, Imlay JA. Superoxide accelerates DNA damage by elevating free-iron levels. *Proc. Natl. Acad. Sci. USA* 1996;93:13635–13640. [PubMed: 8942986]
27. McCord JM, Edeas MA. SOD, oxidative stress and human pathologies: a brief history and a future vision. *Biomed. Pharmacother* 2005;59:139–142. [PubMed: 15862706]
28. Raha S, Robinson BH. Mitochondria, oxygen free radicals, disease and ageing. *Trends Biochem. Sci* 2000;25:502–508. [PubMed: 11050436]
29. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. *Free Radic. Biol. Med* 2007;43:477–503. [PubMed: 17640558]
30. Sawyer DT, Valentine JS. How super is superoxide? *Acc. Chem. Res* 1981;14:393–400.
31. McCord JM, Fridovich I. Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem* 1969;244:6049–6055. [PubMed: 5389100]
32. Benov LT, Fridovich I. *Escherichia coli* expresses a copper- and zinc-containing superoxide dismutase. *J. Biol. Chem* 1994;269:25310–25314. [PubMed: 7929223]
33. Tainer JA, Getzoff ED, Richardson JS, Richardson DC. Structure and mechanism of copper, zinc superoxide dismutase. *Nature* 1983;306:284–287. [PubMed: 6316150]
34. Youn HD, Kim EJ, Roe JH, Hah YC, Kang SO. A novel nickel-containing superoxide dismutase from *Streptomyces* spp. *Biochem. J* 1996;318(Pt 3):889–896. [PubMed: 8836134]
35. Wuerges J, Lee JW, Yim YI, Yim HS, Kang SO, Djinojic CK. Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. *Proc Natl Acad Sci U S A* 2004;101:8569–8574. [PubMed: 15173586]
36. Yost FJ Jr, Fridovich I. An iron-containing superoxide dismutase from *Escherichia coli*. *J. Biol. Chem* 1973;248:4905–4908. [PubMed: 4352182]
37. Lah MS, Dixon MM, Patridge KA, Stallings WC, Fee JA, Ludwig ML. Structure-function in *Escherichia coli* iron superoxide dismutase: comparisons with the manganese enzyme from *Thermus thermophilus*. *Biochemistry* 1995;34:1646–1660. [PubMed: 7849024]
38. Keele BB Jr, McCord JM, Fridovich I. Superoxide dismutase from *Escherichia coli* B. A new manganese-containing enzyme. *J. Biol. Chem* 1970;245:6176–6181. [PubMed: 4921969]
39. Edwards RA, Baker HM, Whittaker MM, Whittaker JW, Jameson GB, Baker EN. Crystal structure of *Escherichia coli* manganese superoxide dismutase at 2.1 Å resolution. *J. Biol. Inorg. Chem* 1998;3:161–171.
40. Weisiger RA, Fridovich I. Mitochondrial superoxide dismutase. Site of synthesis and intramitochondrial localization. *J. Biol. Chem* 1973;248:4793–4798. [PubMed: 4578091]
41. Autor AP. Biosynthesis of mitochondrial manganese superoxide dismutase *Saccharomyces cerevisiae*: Precursor form of mitochondrial superoxide dismutase made in the cytoplasm. *J. Biol. Chem* 1981;257:2713–2718. [PubMed: 7037771]
42. Borgstahl GE, Parge HE, Hickey MJ, Beyer WF Jr, Hallewell RA, Tainer JA. The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* 1992;71:107–118. [PubMed: 1394426]
43. Touati D. Investigating phenotypes resulting from a lack of superoxide dismutase in bacterial null mutants. *Methods Enzymol* 2002;349:145–154. [PubMed: 11912904]
44. Liu XF, Elashvili I, Gralla EB, Valentine JS, Lapinskas P, Culotta VC. Yeast lacking superoxide dismutase. *J. Biol. Chem* 1991;267:18298–18302. [PubMed: 1526970]
45. Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, Wallace DC, Epstein CJ. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat. Genet* 1995;11:376–381. [PubMed: 7493016]
46. Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC. Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc. Natl. Acad. Sci. USA* 1999;96:846–851. [PubMed: 9927656]

47. Lebovitz RM, Zhang H, Vogel H, Cartwright J Jr, Dionne L, Lu N, Huang S, Matzuk MM. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc. Natl. Acad. Sci. USA* 1996;93:9782–9787. [PubMed: 8790408]
48. Duttaroy A, Paul A, Kundu M, Belton A. A Sod2 null mutation confers severely reduced adult life span in *Drosophila*. *Genetics* 2003;165:2295–2299. [PubMed: 14704205]
49. Mockett RJ, Orr WC, Rahmandar JJ, Benes JJ, Radyuk SN, Klichko VI, Sohal RS. Overexpression of Mn-containing superoxide dismutase in transgenic *Drosophila melanogaster*. *Arch. Biochem. Biophys* 1999;371:260–269. [PubMed: 10545213]
50. Hu D, Cao P, Thiels E, Chu CT, Wu GY, Oury TD, Klann E. Hippocampal long-term potentiation, memory, and longevity in mice that overexpress mitochondrial superoxide dismutase. *Neurobiol. Learn Mem* 2007;87:372–384. [PubMed: 17129739]
51. Kuchar J, Hausinger RP. Biosynthesis of metal sites. *Chem. Rev* 2004;104:509–525. [PubMed: 14871133]
52. Hausinger RP. Mechanisms of metal ion incorporation into metalloproteins. *Biofactors* 1990;2:179–184. [PubMed: 2198877]
53. Rosenzweig AC. Metallochaperones: bind and deliver. *Chem. Biol* 2002;9:673–677. [PubMed: 12079778]
54. Mulrooney SB, Hausinger RP. Nickel uptake and utilization by microorganisms. *FEMS Microbiol. Rev* 2003;27:239–261. [PubMed: 12829270]
55. Krebs C, Agar JN, Smith AD, Frazzon J, Dean DR, Huynh BH, Johnson MK. IscA, an alternate scaffold for Fe-S cluster biosynthesis. *Biochemistry* 2001;40:14069–14080. [PubMed: 11705400]
56. Field LS, Luk E, Culotta VC. Copper chaperones: personal escorts for metal ions. *J. Bioenerg. Biomembr* 2002;34:373–379. [PubMed: 12539964]
57. Huffman DL, O'Halloran TV. Function, structure, and mechanism of intracellular copper trafficking proteins. *Annu. Rev. Biochem* 2001;70:677–701. [PubMed: 11395420]
58. Merchant SS, Sagasti A. Precious metal economy. *Cell Metab* 2006;4:99–101. [PubMed: 16890535]
59. Portnoy ME, Rosenzweig AC, Rae T, Huffman DL, O'Halloran TV, Culotta VC. Structure-function analyses of the ATX1 metallochaperone. *J. Biol. Chem* 1999;274:15041–15045. [PubMed: 10329707]
60. Culotta VC, Yang M, O'Halloran TV. Activation of superoxide dismutases: putting the metal to the pedal. *Biochim. Biophys. Acta* 2006;1763:747–758. [PubMed: 16828895]
61. Culotta VC, Klomp LW, Strain J, Casareno RL, Krems B, Gitlin JD. The copper chaperone for superoxide dismutase. *J. Biol. Chem* 1997;272:23469–2372. [PubMed: 9295278]
62. Cobine PA, Pierrel F, Winge DR. Copper trafficking to the mitochondrion and assembly of copper metalloenzymes. *Biochim. Biophys. Acta* 2006;1763:759–772. [PubMed: 16631971]
63. Lamb AL, Torres AS, O'Halloran TV, Rosenzweig AC. Heterodimeric structure of superoxide dismutase in complex with its metallochaperone. *Nat. Struct. Biol* 2001;8:751–755. [PubMed: 11524675]
64. Changela A, Chen K, Xue Y, Holschen J, Outten CE, O'Halloran TV, Mondragón A. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. *Science* 2003;301:1383–1387. [PubMed: 12958362]
65. Carroll MC, Girouard JB, Ulloa JL, Subramaniam JR, Wong PC, Valentine JS, Culotta VC. Mechanisms for activating Cu- and Zn-containing superoxide dismutase in the absence of the CCS Cu chaperone. *Proc. Natl. Acad. Sci. USA* 2004;101:5964–5969. [PubMed: 15069187]
66. Cobine PA, Ojeda LD, Rigby KM, Winge DR. Yeast contain a nonproteinaceous pool of copper in the mitochondrial matrix. *J. Biol. Chem* 2004;279:14447–14455. [PubMed: 14729672]
67. Chivers PTA. Galvanizing story--Protein stability and zinc homeostasis. *J. Bacteriol* 2007;189:2953–2954. [PubMed: 17307845]
68. Feng W, Cai J, Pierce WM, Franklin RB, Maret W, Benz FW, Kang YJ. Metallothionein transfers zinc to mitochondrial aconitase through a direct interaction in mouse hearts. *Biochem. Biophys. Res. Commun* 2005;332:853–858. [PubMed: 15913554]
69. Gifford JL, Walsh MP, Vogel HJ. Structures and metal-ion-binding properties of the Ca²⁺-binding helix-loop-helix EF-hand motifs. *Biochem. J* 2007;405:199–221. [PubMed: 17590154]

70. Günther T. Concentration, compartmentation and metabolic function of intracellular free Mg^{2+} . *Magnes Res* 2006;19:225–236. [PubMed: 17402290]
71. Abdallah FB, Chahine JM. Transferrins, the mechanism of iron release by ovotransferrin. *Eur. J. Biochem* 1999;263:912–920. [PubMed: 10469158]
72. Baker EN, Baker HM. Molecular structure, binding properties and dynamics of lactoferrin. *Cell Mol. Life Sci* 2005;62:2531–2539. [PubMed: 16261257]
73. Mizutani K, Mikami B, Hirose M. Domain closure mechanism in transferrins: new viewpoints about the hinge structure and motion as deduced from high resolution crystal structures of ovotransferrin N-lobe. *J. Mol. Biol* 2001;309:937–947. [PubMed: 11399070]
74. Gerstein M, Anderson BF, Norris GE, Baker EN, Lesk AM, Chothia C. Domain closure in lactoferrin: two hinges produce a see-saw motion between alternative close-packed interfaces. *J. Mol. Biol* 1993;234:357–372. [PubMed: 8230220]
75. Peterson NA, Arcus VL, Anderson BF, Tweedie JW, Jameson GB, Baker EN. “Dilysine trigger” in transferrins probed by mutagenesis of lactoferrin: crystal structures of the R210G, R210E, and R210L mutants of human lactoferrin. *Biochemistry* 2002;41:14167–14175. [PubMed: 12450380]
76. Shi H, Bencze KZ, Stemmler TL, Philpott CC. A cytosolic iron chaperone that delivers iron to ferritin. *Science* 2008;320:1207–1210. [PubMed: 18511687]
77. Beyer WF Jr, Fridovich I. In vivo competition between iron and manganese for occupancy of the active site region of the manganese superoxide dismutase of *Escherichia coli*. *J. Biol. Chem* 1981;266:303–308. [PubMed: 1985901]
78. Whittaker JW, Whittaker MM. Active site spectral studies on manganese superoxide dismutase. *J. Am. Chem. Soc* 1991;113:5528–5540.
79. Whittaker MM, Whittaker JW. Mutagenesis of a proton linkage pathway in *Escherichia coli* manganese superoxide dismutase. *Biochemistry* 1997;36:8923–8931. [PubMed: 9220980]
80. Culotta VC, Yang M, O'Halloran TV. Activation of superoxide dismutases: putting the metal to the pedal. *Biochim. Biophys. Acta* 2006;1763:747–758. [PubMed: 16828895]
81. Luk E, Yang M, Jensen LT, Bourbonnais Y, Culotta VC. Manganese activation of superoxide dismutase 2 in the mitochondria of *Saccharomyces cerevisiae*. *J. Biol. Chem* 2005;280:22715–22720. [PubMed: 15851472]
82. Luk EE, Culotta VC. Manganese superoxide dismutase in *Saccharomyces cerevisiae* acquires its metal co-factor through a pathway involving the Nramp metal transporter, Smf2p. *J. Biol. Chem* 2001;276:47556–47562. [PubMed: 11602606]
83. Luk E, Carroll M, Baker M, Culotta VC. Manganese activation of superoxide dismutase 2 in *Saccharomyces cerevisiae* requires MTM1, a member of the mitochondrial carrier family. *Proc. Natl. Acad. Sci. USA* 2003;100:10353–10357. [PubMed: 12890866]
84. Neupert W, Herrmann JM. Translocation of proteins into mitochondria. *Annu Rev Biochem* 2007;76:723–749. [PubMed: 17263664]
85. Yang M, Cobine PA, Molik S, Naranuntarat A, Lill R, Winge DR, Culotta VC. The effects of mitochondrial iron homeostasis on cofactor specificity of superoxide dismutase 2. *EMBO J* 2006;25:1775–17783. [PubMed: 16601688]
86. Pierrel F, Cobine PA, Winge DR. Metal ion availability in mitochondria. *Biomaterials* 2007;20:675–682. [PubMed: 17225062]
87. Whittaker MM, Whittaker JW. Thermally triggered metal binding by recombinant *Thermus thermophilus* manganese superoxide dismutase, expressed as the apo-enzyme. *J. Biol. Chem* 1999;274:34751–34757. [PubMed: 10574944]
88. Whittaker MM, Whittaker JW. Recombinant superoxide dismutase from a hyperthermophilic archaeon, *Pyrobaculum aerophilum*. *J. Biol. Inorg. Chem* 2000;5:402–408. [PubMed: 10907751]
89. Mizuno K, Whittaker MM, Bächinger HP, Whittaker JW. Calorimetric studies on the tight binding metal interactions of *Escherichia coli* manganese superoxide dismutase. *J. Biol. Chem* 2004;279:27339–27344. [PubMed: 15082717]
90. Ose DE, Fridovich I. Manganese-containing superoxide dismutase from *Escherichia coli*: Reversible resolution and metal replacements. *J. Biol. Chem* 1979;254:360–364.
91. Freiere E. Differential scanning calorimetry. *Methods Molec. Biol* 1995;40:191–218. [PubMed: 7633523]

92. Barone G, Catanzano F, Del Vecchio P, Giancola C, Graziano G. Differential scanning calorimetry as a tool to study protein-ligand interactions. *Pure Appl. Chem* 1995;67:1867–1872.
93. Brandts JF, Lin LN. Study of strong to ultratight protein interactions using differential scanning calorimetry. *Biochemistry* 1990;29:6927–6940. [PubMed: 2204424]
94. Brandts JF, Hu CQ, Lin LN, Mos MT. A simple model for proteins with interacting domains. Applications to scanning calorimetry data. *Biochemistry* 1989;28:8588–8596. [PubMed: 2690944]
95. Lin LN, Mason AB, Woodworth RC, Brandts JF. Calorimetric studies of the N-terminal half-molecule of transferrin and mutant forms modified near the Fe(3+)-binding site. *Biochem J* 1993;293:517–522. [PubMed: 8343132]
96. Edwards RA, Whittaker MM, Whittaker JW, Jameson GB, Baker EN. Distinct metal environment in Fe-substituted manganese superoxide dismutase provides a structural basis of metal specificity. *J. Am. Chem. Soc* 1998;120:9684–9685.
97. Whittaker MM, Mizuno K, Bächinger HP, Whittaker JW. Kinetic analysis of the metal binding mechanism of *Escherichia coli* manganese superoxide dismutase. *Biophys. J* 2006;90:598–607. [PubMed: 16258041]
98. Markley JL. Observation of histidine residues in proteins by nuclear magnetic resonance spectroscopy. *Accounts Chem. Res* 1975;8:70–80.
99. Loudon GM. Mechanistic interpretation of pH-rate profiles. *J. Chem. Ed* 1991;68:973–984.
100. Whittaker MM, Whittaker JW. Conformationally gated metal uptake by apomanganese superoxide dismutase. *Biochemistry* 2008;47:11625–11636. [PubMed: 18841998]
101. Matsumura M, Matthews BW. Stabilization of functional proteins by introduction of multiple disulfide bonds. *Methods Enzymol* 1991;202:336–356. [PubMed: 1784181]
102. Thornton JM. Disulfide bridges in globular proteins. *J. Mol. Biol* 1981;151:261–287. [PubMed: 7338898]
103. Means, GE.; Feeney, RE. *Chemical Modification of Proteins*. Holden-Day; New York: 1971. p. 157-158.
104. Kim J-S, Raines RT. Dibromobimane as a fluorescent cross-linking reagent. *Anal. Biochem* 1995;225:174–176. [PubMed: 7778775]
105. Whittaker MM, Whittaker JW. A glutamate bridge is essential for dimer stability and metal selectivity in manganese superoxide dismutase. *J. Biol. Chem* 1998;273:22188–22193. [PubMed: 9712831]
106. Kraulis P. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr* 1991;24:946–50.
107. Merrit EA, Murphy MEP. Raster3D version 2.0 A program for photorealistic molecular graphics. *Acta Crystallogr* 1994;D50:869–73.

Fig. 1. Cellular biochemistry of superoxide. Potential sources of superoxide free radicals in living cells (including respiratory chain components, enzymes of the citric acid cycle, and other reduced cofactors, metal ions and metabolites) are shown at the top. Deleterious reactions of reactive oxygen species derived from superoxide (including destruction of Fe-S clusters and DNA mutagenesis) are illustrated below

Fig. 2.

Metal delivery mechanisms *in vivo*. (A) Chaperone-independent metal binding of apo-metalloprotein (Apo-MP) to form holo-metalloprotein (MP) product through direct interaction with the free metal ion. (B) Chaperone-dependent metal binding, mediated by formation of a metallochaperone (MC) complex, permitting targeted delivery of the metal ion to a specific apo-metalloprotein *via* a heterodimeric intermediate.

Fig. 3.

Pathways for SOD metallation. (A) Eukaryotic (yeast) pathways for metal delivery to Cu,Zn-SOD (Sod1) and mitochondrial MnSOD (Sod2) are illustrated. Cu and Zn enter the cell through specific transporters. Metal delivery to the Sod1 apoprotein has been shown to involve metallochaperones (including the copper chaperone for superoxide dismutase, CCS). Mn uptake involves a specific transporter (Smf1), and intracellular Mn is trafficked to the vacuole (V). A specific pump (Smf2) mobilizes Mn reserves for delivery to the mitochondrial matrix, where it binds to the Sod2 apoprotein. (B) Prokaryotic pathways for metal delivery to NiSOD (SodN), MnSOD (SodA) and FeSOD (SodB). Specific transporters in the cell membrane (NikD, MntH, FeoB) mediate uptake of the metal ions into the cytosol for delivery to SOD apo-proteins *via* either chaperone-dependent or chaperone-independent mechanisms.

Fig. 4.

Timeline for metal binding by metalloproteins. In principle, metal binding may occur at any point during biosynthesis of the protein, including (A) co-translational metal binding to the nascent polypeptide chain emerging from the ribosome; (B) post-translational metal binding to the isolated, fully folded subunit prior to assembly of the multimer; or (C) post-translational metal binding to the completely folded and assembled multimer.

Fig. 5. Differential scanning calorimetry analysis of metal binding affinity for manganese superoxide dismutase. (A) DSC endotherms for global unfolding of *Escherichia coli* MnSOD complexes, including (1) apo-MnSOD, (2) reduced Mn(II)₂-MnSOD, and (3) oxidized Mn(III)₂-MnSOD. (B) Color-coded thermodynamic box diagram corresponding to structural equilibria represented in the DSC endotherms (A).

Fig. 6. Manganese and iron complexes of MnSOD. (Left) The active site of native *E. coli* Mn₂-MnSOD contains a trigonal bipyramidal 5-coordinate metal center. (Based on PDB ID 1VEW). (Right) The corresponding view of the Fe-substituted enzyme (Fe₂-MnSOD), showing tetragonal 6-coordination of the metal cofactor resulting from ligation by a second solvent-derived hydroxide. The hydroxide adduct blocks the substrate-access gateway to the active site. (Based on PDB ID 1MMM). Prepared with the program MOLSCRIPT [106].

Fig. 7.

Fluorimetric assay for metal uptake by apo-manganese superoxide dismutase. (Top, Right) A single subunit of *E. coli* MnSOD with its buried metal center is shown, with ball-and-stick rendering of the clustered tryptophan side chains. (Based on PDB ID 1VEW) (Top, Left) Jablonski diagram illustrating the predicted fluorescence quenching mechanism for tryptophan residues in the presence of a bound metal ion. EX, excitation; EM, emission; Q, quenching by Förster energy transfer and nonradiative decay. (Bottom) Fluorescence timecourse for Co²⁺ binding by apo-MnSOD in 20 mM MOPS (pH 7.6), 37°C. Resolution of initial amplitude (A₀) and total amplitude (A_{TOT}) for the biphasic uptake kinetics is shown.

Fig. 8. Temperature and pH dependence of apo-MnSOD metal uptake. (Top) Temperature profile for ratio of initial to total amplitude (A_0/A_{TOT}) measured from the biphasic kinetics of Co^{2+} uptake by *E. coli* apo-MnSOD in 20 mM MOPS pH 7. (Bottom) pH dependence for ratio of initial to total amplitude (A_0/A_{TOT}) for uptake of Co^{2+} by *E. coli* apo-MnSOD at 37°C .

Fig. 9.

Hypothetical models for gated metal binding mechanism. (A) Conformational gating associated with a temperature-dependent conformational equilibrium between closed and open states. Metal binding occurs exclusively in the open state to irreversibly form the holoprotein product. (B) Structurally gating associated with temperature-dependent dimer dissociation, with metal binding to the isolated monomeric subunits followed by irreversible assembly of the dimeric holoprotein product.

Fig. 10.

Covalently cross-linked MnSOD variants. Disulfide engineering methods have been used to introduce cysteine pairs into MnSOD, which were covalently cross-linked to form disulfides by oxidation or bridged by treatment with a bifunctional thiol reagent (dBmBr). The locations of the cross-links are mapped onto the native MnSOD structure (PDB ID 1VEW). Prepared with the programs MOLSCRIPT [106] and RASTER3D [107].

Fig. 11. Hypothetical structures for apo-MnSOD conformational gating states. (A) Closed gate conformation for apo-MnSOD, based on the structure of the metallated holoenzyme (PDB 1vew). The dashed line represents the subunit interface. His171 serves as a metal ligand in the holoenzyme. Rotation of this side chain with reorientation of the interface-spanning Glu170 from the opposing subunit would open a large access channel into the metal binding region in the interior of the protein (B). Two additional residues, Tyr173 and Arg180, may serve to stabilize the protein in the open conformation. Prepared with the programs MOLSCRIPT [106] and RASTER3D [107].