METAL IONS IN BIOLOGICAL SYSTEMS

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Interrelations Between Free Radicals and Metal Ions in Life Processes

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Preface to the Series

Recently, the importance of metal ions to the vital functions of living organisms, hence their health and well-being, has become increasingly apparent. As a result, the long-neglected field of "bioinorganic chemistry" is now developing at a rapid pace. The research centers on the synthesis, stability, formation, structure, and reactivity of biological metal ion-containing compounds of low and high molecular weight. The metabolism and transport of metal ions and their complexes is being studied, and new models for complicated natural structures and processes are being devised and tested. The focal point of our attention is the connection between the chemistry of metal ions and their role for life.

No doubt, we are only at the brink of this process. Thus, it is with the intention of linking coordination chemistry and biochemistry in their widest sense that the *Metal Ions in Biological Systems* series reflects the growing field of "bioinorganic chemistry". We hope, also, that this series will help to break down the barriers between the historically separate spheres of chemistry, biochemistry, biology, medicine, and physics, with the expectation that a good deal of future outstanding discoveries will be made in the interdisciplinary areas of science.

Should this series prove a stimulus for new activities in this fascinating "field", it would well serve its purpose and would be a satisfactory result for the efforts spent by the authors.

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Biological Chemistry of Copper-Zinc Superoxide Dismutase and Its Link to Amyotrophic Lateral Sclerosis

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1. INTRODUCTION

The evolution of photosynthesis on earth infused the atmosphere with increasing amounts of O_2. Beginning around 550 million years ago, sufficient O_2 was generated to change the nature of the atmosphere from reducing to oxidizing and thus to change the nature of life itself [1]. Indeed, this change may have fueled the evolutionary explosion that

characterized the Cambrian era. Most of the organisms that survived the transition and adapted to (indeed, thrived on) an oxidizing atmosphere such as we have today were those that "learned" how to use reduction of dioxygen to water ($O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$) to produce energy in a form that could be stored. However, like most good things, oxygen utilization has its down side: partially reduced species derived from dioxygen can be highly reactive and toxic. So, concurrent with the use of O_2, organisms evolved mechanisms to protect themselves from the byproducts of oxygen utilization.

Earthly aerobic life forms have evolved several systems that work together to defend against the deleterious side effects of oxygen metabolism. These systems include (1) enzymes that react directly with the intermediate reduction products to remove them, (2) systems that shelter redox active metal ions from reactions with cellular components, (3) small antioxidant molecules that intercept radicals, and (4) elaborate repair and degradation systems that identify and repair or recycle damaged macromolecular components.

This chapter focuses on the first type of system, in fact on a single enzyme—the antioxidant enzyme copper-zinc superoxide dismutase (CuZnSOD). Superoxide dismutases (SODs) use metal ions to catalyze the disproportionation of O_2^- (superoxide) via reactions (1) and (2).

$$O_2^- + M^{n+} \longrightarrow O_2 + M^{(n-1)+} \quad (1)$$

$$O_2^- + M^{(n-1)+} + 2H^+ \longrightarrow H_2O_2 + M^{n+} \quad (2)$$

SODs constitute part of the primary line of defense against what are known as reactive oxygen species (ROS) such as superoxide (O_2^-, the one-electron reduction product of dioxygen), hydrogen peroxide (H_2O_2, the two-electron reduction product of dioxygen), and hydroxyl radical ($^\bullet OH$, resulting from one-electron reduction of hydrogen peroxide followed by O-O bond cleavage to yield $^\bullet OH$ and OH^-). Sometimes other reactive compounds derived from these three are also included under the label ROS, such as peroxynitrite ($ONOO^-$) and lipid hydroperoxides. SODs, together with catalases and peroxidases (which catalyze removal of H_2O_2), severely limit production of all these products by removing O_2^- and H_2O_2.

Erythrocuprein, isolated from bovine blood, was found to possess SOD activity in 1969 and was therefore renamed CuZnSOD [2]. Since that time many superoxide dismutases have been isolated, and some form of SOD has been found in every aerobic and aerotolerant organism.

There are two major forms of CuZnSOD: the dimeric intracellular form present in abundant quantities in the cytosol and nucleus of almost all eukaryotic organisms, and a higher molecular weight, tetrameric, extracellular homolog of much lower abundance (ECSOD) [3]. The glycosylated ECSOD is located in the interstitial spaces of tissue as well as plasma and is bound mostly to heparin sulfate proteoglycans. To date all eukaryotes have been found to contain cytosolic CuZnSOD, with the notable exception of some marine arthropods which seem to have replaced it with a cytosolic manganese SOD [4].

Plants contain cytosolic and chloroplast-specific (plastidic) CuZnSODs and usually contain multiple isozymes [5]. Several reports have demonstrated a peroxisomal localization for CuZnSODs as well. Sunflower cotyledons provide an interesting curiosity in that they do not seem to have a mitochondrial MnSOD but instead have CuZnSOD localized to this compartment [6].

Procaryotic homologs of CuZnSOD have also been found in the periplasm of many bacteria, indicating a remarkable degree of conservation of this enzyme throughout evolution [7]. There have even been published reports of viral CuZnSOD genes, supporting the hypothesis that this gene is critical for aerobic life [8].

Another, evolutionarily unrelated, family of SODs uses manganese or iron as the metal cofactor (MnSOD and FeSOD). MnSOD is found as a tetramer in the matrix of mitochondria, and both MnSOD and FeSOD (dimeric or tetrameric) are widely distributed in prokaryotes. These SODs are also well studied, both biologically and physically, but the details are beyond the scope of this chapter (for access to this literature, see [9–13]). Another, more recently discovered SOD family uses nickel as the catalytic metal ion (NiSOD) [14–16].

It can be truthfully stated that CuZnSOD is one of the most thoroughly characterized proteins in the scientific literature, and thousands of research articles describe properties ranging from physical characterization to biological activity. Any chapter reviewing CuZnSOD must therefore of necessity be selective in its coverage, and in this review we have chosen to present an overview of the biophysical properties of CuZnSOD followed by a review of some of the more recent biological results, particularly those relating to biological function, including a discussion of the role of this enzyme in the human neurodegenerative disease amyotrophic lateral sclerosis (ALS). In the sections addressing the biological function of CuZnSOD, we have somewhat broadened the scope to include other SODs, particularly the MnSOD, since the SODs are closely linked by a common function.

A large number of excellent reviews of various aspects of SOD structure and function have been published over the years, to which the reader is referred for more comprehensive coverage. For biophysical characterization, see [17–23]. For discussion of SOD mechanisms, see [24]. For more information on basic biological function, see [25–29]; for SOD in yeast, see [30,31]; for a review of recent developments in biological damage due to reactive oxygen species, see [32]; for ALS and the role of SOD, see [33,34] and Sec. 5 of this chapter.

2. BIOPHYSICAL PROPERTIES OF WILD-TYPE SUPEROXIDE DISMUTASE

A wealth of knowledge regarding structure-function relationships in CuZnSOD lies buried in stacks of journals dating back to the original isolation of erythrocuprein in 1939 [35]. Yet, as researchers periodically discover, the textbooks must be rewritten and dogma put to the test. The recent discovery that mutations in CuZnSOD cause familial amyotrophic lateral sclerosis (FALS) [36,37] has revitalized research routes down what was previously thought by many to be a too-well-trodden path. At first glance it seems reasonable that mutations in CuZnSOD might be disease causing, based on knowledge of its function in protecting against damage in vivo by ROS. But this initial conclusion must be reconsidered when it is confronted by the evidence that the ALS phenotype is due to a gain of function rather than a loss of SOD activity [33,38–40]. This new evidence has become the impetus to explore avenues of fundamental research on CuZnSOD that were not pursued before in order to fill in the chemical details and provide an explanation for the disease causing properties.

2.1. Protein Structure

While ECSOD is tetrameric [41] and the periplasmic CuZnSOD from *Escherichia coli* is monomeric [42], cytoplasmic CuZnSOD is invariably

a dimer of identical subunits. (The only exception reported to date is the monomeric isozyme IV from *Oryzae sativa* (rice) [43].) Each subunit of cytoplasmic CuZnSOD is approximately 16 kD, contains around 150 amino acid residues, and binds one zinc ion and one catalytic copper ion [22]. We will use the amino acid numbering for the human CuZnSOD, which has 153 amino acids, throughout this chapter (see Fig. 1 for an alignment of human, bovine, and yeast CuZnSOD sequences).

The overall tertiary fold is an immunoglobulin-like β barrel, consisting of eight antiparallel β sheets with a conserved disulfide bond [44,45]. The dimer is held together by hydrophobic contacts that cover approximately 50 Å^2 [45]. High-resolution crystal structures are available for the human [46,47], yeast [48–51], bovine [45,52–58], frog [56,59,60], and spinach [61] enzymes. They demonstrate the conservation of protein fold as well as amino acid sequence [62,63]. The wealth of crystallographic data has been recently reviewed by Bertini et al. [17] and the structure of the human wild type CuZnSOD dimer is shown in Fig. 2.

2.2. Nature of the Dimer Interaction

While the intersubunit contacts are quite strong, there is evidence that, at least in vitro, the subunits can be separated. The bovine, yeast, and swordfish enzymes were shown to be monomerized in 8 M urea [64,65], and the wheat germ subunits were reported to dissociate in sodium dodecyl sulfate (SDS) [66,67]. Dissociation was proposed to be dependent on the metallation state of the protein [68,69], although these results were later questioned [70]. It has also been reported that simple dilution is enough to significantly effect the monomer/dimer equilibrium [71]. Guanidinium hydrochloride [72,73] and SDS also influence the monomer/dimer equilibrium, as do succinylation [74], point mutations at sites not in the dimer interface [75], and temperature [71]. Introduction of charged residues into the hydrophobic interface using site-directed mutagenesis [76–79] was found to be sufficient to prevent dimerization of the subunits of CuZnSOD, but the monomeric mutant enzyme was found to have very low SOD activity.

Related to the monomer/dimer equilibrium is the phenomenon of intersubunit communication or cooperativity, which is supported by

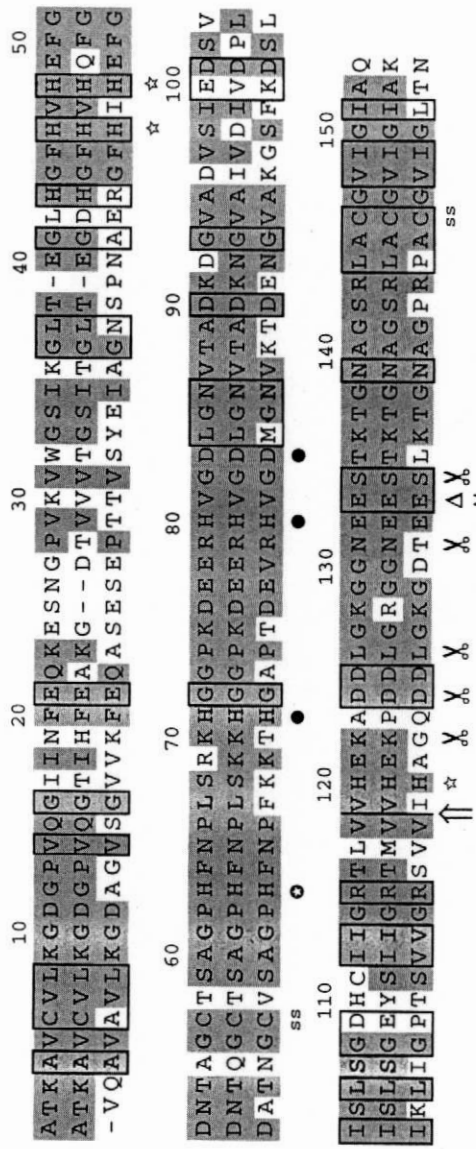
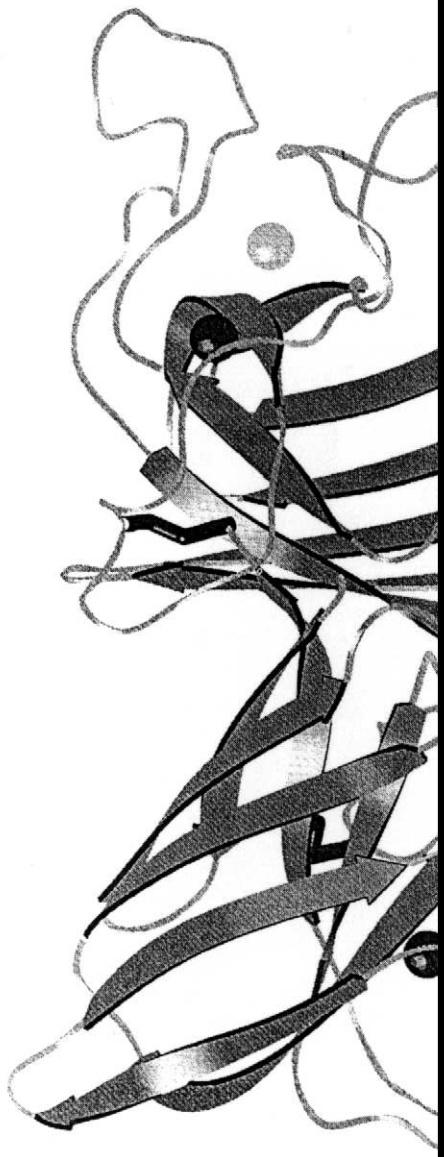


FIG. 1. Sequence alignment of human, bovine, and yeast CuZnSOD. Shaded areas indicate amino acid identity in at least two sequences. Numbering is according to the human sequence. Boxed areas indicate positions at which ALS-causing missense mutations have been found in the human gene. (●) Amino acids involved in zinc binding, (☆) copper binding amino acids. (Note that His63 is a ligand for both copper and zinc.) (ss) Position of the two cysteines involved in the disulfide bridge. (✂) Position of ALS mutations that result in a premature stop and truncation of the protein. (⇑) An ALS mutation that results in an insertion of the tripeptide FLE. (Δ) An ALS mutation that results in the deletion of one amino acid.



several lines of evidence, at least in the case of the bovine, yeast, and human enzymes. Studies using nuclear magnetic resonance (NMR) [80], differential scanning colorimetry (DSC) [81], pulse radiolysis [82], and metal titration [83–85] suggested that the two subunits communicated with each other and that structural changes in one subunit could affect the conformation of the other subunit.

Computational studies have predicted that an inherent subunit asymmetry should exist in dimeric CuZnSOD [86,87]. This theoretical prediction is challenged by the published crystal structures, which show equivalent subunits for all species. However, it is now believed that the yeast enzyme possesses significant asymmetry when stripped of its metals [88,89], and one human FALS-causing mutant was shown both in solution and in the crystalline state to exhibit significant differences between the subunits [90].

The teleological reason that CuZnSOD forms a dimer is still a matter of debate. The fact that the monomer prepared by site-directed mutagenesis is not SOD-active may indicate that dimer contact is crucial for proper formation of the active site. However, the *E. coli* enzyme is monomeric and active [91,92]. One recent publication regarding the structure and stability of *E. coli* CuZnSOD suggests that the β-barrel fold is enough to stabilize the structure of CuZnSOD, but the additional stability toward heat and pH denaturation is conferred by dimerization [93]. It is also possible that the CuZnSOD protects its amino and carboxy termini from proteolysis by burying them in the dimer interface.

The importance of dimerization in generating the final electrostatic field that attracts superoxide to the active site is also unknown. Dimerization may maximize the positive surface of the protein, ensur-

FIG. 2. Three dimensional structure of the human wild-type CuZnSOD. Note that the basic fold is an 8-stranded β barrel. β strands are indicated by broad arrows and loops by ropes. The lighter sphere is the zinc ion; the darker one is the copper ion. The view we have chosen shows that the copper ion is located deeper in the protein and that the zinc ion is more peripherally located. However, the copper ion has one coordination site accessible to small anions through the narrow active site channel while the zinc ion has no open coordination sites. It serves to stabilize the neighboring loop structure. The disulfide bond, which also serves to stabilize a loop, is shown in black.

ing optimal collisions with its negative substrate. That the monomeric point mutant is inactive despite an apparently identical structure (determined by NMR) supports the last idea [78], but the existence of a fully active monomeric protein from *E. coli* tends to refute it [42].

Overall, CuZnSOD is a remarkably stable protein. The melting temperature, as gauged by DSC, can be as high as 100°C, in the case of the bovine enzyme, and the protein is denatured by neither 8 M urea nor 1% SDS [94]. Recombinant human CuZnSOD is not proteolytically degraded by trypsin, aminopeptidase M, or serum [95]. The structural attributes that result in this unusual stability are unknown.

2.3. Structural Heterogeneity

It has been known for some time that CuZnSOD from a variety of sources produces a pattern of multiple bands on nondenaturing polyacrylamide gels [96,97]. Little is known about the nature of these electromorphs, but there are many theories as to their origins. In some instances, further purification of some of these individual bands, followed by another round of electrophoresis, yielded the same pattern of multiple bands, leading to the suggestion that the band contained forms capable of equilibrating with each other [98,99].

An early suggestion of labile sulfur bound to CuZnSOD was proposed because some preparations of CuZnSOD displayed a 320-nm UV band indicative of thiolate charge transfer [100]. Another thought was that the different forms represented covalently modified derivatives of the enzyme, perhaps the products of oxidative modification or glycation [101–107]. Such conclusions were based on observations of the increased heterogeneity when CuZnSOD was exposed to conditions that increase such phenomena. It is also possible that the multiple bands represent different metallation states of the protein, i.e., apo- versus holo-protein [85,108,109]. However, other evidence suggests that they are the result of folding isomers of the enzyme [98,99]. The discrepancy between total and electron paramagnetic resonance (EPR)-detectable copper [110,111] and the presence of conformational sub-states detected using EPR [112] support this last theory. Based on the large amount of evidence of heterogeneity, it seems possible that CuZnSOD has several quasi-stable structures, each differing from the others minutely.

2.4. Cysteines

The disulfide bond, which is conserved in all eukaryotic CuZnSODs, links Cys57 and Cys146 (see Figs. 1 and 2). The mammalian CuZnSODs contain a third cysteine, Cys6, that is buried in the dimer interface, while the human protein possesses yet another cysteine, Cys111, that is exposed on the surface of the protein [62,63]. The buried Cys6 in the human and bovine proteins has been mutagenized to an alanine in order to create a more thermostable enzyme. The human enzyme is also stabilized by mutagenesis of the surface Cys111 to serine [57,113,114].

The fact that CuZnSOD possesses a disulfide bond is interesting in itself [115]. Such bonds stabilize protein structures, and they are common in secreted proteins but are not commonly found in intracellular proteins. One possible explanation is that it plays a role in the folding pathway of CuZnSOD.

The role of the other cysteines in the CuZnSOD sequence is equally intriguing. While free, nondisulfide cysteines are common as ligands in metalloproteins, there is no evidence to support the notion that they function in this capacity in CuZnSOD. Also of questionable relevance is the fact that some proteins contain redox-active thiol groups that have catalytic functions. Surface-exposed thiols have even been proposed to possess a novel function—the reversible binding of nitric oxide [116]. Since human CuZnSOD possesses both a buried and an exposed thiol, it will be interesting to see whether new discoveries will be made about their functions.

2.5. Metal Binding Sites

The Zn^{2+} ion occupies a tetrahedral binding site in a loop consisting of residues 63–83 (human CuZnSOD numbering). Zinc is ligated to three nitrogens from histidyl imidazole rings (His63, His71, His80) and one carboxylic oxygen from aspartate (Asp83) (Fig. 3). In the oxidized form of the enzyme, His63 forms an imidazolate bridge to the bound Cu^{2+} ion, which is tetragonally ligated to four histidines (His46, His48, His63, His120). In addition to the direct imidazolate bridge between the Cu^{2+} and Zn^{2+} ions, there is a bridge formed by hydrogen bonds that

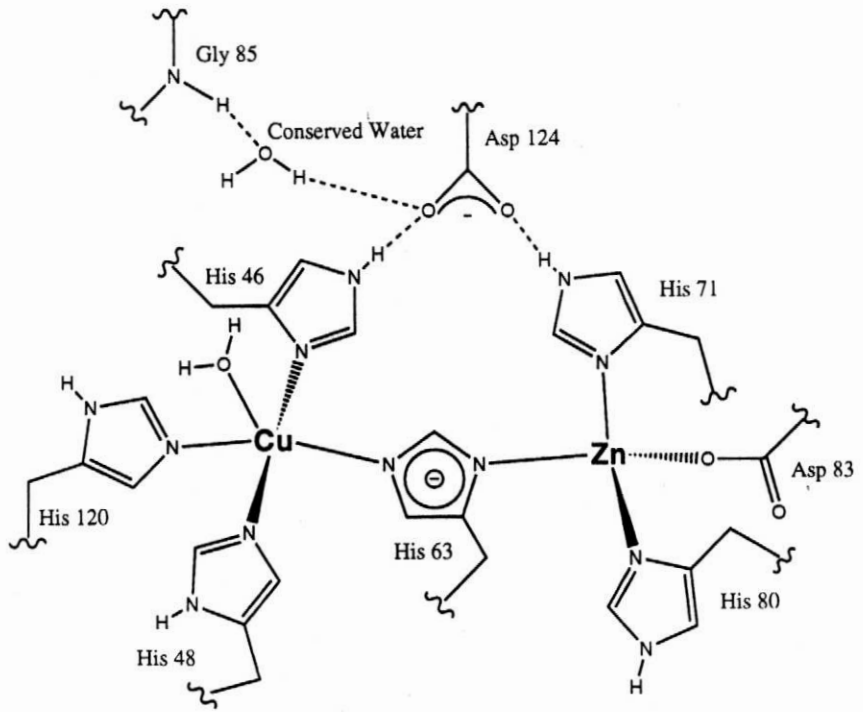


FIG. 3. The active site region of CuZnSOD. Note the direct connection between the two metal ions through the imidazolate bridge formed by His63, as well as an indirect connection via the secondary bridge formed by Asp124 which connects two histidine ligands, one for the zinc and one for the copper ion. The water coordinated to the copper ion is replaced by superoxide anion during catalysis. Hydrogen bonds are indicated by dotted lines, and connections to the protein backbone by squiggles. See text for more discussion.

links the two metal binding sites. This hydrogen bonding network connects His71 of the zinc site with His46 of the copper site via the carboxylate group on Asp124 [45]. This secondary bridge seems to be more vital to the integrity of the zinc binding site because site-directed mutants modified at position 124 are zinc-deficient but copper-replete [117]. In solution and in one reported crystal structure, reduction of Cu^{2+} to Cu^{+} results in breakage of the imidazolate bridge [51,118–123] with the Cu^{+} ion moving away from His63 [51].

The zinc in wild-type CuZnSOD can be replaced by Co^{2+} with

neither loss in activity [124–126] nor significant perturbation of the protein structure [55]. It can similarly be replaced, with retention of some or all SOD activity, by Cd^{2+} [127], Cu^{2+} [128], Hg^{2+} [129], Ni^{2+} [130], and VO^{2+} [131], providing a wealth of structural probes for the zinc site. The copper can be replaced with Co^{2+} [132], Zn^{2+} [80], Cd^{2+} [133], Ni^{2+} [134], or Ag^{+} [135], although none of these copper-substituted CuZnSOD derivatives is SOD-active.

2.6. In Vivo Metallation State

CuZnSOD isolated from yeast exposed to nontoxic levels of Ag^{+} has been found to contain silver, indicating that the metallation state of CuZnSOD can be altered in vivo [136]. Other studies in yeast demonstrated that CuZnSOD can buffer toxic levels of copper [137] and that the copper-binding protein, metallothionein, can functionally substitute for CuZnSOD [138]. Many studies on dietary copper and zinc deficiency in whole organisms have shown significant alterations in the SOD activity of intracellular CuZnSOD [129,139–142]. But the question has always remained whether this effect is due to alterations in metallation states as opposed to CuZnSOD protein levels.

Several studies have concluded that CuZnSOD exists in vivo as both a metallated pool and a metal-deficient pool. In the yeast *Saccharomyces cerevisiae* grown under anaerobic conditions, it was shown that a significant portion of the CuZnSOD is inactive and that it can be reactivated by the addition of copper ions to cell extracts [143]. Similar experiments in human K562 cells led to the conclusion that the same is true in this mammalian system if the cells are undifferentiated [144, 145]. More compelling data were provided by a group studying human lymphoblasts who showed that ^{64}Cu incorporation into an apoprotein pool of CuZnSOD could be achieved by addition of the copper isotope to intact cells and that this incorporation was accompanied by an increase in SOD activity [146]. Similar results were obtained in the presence of cycloheximide, demonstrating that it was not de novo synthesis that accounted for the increased copper binding and SOD activity. A separate study of CuZnSOD isolation under conditions of copper deficiency led to the conclusion that the isolated protein was both copper- and zinc-deficient, suggesting that metal ions may be inserted into the

apoprotein in a cooperative fashion. It was also observed in the same study that the SOD activity in lysates from copper-deficient cells was very low [108].

This line of research has recently gained momentum due to the discovery of copper chaperones that play an important role in facilitating proper metal ion insertion into CuZnSOD apoprotein within yeast and human cells. In the case of *S. cerevisiae*, the relevant gene is *LYS7* and its human homolog is *ccs1* (Copper Chaperone for SOD) [147]. Lys7 and CCS are multidomain proteins with one domain that is homologous to the CuZnSOD monomer. It seems likely that this domain recognizes apoCuZnSOD and, perhaps, like a classic protein chaperone [148], modifies the protein structure to make it more amenable to metal binding.

3. ENZYMATIC ACTIVITY

3.1. Redox Reactivity

CuZnSOD is remarkably specific with respect to its reactivity, and the rates of reaction of both the oxidized, cupric form and the reduced, cuprous form with O_2^- appear to have been optimized, with rate constants for both approaching the diffusion limits [149]; see reactions (3) and (5). At the same time, rates of reactions with other potential substrates such as H_2O_2 [150,151], NADH, and ascorbate [152] appear to have been minimized.

CuZnSOD activity is high and nearly constant over a wide pH range (pH = 5–9). Removal of Zn^{2+} makes the enzyme markedly pH-dependent although it does not alter the maximal activity [153]. The zinc-deficient enzyme also reacts more readily with peroxynitrite than does the native enzyme [154]. These data demand a rethinking of the role of zinc in this protein, because it is clearly not only a structural ion that confers stability but also a regulatory ion that modulates the reactivity of copper in the enzyme. Ironically, the belated awareness of this role for zinc is in keeping with the fact that zinc was not even known to be a cofactor in CuZnSOD until 31 years after the protein's initial isolation [35,155].

The products of the SOD reaction, O_2, and H_2O_2, can also react with CuZnSOD, although the reactions are much slower than the reactions of the enzyme with O_2^-. Thus O_2 reacts slowly with reduced, cuprous CuZnSOD forming the oxidized, cupric enzyme and superoxide; see reactions (3) and (4) [156].

$$\text{E-Cu}^{2+} + O_2^- \longrightarrow \text{E-Cu}^{+} + O_2 \quad k = 2 \times 10^9\ \text{M}^{-1}\text{s}^{-1} \tag{3}$$

$$\text{E-Cu}^{+} + O_2 \longrightarrow \text{E-Cu}^{2+} + O_2^- \quad k = 1.5\ \text{M}^{-1}\text{s}^{-1}\ [156] \tag{4}$$

Hydrogen peroxide can function both as a reductant and as an oxidant of CuZnSOD. The oxidized enzyme is reduced by the addition of H_2O_2 [157,158], and the resulting reduced CuZnSOD can then react further with H_2O_2, albeit slowly, to form hydroxyl radical or a hydroxyl-like species [159,160] that is capable of hydroxylating substrates and of irreversibly inactivating the enzyme [161] by oxidizing a single histidyl residue [162]; see reactions (5)–(7).

$$\text{E-Cu}^{+} + O_2^- + 2\text{H}^+ \longrightarrow \text{E-Cu}^{2+} + H_2O_2 \quad k = 2 \times 10^9\ \text{M}^{-1}\text{s}^{-1} \tag{5}$$

$$\text{E-Cu}^{2+} + H_2O_2 \longrightarrow \text{E-Cu}^{+} + O_2^- + 2\text{H}^+ \quad k = 0.56\ \text{M}^{-1}\text{s}^{-1}\ [158] \tag{6}$$

$$\text{E-Cu}^{+} + HO_2^- + \text{H}^+ \longrightarrow \text{E-Cu}^{2+} + \text{OH}^- + {}^\bullet\text{OH} \quad k = 2.6 \times 10^3\ \text{M}^{-1}\text{s}^{-1}\ [160] \tag{7}$$

CuZnSOD also reacts with peroxynitrite resulting in nitrosylation of tyrosine residues [163,164]. As stated above, this activity can be increased by the removal of zinc from the protein.

$$\text{E-Cu}^{2+} + \text{OONO}^- + \text{H}^+ \longrightarrow \text{E-Cu}^{2+}(\text{OH}^-) + NO_2^+ \tag{8}$$

Hydrosulfide anion, HS^- [165,166], is oxidized by CuZnSOD producing hydrosulfide radical ($HS^\bullet$). $HS^\bullet$ can then recombine with another $HS^\bullet$ or with HS^- to form elemental sulfur (S^0) or polysulfide anion (S_n^{2-}). The oxidized enzyme is then regenerated by reaction with O_2 [165,166]. Reactions of CuZnSOD with NO to give NO^- have also been reported [167]; see reactions (9)–(11).

$$\text{E-Cu}^{2+} + HS^- \longrightarrow \text{E-Cu}^{+} + HS^\bullet \tag{9}$$

$$\text{E-Cu}^{+} + \text{NO} \longrightarrow \text{E-Cu}^{2+} + \text{NO}^- \tag{10}$$

3.2. Dismutase Mechanism

CuZnSOD scavenges superoxide anion in a nearly pH-independent manner over a wide range of pH. The catalytic rate constant has been

determined to be $2 \times 10^9\ M^{-1}\ s^{-1}$, which is extremely high for an enzyme and approaches the diffusion-controlled limit. The protein surface surrounding the active site channel is highly positively charged [168,169]. It is this positively charged electrostatic field that is postulated to steer the superoxide substrate into the active site. Altering the electrostatic field by covalent modification [170], ionic strength manipulation [171], or site-directed mutagenesis can diminish [172] and, remarkably, even enhance [173] the activity of the enzyme. Even the reaction with peroxide, which reacts in the form of the HO_2^- anion, is believed to be electrostatically guided [174].

The superoxide anion is believed to bind to the axial position on the roughly tetragonal Cu^{2+}, replacing a weakly coordinated water molecule, forming a hydrogen bond with Arg143. Electron transfer to the Cu^{2+} ion results in breaking of the imidazolate bridge and release of dioxygen. Another superoxide then reacts with the trigonal Cu^+,

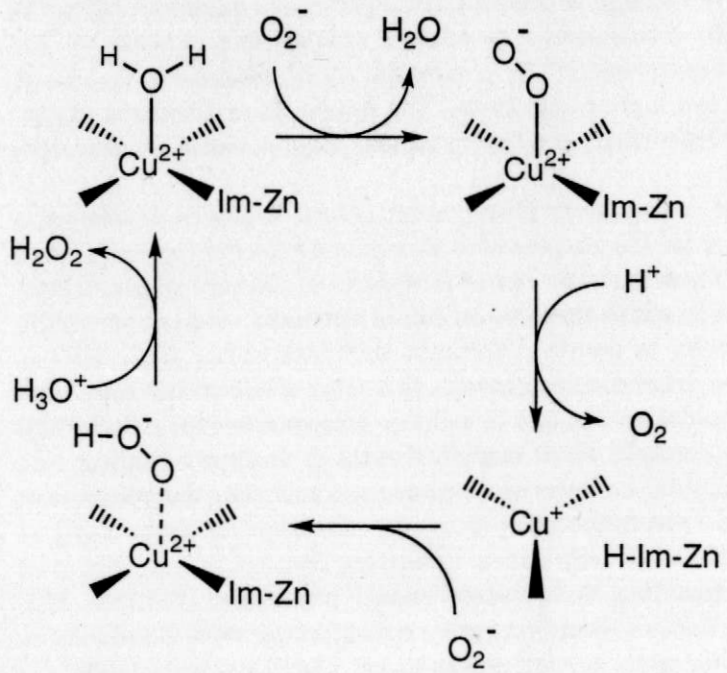


FIG. 4. Mechanism of superoxide dismutation by CuZnSOD. "Im" stands for the bridging imidazolate (His63). See text for more detailed explanations.

oxidizing it to form Cu^{2+}. The imidazolate bridge between copper and zinc is reformed and peroxide is released. The details of the timing of the proton transfers are unknown, but evidence suggests that the role of the imidazolate bridge (and consequently the zinc ion as well) is to ensure that the peroxide ligand is only weakly bound in an axial position, ensuring rapid release of the peroxide product [175,176] (see Fig. 4). Under normal conditions of low superoxide concentrations, the imidazolate bridge between the zinc and copper sites is broken upon reduction. This step is relatively slow, however, and requires significant atomic rearrangement; it is not expected to occur in the event of saturating substrate concentrations [122]. In the latter case the imidazolate bridge is expected to remain intact during catalysis; however, this situation is unlikely to occur in vivo because CuZnSOD exists in vast excess of its substrate inside of a normal cell.

4. EVIDENCE CONCERNING BIOLOGICAL FUNCTION

4.1. Historical Background

Almost from the moment that the SOD activity of CuZnSOD was first announced by McCord and Fridovich in 1969 [2], this enzyme has been widely recognized as an important antioxidant enzyme, and its high SOD activity and its ubiquitous presence in aerobic organisms strongly support this conclusion. Nevertheless, an early controversy in the field arose from the concerns of some chemists (including one of the present authors), who were reluctant to assign to superoxide an extremely deleterious role in causing generalized damage without knowledge of the specific chemical reactions that cause superoxide to be toxic in vivo [177]. Much of this concern arose as detailed information concerning the chemical reactivity of superoxide accumulated. In particular (1) that the rate of spontaneous disproportionation of superoxide is fast and it therefore will not accumulate to high concentrations, even in the absence of an SOD; (2) that cupric ion itself is an excellent catalyst of superoxide disproportionation, albeit only at low pH, suggesting the possibility that the SOD activity of CuZnSOD might be artifactual; and (3) that superoxide is usually not capable of fast oxidations of substrates because a proton is needed before it can accept an electron from a substrate (reaction (11)), and it is not strongly basic in water [24].

$$O_2^- + e^- + HX \longrightarrow HO_2^- + X^- \quad (11)$$

Moreover, the explanation of superoxide toxicity, widely accepted by some at that time, that it acted as a reductant of redox metal ions in the Fenton reaction (reactions (12) and (13)) seemed improbable to others because other reducing agents such as ascorbate could also play that role and yet were not considered to be highly toxic.

$$M^{n+} + O_2^- \longrightarrow M^{(n-1)+} + O_2 \quad (12)$$

$$M^{(n-1)+} + H_2O_2 \longrightarrow M^{n+} + OH^- + {}^{\bullet}OH \quad (13)$$

Time has provided much experimental evidence to support the hypothesis originally put forward by the enzymologists that CuZnSOD is indeed a central antioxidant enzyme. The corollary that superoxide is an important endogenously generated toxin also is by and large true. But the chemists were also right: Superoxide does not cause random damage but reacts with certain vulnerable targets, particularly exposed 4Fe-4S clusters, which are nonetheless extremely important for survival. Superoxide may also participate in Fenton-type reactions, but it is likely that the damage here is also site-specific, with hydroxyl radical, $^{\bullet}OH$, being generated and reacting in the close vicinity of metal ions bound more or less specifically to biological molecules [178]. These conclusions are based on much work from many laboratories working with different organisms, some of which we will review in the following sections.

Although this chapter focuses primarily on CuZnSOD, it is important in any discussion of biological function that the other SODs be considered as well because they play a vital role in concert with CuZnSOD in vivo, MnSOD, found in the mitochondria of eukaryotes, will therefore be covered in some detail in the following section. The prokaryotic FeSOD and MnSOD, and the CuZnSOD found in chloroplasts of plants, will not be thoroughly discussed here, although their importance to aerobic life is unquestionable.

4.2. Genetic Manipulation of SOD Levels in Whole Organisms

4.2.1. Knockouts

SOD gene knockouts were made first in bacteria [179,180] and then in yeast [181–185]. *Escherichia coli* lacking SOD were found to grow slowly in air, to have an air-dependent auxotropy for branched chain amino acids, and to be very sensitive to redox cycling drugs such as paraquat or phenazine methosulfate. The phenotype of *Saccharomyces cerevisiae* that lack CuZnSOD (*sod1*Δ) was found to be similar though not identical. While the gene is not strictly essential, the strains grow poorly in air, are extremely sensitive to redox-cycling drugs, have air-dependent auxotrophies for cysteine or methionine and for lysine, and will not grow in 100% oxygen. *S. cerevisiae* lacking the mitochondrial MnSOD (*sod2*Δ), on the other hand, grow normally in air, but are sensitive to redox cycling drugs, will not grow in 100% oxygen, and have difficulty growing in nonfermentable carbon sources (that require respiration for their utilization). (Yeast are normally grown in medium containing glucose, the substrate for glycolysis, as the carbon source.)

Indicators of oxidative damage tend to increase in older individuals or populations, implying that oxidative damage plays a role in the aging process, and it has been suggested that it is the main cause of aging [186]. While this hypothesis remains to be proven, it seems likely that oxidative damage is one of several (or many) causes of aging. To study the role of antioxidant proteins in this process, "knockout" mutants and "overexpressors" have been made and characterized in yeast as well as a few higher organisms. The results from knockout experiments have been clear; the overexpressor experimental results were less so.

In yeast, a stationary phase model system has been developed in our laboratory for the purpose of studying oxidative damage associated with aging. Yeast that run out of food enter stationary phase, a nongrowing state in which they live on stored nutrients, and can survive in water for weeks to months. Mutants that lack either CuZnSOD or MnSOD show dramatic decreases in this "chronological life span" and evidence of oxidative damage to cellular components [187]. CuZnSOD mutants, for example, show increased rates of nuclear mutation, and MnSOD mutants show lowered aconitase and succinate dehydrogenase activities and a precipitous drop in oxygen consumption, particularly as they enter into stationary phase, indicating damage to mitochondrial components resulting in decreased energy production [187,188]. Mutants lacking CuZnSOD in *Drosophila melanogaster* were found to have a shortened life span, as well as decreased lifetime activity [189].

Recently, transgenic mice lacking CuZnSOD or MnSOD have been constructed [39,190–192]. Rather surprisingly, the CuZnSOD-minus mice grew and developed normally. In fact, they were little affected,

unless injury occurred. Even traditional indicators of oxidative damage (protein carbonyls, lipid peroxidation, and GSSG/GSH ratio) were unaffected, at least in brain. Because these investigators were focused on determining whether lack of CuZnSOD activity could be a cause of the paralytic disease ALS, they assayed motor neuron function and found that axonal regrowth following injury was markedly impaired [39]. Another group, working with a different strain of *sod1*- mice, reported that the main effect of the removal of active CuZnSOD was a drastic decrease in female fertility. In *sod1* −/− mothers, embryos died shortly after implantation; in heterozygous (−/+) or wild-type mothers, this effect was not observed [190], indicating it is a maternal effect.

Mice lacking MnSOD, on the other hand, were severely impaired. They developed normally in utero but died shortly after birth. Two groups have constructed such mice. At birth, the mice made by Epstein and coworkers [191] appeared almost normal, except they were pale, hypothermic (cooler than normal), and tired easily. However, their condition deteriorated rapidly following birth and they all died within 10 days. At autopsy, enlarged hearts and lipid deposits in muscle and liver were observed. Mitochondria were ultrastructurally normal, but the activities of aconitase and succinate dehydrogenase were quite low. The second group constructed MnSOD knockout mice in a different strain background and found a somewhat different phenotype [192]. Their mice survived for 3 weeks, and exhibited severe anemia and neurological symptoms (degeneration of large CNS neurons, weakness, and circling behavior). At later stages, mitochondrial structural damage was evident. Interestingly, in these mice CuZnSOD levels were increased by about 25%. Overall, the affected cell types are those with obligatory requirements for high levels of oxidative metabolism—cardiac myocytes, neurons, hepatocytes, and hematopoietic cells. When the mice from the Epstein laboratory were treated with SOD mimics, the early death was somewhat delayed, and neurological symptoms and spongiform degeneration of the cortex and specific brain stem nuclei were observed [193].

Mice lacking ECSOD have also been constructed and the effect of its absence was mild. Homozygotes developed normally, and no changes were observed in a wide range of other antioxidant enzymes. The ECSOD null mutant mice were more sensitive to 99% oxygen, accumulating lung damage faster and dying sooner than their wild-type counterparts [194].

It is interesting to contrast the effects of SOD knockouts in yeast with those of mammals. MnSOD is apparently much more important in mice, while the CuZnSOD is more important in yeast. The small magnitude of the effect of the absence of MnSOD in yeast may be an artifact of the somewhat artificial conditions in which most lab yeast are grown—they always have plenty of glucose and other nutrients and thus do not need to respire, enter stationary phase, or sporulate, all processes that absolutely require mitochondrial respiration. Nevertheless, it is still true that the CuZnSOD is far more important to the survival of yeast, possibly because each yeast cell in real life could be directly exposed to the air at any time, while murine cells are mostly well protected by physical barriers from direct contact with air. Interestingly, when fetal fibroblast cells are cultured from knockout mice, the phenotype is more like that observed in yeast. *sod1*Δ cells are difficult to culture (most die soon after plating), and far more sensitive to the redox cycling drug paraquat, while *sod2*Δ yeast are easier to culture and not as sensitive to paraquat, although they are more sensitive than wild-type cells [195]. Mice heterozygous for MnSOD deletion are grossly normal but were found to be more susceptible to cerebral infarction following focal cerebral ischemia [196].

In cell culture models it was shown that downregulation of CuZnSOD by antisense RNA in neurons resulted in increased apoptotic death after serum withdrawal. Also microinjection of CuZnSOD protein was protective in the same system. Other studies showed that CuZnSOD overexpression in another neuronal cell culture system resulted in protection against this same type of induced apoptosis [197].

4.2.2. Overexpressors

There has been interest in the possible consequences of too much CuZnSOD ever since CuZnSOD was found to be located on human chromosome 21, an extra copy (or partial copy) of which causes Down syndrome. CuZnSOD levels in many Down patients are approximately 150% of normal, as might be expected [198]. It was reported around 10 years ago that transgenic mice overexpressing human CuZnSOD had altered neuromuscular junctions in the tongue, similar to those seen in Down patients [199]. Decreased blood levels of serotonin were also observed in the mice, as in patients, and attributed to more active uptake of serotonin by platelets; reuptake of neurotransmitters in the brain

may be similarly affected [200]. More recently, ultrastructural abnormalities in the thymus similar to those found in Down patients have been observed in transgenic mice overexpressing human CuZnSOD. It was suggested that these abnormalities may account for some of the immune system defects in Down syndrome [201]. It is clear, however, that an altered CuZnSOD level is not the whole picture. There are many other genes on chromosome 21 that are also duplicated and that may have effects, including a gene implicated in familial Alzheimer's disease. In addition, CuZnSOD levels are not always elevated in patients. Down syndrome can be caused by partial duplications of chromosome 21, and often these cases show normal CuZnSOD levels [202]. Thus, while CuZnSOD overexpression may account for some of the features of Down syndrome, we must look elsewhere for the whole story.

A longstanding theory, which nevertheless remains to be definitively proven or disproven, holds that accumulation of oxidative damage is a primary cause of aging [186,203,204]. Workers have investigated the possibility that alterations in CuZnSOD can alter aging in several different organisms. It is clear that a lack of CuZnSOD can have drastic consequences on life span, in, e.g., *Drosophila* [189], and yeast in stationary phase (chronological life span) [187]. Mice without MnSOD die within a few days of birth (see above), but an effect on life span of CuZnSOD-lacking mice has not been reported.

What is less clear is whether overexpression of CuZnSOD and/or other antioxidant enzymes can enhance life span or even improve resistance to oxidative stress. Reveillaud et al. [205] reported that overexpression of bovine CuZnSOD in *Drosophila* led to increased resistance to paraquat and to a slight life span extension. Orr and Sohol reported that overexpression of CuZnSOD only led to increased life span if catalase was also overexpressed [206]. More recently, Sun and Tower [207] found that turning on extra CuZnSOD activity in adult *Drosophila* (as opposed to having it present during the larval stages as well) can extend mean life span up to 20% [208]. Overexpression of MnSOD in mice was reported to protect against adriamycin-mediated cardiac damage [208].

On the other hand, there is some evidence that overexpression of SOD by itself is slightly deleterious, resulting in increased susceptibility to oxidative damage, and that simultaneous overexpression of either catalase or glutathione peroxidase (enzymes that degrade hydrogen peroxide) can prevent this effect. The first observation that extra SOD activity could be harmful was made in *E. coli* overexpressing MnSOD [209]. In transgenic tobacco plants, overexpression of chloroplastic CuZnSOD caused increased resistance to oxidative stress. However, ascorbate peroxidase was also elevated in these strains, indicating that the increased protection may have resulted from the double overexpression, rather than just overexpression of the SOD [210,211]. A similar observation was made in cultured mammalian cells overexpressing CuZnSOD. It was noted that the degree of resistance to oxidative stress correlated with the balance between CuZnSOD and glutathione peroxidase (either cotransfected or endogenously induced), rather than with the absolute levels of CuZnSOD [212]. Careful examination of mammalian cells transfected with human SOD showed that the transfection caused a variety of changes in different clones in the expression of other antioxidant enzymes, particularly glutathione peroxidase, and some enzymes not normally associated with oxygen metabolism [213]. On the other hand, in transfected cell lines without elevated Gpx, the resistance to paraquat correlated directly with the SOD level [214]. Thus it is evident that it is not sufficient to consider a single antioxidant enzyme in isolation. The absolute levels and the balance between the different activities are probably both important.

4.3. CuZnSOD as a Component of Antioxidant Systems

Normal metabolic processes involving oxygen necessarily generate O_2^- and/or H_2O_2; if redox-active metal ions are present, then they can catalyze the production of the extremely reactive hydroxyl radical ($^{\bullet}OH$) or other reactive species from these reactants. Even a process as efficient in its use of oxygen as mitochondrial respiration leaks reactive oxygen species at a measurable rate, which has been estimated (from in vitro experiments) at from 1% to 4% of the oxygen utilized. Other cellular reactions also generate superoxide of H_2O_2 as product. Antioxidant enzymes as well as small-molecule antioxidants are strategically located to deal with these leaks.

CuZnSOD and MnSOD are structurally unrelated enzymes that nevertheless catalyze exactly the same reaction. MnSOD is located in the mitochondrial matrix and CuZnSOD in the cytoplasm and nucleus;

thus these two SODs cover most of the aqueous compartments in the cell. CuZnSOD has also been reported to be found in the intermembrane space of mitochondria [9] (see also [215]). Because hydrogen peroxide is one of the products of superoxide dismutation, enzymes that degrade H_2O_2 are usually located in close proximity: catalase and/or glutathione peroxidase in the cytoplasm, glutathione peroxidase (or cytochrome c peroxidase in yeast) in the intermembrane space of mitochondria. In addition, large amounts of catalase are found in peroxisomes, where heavy generation of H_2O_2 occurs without intermediate production of O_2^-.

There are no known antioxidant enzymes in the lipid compartments; protection is provided by small-molecule antioxidants—primarily vitamin E (or α-tocopherol) and the reduced form of coenzyme Q (QH_2 or ubiquinol). In mammalian systems these are kept reduced by the ascorbic acid cycle. Membranes are quite vulnerable to oxidative damage, particularly if they contain, as most membranes do, polyunsaturated fatty acids. Small-molecule antioxidants in the aqueous compartments are ascorbic acid, glutathione (GSH), and uric acid (in plasma). Ascorbic acid and GSH have enzyme systems that keep them reduced. GSH reductase reduces the oxidized form of glutathione (GSSG) to two molecules of GSH at the expense of NADPH. Thus the system that produces NADPH is important [216].

4.4. Connections Between Metal Metabolism and CuZnSOD

In yeast, lack of CuZnSOD (*sod1*Δ) causes a severe phenotype, which is described above. Perhaps surprisingly, however, strains of yeast lacking both SODs can be constructed. This presents a useful system for finding out what other cellular components can be involved in antioxidant protection. A number of genetic suppressors of the defects observed in yeast lacking SOD have been isolated and are summarized in Table 1 (see also [30,31]). The gene products involved are located in various cellular compartments and organelles, but they have in common an involvement in metal metabolism, particularly copper or manganese metabolism. Simply adding either copper or manganese to the culture medium significantly improves the growth of these strains, and

TABLE 1

Mutations that Rescue the Phenotype of Yeast Lacking SOD

Gene	Rescue by	Mechanism	Reference
CUP1	Overexpr.	Gene for copper metallothionein (MT); required for rescue of *sod1*Δ yeast by excess copper in the medium; MT with bound copper has SOD-like, or superoxide scavenging, activity in vitro, and may have similar activity in vivo as well.	138
bsd2	Deletion	Located in the endoplamic reticulum, regulates translocation to the cell surface of *SMF1* and *SMF2* metal transporters; deletion results in increased intracellular copper.	217
pmr1	Deletion	P-type ATPase in Golgi involved in Mn transport; deletion results in increased Mn accumulation in cytoplasm, which is required for rescue.	220
ATX1	Overexpr.	Copper chaperone in cytoplasm carries copper from *CTR1* copper transporter at plasma membrane to *CCC2* copper transporter into secretory pathway; not clear why overexpression rescues *sod1*Δ yeast, may deliver copper to other site(s) as well.	218
ATX2	Overexpr.	Golgi membrane protein involved in Mn metabolism; overexpression results in increased Mn accumulation, which is required for rescue.	219
coq3, *atp2*, other *pet* mutants	Deletion	Nuclear petite (respiration-deficient) mutations; lack of respiration decreases generation of superoxide.	187

these suppressors appear to cause similar changes in intracellular metal ion levels.

Out of this work has also come the discovery of copper chaperones—small soluble proteins that accept copper at the plasma membrane and deliver it to various cellular targets (reviewed in [221]). The yeast work led directly to the isolation of similar human proteins, indicating that these functions are widespread if not universal. Of particular relevance to the topic of this review was the identification of the gene for a protein that delivers copper to CuZnSOD [147]. In yeast, this gene is *LYS7*, and the phenotype of *lys7* mutants is due to their lack of CuZnSOD activity. Thus, even a protein such as CuZnSOD, which is soluble and cytoplasmic and easy to metallate in vitro, requires special delivery of copper in order to be active. This discovery may also have relevance for the familial forms of the neurodegenerative disease ALS (discussed in Sec. 5).

4.5. Targets of Superoxide Damage

O_2^- is a key player in hydroxyl radical generation in vivo because its dismutation is the primary source of cellular H_2O_2; H_2O_2 in the presence of redox-active metal ions (iron or copper) and a reducing agent can then be further reduced to form the dangerously reactive hydroxyl radical (the Fenton reaction). While O_2^- may act as a reducing agent for metal ions in vivo, many other cellular reducing agents can also perform this role, leaving one to wonder exactly why superoxide itself appeared so toxic. In addition, superoxide on a chemical level is a rather sluggish reactant.

Until recently, there was little hard evidence for toxicity due to direct reactions of O_2^- itself. For one thing, because of the spontaneous dismutation of superoxide to H_2O_2 and O_2, it is difficult to experimentally separate the effects of O_2^- from those of H_2O_2. However, in the past few years, specific targets for superoxide-mediated damage have been identified. Certain iron-sulfur cluster-containing enzymes with exposed 4Fe-4S clusters have been shown to be directly inactivated by superoxide in vivo as well as in vitro. Superoxide partially disassembles the 4Fe-4S cluster, inactivating the enzyme and releasing iron. These targets include the tricarboxylic acid cycle enzyme aconitase and dihydroxyacid dehydratase, an enzyme involved in branched chain amino acid synthesis in *E. coli* [222,223]. The sensitivity of aconitase to inactivation by superoxide, as well as its ubiquitous presence in respiration-competent organisms, has led to its use as a sensor for superoxide in vivo [224].

Elevated levels of oxidative stress have long been known to result in oxidation of DNA, and recent results suggest that free intracellular iron is involved in this oxidation [225,226]. A widely accepted theory is that "free" iron may bind loosely to DNA, where it can act as a catalyst for the generation, from hydrogen peroxide, of a very reactive species that then reacts with and modifies DNA in the immediate vicinity. Among the species suggested to attack DNA are hydroxyl radical, an iron ferryl radical, or an iron-bound hydroxyl radical [227]. Increases in cellular iron have recently been reported to cause iron deposition in the nucleus [225]. Cellular reductants such as superoxide, ascorbate, and NADH may then reduce the bound iron to the ferrous state where Fenton chemistry may then occur in close proximity to DNA bases and sugars [226]. This type of chemistry is also possible for copper ions adventitiously bound to DNA, which has been observed in vitro with NADH as the reductant [228] and would be expected to result in modified bases and/or single-strand breaks in the local DNA. That such accumulation of iron is dangerous is evidenced by the phenotype of yeast *yfh1* mutants. These mutants lack the yeast equivalent of the human protein responsible for Friedreich's ataxia (frataxin) and accumulate iron in their mitochondria. They invariably lose mitochondrial function due to damage to the mitochondrial DNA and are hypersensitive to added hydrogen peroxide [229].

The astute reader will have noticed that another effect of the inactivation of iron-sulfur cluster enzymes is the release of "free" iron into the cellular fluids. Because this free iron can then participate in the Fenton reaction, leading to further damage at sites close to the iron ion, it can be very dangerous. Such a mechanism has been proposed and discussed by Liochev and Fridovich [230]. (See also Chaps. 1 and 3 of this volume.)

In conclusion, it now appears that the toxicity of superoxide is due primarily to two factors: (1) its role as the main precursor of mitochondrially generated H_2O_2 and (2) its ability to react with exposed 4Fe-4S clusters. The second factor may be critically important as it leads to release of free iron as well as inactivation of certain key enzymes.

4.6. Role of SOD in Apoptosis and Cell Death

There has recently been a great surge of interest in programmed cell death, also known as apoptosis. In response to various kinds of signals, both natural and damage-induced, most if not all kinds of cells can enter a pathway in which they essentially self-destruct in a manner designed to minimize damage to surrounding cells and to promote easy cleanup of the debris. Apoptosis is important during normal development of multicellular organisms, when certain cells are programmed to die during remodeling of various tissues, as well as being a defense mechanism against cancer. Undesired apoptosis has been implicated in some neurological diseases and other pathological states. There is a large and fascinating literature on this subject, which is, unfortunately, somewhat distant from the focus of this chapter. The reader is referred to some recent reviews for further information [231–233].

In recent years, it has become apparent that oxidative signals can cause apoptosis and that SOD levels can influence the degree to which these signals are effective. For example, an early result showed that overexpression of wild-type CuZnSOD in a neural cell line caused increased resistance to induction of apoptosis by two different signals—either withdrawal of growth factors or treatment with a calcium ionophore [234]. It has also been shown that mitochondrial changes are intimately involved in the pathway; one of the early steps in the committed pathway to apoptosis is the release of some mitochondrial contents into the cytoplasm—specifically cytochrome c and calcium (reviewed in [235,236]).

Interestingly, a death pathway may be a very early feature of eukaryotes. There are indications that even as simple a eukaryote as *S. cerevisiae* has primitive features of such a pathway, although death in this organism does not show all the defining hallmarks of apoptosis as seen in mammalian cells. As is described above, yeast mutants that lack CuZnSOD die quickly after entering stationary phase. The human antiapoptotic protein Bcl-2 [237] is partially able to prevent this death [238], indicating some relationship between the human and yeast pathways. However, it should be noted in passing that yeast do not contain an obvious homolog of Bcl-2. The evidence for a death program in yeast has been augmented by other investigators. Two different groups [239,240] have shown that some of the typical morphological changes

can be observed in dying yeast, and a third group [241] showed that cytochrome c release and decreased cytochrome oxidase activity was modulated in yeast by Bcl-2 family members, in a manner parallel to that seen in mammalian cells.

5. ROLE OF MUTANT HUMAN CuZnSUPEROXIDE DISMUTASE IN AMYOTROPHIC LATERAL SCLEROSIS

5.1. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease) is a neurodegenerative disease, with a mean age of onset of 55 years, characterized by the slow loss of large motor neurons in the spinal cord and brain [242]. In the vast majority of cases, the disease is sporadic and has no known cause (sporadic ALS, or SALS). There are, however, some encouraging data that suggest the involvement of defectively spliced glutamate transporters and glutamate excitotoxicity [243]. In approximately 10% of cases, ALS is inherited and approximately one-fifth of those familial ALS (FALS) cases are associated with mutations in *sod1*, the gene that encodes human CuZnSOD [244]. There is also evidence that a small percentage of SALS cases may also be the result of somatic or inherited mutations in *sod1* [245]. In the latter case, it may be poorly documented family histories that lead to the classification as SALS and not FALS. While most FALS-causing *sod1* mutations are dominant, requiring only one copy of the mutant gene for the ALS phenotype, two mutations have been reported in homozygous patients that apparently leave heterozygotes unaffected [246,247].

5.2. Gain-of-Function Mechanism for FALS Mutant CuZnSODs

Several studies support the conclusion that FALS mutations in CuZnSOD are dominant and exert their effects due to a gain of function: (1) Several of the FALS mutant human CuZnSOD genes (A4V, L38V, G93A, G93C, G37R, G41D, and G85R, but not H46R and H48Q which have no SOD activity) have been found to rescue the oxygen-sensitive

phenotype of *sod1Δ* yeast (see Sec. 4 above), leading to the conclusion that most FALS mutant CuZnSODs are capable of full or nearly full functionality in yeast [234,248]. (2) FALS mutant CuZnSOD genes (A4V, G93A, L38V, G93C, G37R) introduced into cultured neuronal cells promoted apoptosis where the wild-type human CuZnSOD gene was antiapoptotic, despite higher-than-normal total SOD activity in all cases [248]. (3) Constitutive expression of the FALS mutant G93A but not wild-type human CuZnSOD in a human neuroblastoma cell line induced a loss of mitochondrial membrane potential and an increase in cytosolic calcium concentration [249]. (4) Transgenic mice overexpressing FALS mutant CuZnSODs (A4V, G93A, G85R, G37R) developed a motor neuron degenerative syndrome despite normal or above-normal SOD activities [250–253], whereas transgenic mice overexpressing wild-type human CuZnSOD [250,252,253] as well as mice with no CuZnSOD did not [39].

5.3. Structural Properties of FALS Mutant CuZnSODs

Since the initial study that reported mutations in *sod1*, the number of different mutations has risen from 11 to almost 60 [34,37,244,245,247, 254–256]. When mapped onto the three-dimensional structure of human CuZnSOD these mutations tend to cluster at the dimer interface and in loop regions at the ends of β strands [36,107,257]; however, there is no real pattern to the distribution of mutations. Figure 1 illustrates the positions of FALS-mutated residues in the primary structure of human CuZnSOD. The alignment of human, bovine, and yeast sequences shows that not all of the positions are conserved from species to species. Six of the reported mutations cause premature stop codons that result in proteins that are truncated by 20–33 amino acids. Since all regions of the protein contain ALS-causing mutations, one can speculate that the structure of CuZnSOD is exquisitely sensitive to even small perturbations in the protein fold.

5.3.1. X-Ray Structure of Human G37R CuZnSOD

To date only one crystal structure for a mutant CuZnSOD has been published. In addition to high-resolution crystallography, human G37R has been studied extensively both in vivo and in vitro. Mice that are transgenic for G37R develop motor neuron disease [258]. The mutant protein has been reported to have full specific activity but a twofold reduction in biological half-life relative to wild-type [38]. G37R is also one of the mutations that was shown to enhance apoptosis in a dominant fashion in neural cell culture [234].

The x-ray structure of the human G37R was determined and analyzed to 1.9 Å resolution [90]. The structure of G37R CuZnSOD shows typical β-barrel topology consistent with known CuZnSOD structures and there are no gross deviations from the wild-type and thermostable mutant human CuZnSOD protein coordinates in the Protein Data Bank (pdb 1 spd, pdb 1 sos) [36,47]. While human CuZnSOD expressed in *E. coli* is not N-acetylated as it is in human cells, human G37R CuZnSOD is properly modified by the yeast expression system [40,259]. The replacement of glycine by arginine causes remarkably little rearrangement of the protein backbone relative to wild type.

The only major change in the structure is that the two subunits in the G37R dimer have distinct environments and are different in structure at their copper binding sites. In one subunit, the bridging imidazolate (His63) coordinates both metal ions and the metal ion in the copper site has a four-coordinate ligand geometry suggesting an oxidized copper site. The other subunit shows a distorted trigonal planar geometry at the copper site and no electron density between the copper ion and His63. This is consistent with a reduced copper site. The subunit asymmetry seen in this crystal structure may indicate that mutations can affect subunit communication and the symmetrical nature of the human dimer.

5.3.2. Truncation Mutations

While the majority of FALS-causing mutations are single substitutions of one amino acid residue for another, six of the reported mutations result in proteins truncated by as many as 33 amino acids. These proteins are missing part or all of the last β strand, which is involved in dimer contact; it is difficult to imagine that they are long-lived enough to cause disease. Indeed, several studies have failed to find evidence that truncated protein is even synthesized [260–262]. How these mutations result in the same gain of function as the others is

enigmatic, and it is precisely this question that seems to derail otherwise elegant theories as to why *sod1* mutations cause this disease. It may well be that these mutations hold the key to uncovering the pathogenicity of FALS mutants.

5.4. Hypotheses for Toxicity of Mutant CuZnSOD

5.4.1. Protein Aggregation and Neurofilament Abnormalities

Protein aggregation has been implicated in CuZnSOD-associated FALS in several studies. Human FALS mutant CuZnSODs, but not wild-type human CuZnSOD, was observed to aggregate when expressed in cultured spinal motor neurons [263]. In a yeast two-hybrid assay, FALS mutant CuZnSOD was found to bind to two proteins, lysyl-tRNA synthetase and translocon-associated protein delta, whereas wild-type did not [264]. It is also interesting to note that expression of many of the FALS mutant human CuZnSODs in *E. coli* led to formation of inclusion bodies, whereas expression of the wild type did not [288]. Aggregation might also explain the toxicity of the unstable truncation mutations.

Swelling of the axon of the motor neuron and neurofilament abnormalities are frequently associated with ALS and have been observed in the transgenic mice expressing FALS mutant CuZnSODs as well [265–267]. The hypothesis that the deleterious effect of the FALS mutant CuZnSODs cause neurofilament abnormalities is further supported by the recent demonstration of impaired axonal transport in the ventral roots of human G93A CuZnSOD transgenic mice and the appearance of neurofilament inclusions and vacuoles in vulnerable motor neurons [268].

5.4.2. Alterations in Metal Binding Properties

Since CuZnSOD is an abundant component of eukaryotic cells and it is both a copper- and a zinc-binding protein, one cannot rule out its involvement in metal metabolism. Both copper and zinc have been implicated in neurotoxicity [269,270], and alterations in their intracellular levels or partitioning could potentially mediate motor neuron death in ALS. Several ALS mutations were found to alter the characteristic metal ion binding properties of wild-type CuZnSODs in the case of both yeast FALS analogs [248,271] and the mutant human enzymes

[271–273]. Also, non-protein-bound copper was found to elute close to mutant SOD protein during purification from ALS erythrocytes. This suggested that at least for G37R and H46R copper could leak out during the purification process [260].

Refuting this idea is a study that demonstrated copper replete protein isolated from patients with the D90A mutation [274]. Total blood and plasma copper and zinc levels were shown to be virtually the same in FALS patients and nonneurological controls, and no increase in "free" copper was seen in FALS erythrocytes [275]. Although this rules out gross changes in organismal metal metabolism, it should be noted that blood is not cerebrospinal fluid and erythrocytes are not neurons, so these data do not rule out localized changes in the nervous system or changes in intracellular localization or state of chelation for copper and zinc. It is also possible that relatively small changes in the intracellular levels of these metals could result in toxicity and that this might go unnoticed due to the insensitivity of the experimental method.

5.4.3. Catalysis of Oxidations by Hydrogen Peroxide

Copper is strongly implicated in the pathogenic mechanism of FALS mutant CuZnSODs. Copper chelators provide a modicum of protection from FALS mutant-induced death in both a cell culture model of FALS [234] and in the FALS SOD-expressing transgenic mice [276]. As was stated before in Sec. 3, CuZnSOD is remarkably specific for superoxide. It is possible that these mutants have lost this specificity and are able to react with other favorable substrates to a greater extent than is the wild-type protein. This theory fits with the dominant gain of function that is the working hypothesis for disease initiation.

Wild-type CuZnSOD can react with hydrogen peroxide to generate hydroxyl radical ($^{\bullet}OH$) or a hydroxyl radical-like species, which is decidedly capable of damaging cellular components. Fortunately, nature has designed CuZnSOD to minimize this thermodynamically favorable but biologically undesirable reaction [159,277]. This reaction can be monitored using the spin trap 5,5′-dimethyl-1-pyrroline *N*-oxide as an acceptor for the hydroxyl radical. In this fashion it was shown that two human FALS mutants, A4V and G93A, possessed higher peroxidative activities than did wild-type human CuZnSOD [40,278,279], while in vitro this mechanism uses peroxide as a reductant of the oxidized enzyme—a reaction that is known to be quite slow [158]. In vivo, other

cellular reductants are likely to play a part as well, especially considering the fact that the actual levels of intracellular hydrogen peroxide are likely to be quite low. A recent study observed a superoxide-dependent peroxidase activity for the H48Q FALS CuZnSOD mutant enzyme. The same activity was not observed for the G93A, G93R, or E100G mutants [280]. The possibility that ascorbate could act as a cellular reductant for FALS mutants is also intriguing [271].

While this theory is not proven it must be noted that there is an increasing body of evidence for excess oxidative damage in tissues of ALS patients. Oxidatively damaged nucleic acids, proteins, and lipids have recently been reported in tissue samples obtained from both sporadic ALS and SOD-associated FALS patients [281]. Moreover, vitamin E was shown to have a beneficial effect on transgenic mice expressing G93A mutant CuZnSOD [282].

5.4.4. Catalysis of Tyrosine Nitration

As previously mentioned, CuZnSOD has the ability to react with peroxynitrite to catalyze the nitration of tyrosine residues. Peroxynitrite is formed by the rapid reaction of superoxide with nitric oxide in vivo and it is possible that the toxicity of mutant CuZnSODs is mediated through the further reaction of this powerful oxidant with the enzyme to nitrate protein-bound tyrosine residues [283].

Supporting this hypothesis is evidence from G37R transgenic mice that exhibit a two- to threefold elevation in free nitrotyrosine levels in spinal cord tissue relative to normal mice or mice expressing high levels of wild-type human enzyme [284]. This is also supported by a study that demonstrated increased 3-nitrotyrosine immunoreactivity in the motor neurons of both sporadic and familial ALS patients [285,286]. The peroxynitrite-dependent nitration activity is also enhanced in FALS mutants that have been depleted of zinc [272]. The same group also showed that CuZnSOD was capable of nitrating neurofilament L, providing a likely target within motor neurons [154].

5.5. Conclusions

While there is no consensus regarding the factor or factors that contribute to the pathogenicity of mutant CuZnSODs, clear progress has been made in revealing the more subtle characteristics of this well-studied enzyme. Biophysical characterization of mutant and wild-type CuZnSODs continues to shed light on the structural requirements for proper function just as biological studies have continued to expand the definition of proper function. An illustration of the complexity of the problem is the transgenic mouse model. To date four lines of mice with four different mutations have been studied. What has been determined is that each mutation seems to have a different pathological course with the same end-result — death by motor neuron disease. It is possible that there are multiple properties of the mutant enzymes that contribute to the toxicity, including SOD, peroxidative, and nitration activities, as well as protein stability. The inability to correlate properties of individual mutations with disease severity coupled with heterogeneity within FALS families suggests the possibility of other as-yet-unidentified factors that contribute to the progression of the disease. One study advanced the idea that apolipoprotein E genotyping may play a role [287] in SALS. This line of research may prove beneficial for the FALS community as well.

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ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
CNS	central nervous system
CuZnSOD	copper, zinc superoxide dismutase
DSC	differential scanning calorimetry
ECSOD	extracellular superoxide dismutase
EPR	electron paramagnetic resonance
FALS	familial amyotrophic lateral sclerosis (inherited form of the disease)
GSH	reduced glutathione

GSSG	oxidized glutathione
MnSOD	manganese superoxide dismutase
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NMR	nuclear magnetic resonance
QH_2	reduced coenzyme Q, or ubiquinol
ROS	reactive oxygen species
SALS	sporadic amyotrophic lateral sclerosis
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
*sod1*Δ	gene deletion of CuZnSOD
*sod2*Δ	gene deletion of MnSOD

REFERENCES

1. M. Dole, *J. Gen. Physiol.*, *49*, 5–27 (1965).
2. J. M. McCord and I. Fridovich, *J. Biol. Chem.*, *244*, 6049–6055 (1969).
3. S. Marklund, *Acta Chem. Scand.*, *27*, 1458–1460 (1973).
4. M. Brouwer, T. H. Brouwer, W. Grater, J. J. Enghild, and I. B. Thogersen, *Biochemistry*, *36*, 13381–13388 (1997).
5. J. G. Scandalios, in *Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (J. G. Scandalios, ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997, pp. 527–568.
6. F. J. Corpas, L. M. Sandalios, L. A. Del Rio, and R. N. Trelease, *New Phytol.*, *138*, 307–314 (1998).
7. J. S. Kroll, P. R. Langford, K. E. Wilks, and A. D. Keil, *Microbiology*, *141*, 2271–2279 (1995).
8. M. D. Tomalski, R. Eldridge, and L. K. Miller, *Virology*, *184*, 149–161 (1991).
9. A. F. Miller and D. L. Sorkin, *Comments Mol. Cell. Biophys.*, *9*, 1–48 (1997).
10. M. M. Whittaker and J. W. Whittaker, *Biochemistry*, *36*, 8923–8931 (1997).
11. M. M. Whittaker and J. W. Whittaker, *Biochemistry*, *35*, 6762–6770 (1996).

12. D. Touati, in *Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (J. G. Scandalios, ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997, pp. 447–493.
13. J. A. Fee, in *Superoxide and Superoxide Dismutases* (A. M. Michelson, J. M. McCord, and I. Fridovich, eds.), Academic Press, New York, 1977, pp. 173–192.
14. H. D. Youn, H. Youn, J. W. Lee, Y. I. Yim, J. K. Lee, Y. C. Hah, and S. O. Kang, *Arch. Biochem. Biophys.*, *334*, 341–348 (1996).
15. H. D. Youn, E. J. Kim, J. H. Roe, Y. C. Hah, and S. O. Kang, *Biochem. J.*, *318*, 889–896 (1996).
16. E. J. Kim, H. P. Kim, Y. C. Hah, and J. H. Roe, *Eur. J. Biochem.*, *241*, 178–185 (1996).
17. I. Bertini, S. Mangani, and M. S. Viezzoli, *Adv. Inorg. Chem.*, *45*, 127–250 (1998).
18. I. Bertini, L. Banci, and M. Piccioli, *Coord. Chem. Rev.*, *100*, 67–103 (1990).
19. J. V. Bannister, W. H. Bannister, and G. Rotilio, *CRC Crit. Rev. Biochem.*, *22*, 110–180 (1987).
20. G. Rotilio, L. Calabrese, A. Rigo, and E. M. Fielden, *Adv. Exp. Med. Biol.*, *148*, 155–168 (1982).
21. H. M. Steinman, in *Superoxide Dismutase*, Vol. 1 (L. W. Oberley, ed.), CRC Press, Boca Raton, 1982, pp. 11–68.
22. J. S. Valentine and M. W. Pantoliano, in *Copper Proteins* (T. G. Spiro, ed.), John Wiley and Sons, New York, 1981, pp. 292–358.
23. J. S. Valentine, in *Bioinorganic Chemistry* (I. Bertini, H. B. Gray, S. J. Lippard, and J. S. Valentine, eds.), University Science Books, Mill Valley, CA, 1994, pp. 253–313.
24. D. E. Cabelli, D. Riley, J. A. Rodriguez, J. S. Valentine, and H. Zhu, in *Biomimetic Oxidations Catalyzed by Transition Metal Complexes* (B. Meunier, ed.), Imperial College Press (in press).
25. I. Fridovich, *J. Biol. Chem.*, *272*, 18515–18517 (1997).
26. I. Fridovich, *Annu. Rev. Biochem.*, *64*, 97–112 (1995).
27. I. Fridovich, *Adv. Enzymol. Rel. Areas Mol. Biol.*, *58*, 61–97 (1986).
28. I. Fridovich, *Adv. Exp. Biol. Med.*, *74*, 530–539 (1976).
29. I. Fridovich, *Annu. Rev. Biochem.*, *44*, 147–159 (1975).
30. E. B. Gralla and D. J. Kosman, *Adv. Genet.*, *30*, 251–319 (1992).

31. E. B. Gralla, in *Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (J. G. Scandalios, ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997, pp. 495–525.
32. J. S. Valentine, D. L. Wertz, T. J. Lyons, L.-L. Liou, J. J. Goto, and E. B. Gralla, *Curr. Op. Chem. Biol.*, *2*, 253–262 (1998).
33. R. H. Brown, Jr., *Curr. Opin. Neurobiol.*, *5*, 841–846 (1995).
34. T. Siddique, D. Nijhawan, and A. Hentati, *J. Neural Transm. Suppl.*, *49*, 219–233 (1997).
35. T. Mann and D. Keilin, *Proc. Roy. Soc.*, *B126*, 303 (1939).
36. H.-X. Deng, A. Hentati, J. H. Tainer, Z. Iqbal, A. Cayabyab, W.-Y. Hung, E. D. Getzoff, P. Hu, B. Herzfeldt, R. P. Roos, C. Warner, G. Deng, E. Soriano, C. Smyth, H. E. Parge, A. Ahmed, A. D. Roses, R. A. Hallewell, M. Pericak-Vance, and T. Siddique, *Science*, *261*, 1047–1051 (1993).
37. D. R. Rosen, T. Siddique, D. Patterson, D. A. Figlewicz, P. Sapp, A. Hentati, D. Donaldson, J. Goto, J. P. O'Regan, H.-X. Deng, Z. Rahmani, A. Krizus, D. McKenna-Yasek, A. Cayabyab, S. M. Gaston, R. Berger, R. E. Tanzi, J. J. Halperin, B. Herzfeldt, R. Van den Bergh, W.-Y. Hung, T. Bird, G. Deng, D. W. Mulder, C. Smyth, N. G. Laing, E. Soriano, M. A. Pericak-Vance, J. Haines, G. A. Rouleau, J. S. Gusella, H. R. Horvitz, and R. H. Brown, Jr., *Nature*, *362*, 59–62 (1993).
38. D. R. Borchelt, M. K. Lee, H. S. Slunt, M. Guarnieri, Z. S. Xu, P. C. Wong, R. H. Brown, D. L. Price, S. S. Sisodia, and D. W. Cleveland, *Proc. Natl. Acad. Sci. USA*, *91*, 8292–8296 (1994).
39. A. G. Reaume, J. L. Elliott, E. K. Hoffman, N. W. Kowall, R. J. Ferrante, D. F. Siwek, H. M. Wilcox, D. G. Flood, M. F. Beal, R. H. J. Brown, R. W. Scott, and W. D. Snider, *Nature Genet.*, *13*, 43–47 (1996).
40. M. Wiedau-Pazos, J. J. Goto, S. Rabizadeh, E. B. Gralla, J. A. Roe, M. K. Lee, J. S. Valentine, and D. E. Bredesen, *Science*, *271*, 515–518 (1996).
41. T. D. Oury, J. D. Crapo, Z. Valnickova, and J. J. Enghild, *Biochem. J.*, *317*, 51–57 (1996).
42. A. Battistoni and G. Rotilio, *FEBS Lett.*, *374*, 199–202 (1995).
43. S. Kanematsu and K. Asada, *Plant Cell Physiol.*, *30*, 381–391 (1989).

44. J. S. Richardson, D. C. Richardson, K. A. Thomas, E. W. Silverton, and D. R. Davies, *J. Mol. Biol.*, *102*, 221–235 (1976).
45. J. A. Tainer, E. D. Getzoff, K. M. Beem, J. S. Richardson, and D. C. Richardson, *J. Mol. Biol.*, *160*, 181–217 (1982).
46. H. E. Parge, E. D. Getzoff, C. S. Scandella, R. A. Hallewell, and J. A. Tainer, *J. Biol. Chem.*, *261*, 16215–16218 (1986).
47. H. E. Parge, R. A. Hallewell, and J. A. Tainer, *Proc. Natl. Acad. Sci. USA*, *89*, 6109– 6113 (1992).
48. K. Djinovic, G. Gatti, A. Coda, L. Antolini, G. Pelosi, A. Desideri, M. Falconi, F. Marmocchi, G. Rotilio, and M. Bolognesi, *J. Mol. Biol.*, *225*, 791–809 (1992).
49. K. Djinovic, G. Gatti, A. Coda, L. Antolini, G. Pelosi, A. Desideri, M. Falconi, F. Marmocchi, G. Rotilio, and M. Bolognesi, *Acta Crystallogr.*, *B47*, 918–927 (1991).
50. F. Frigerio, M. Falconi, G. Gatti, M. Bolognesi, A. Desideri, F. Marmocchi, and G. Rotilio, *Biochem. Biophys. Res. Commun.*, *160*, 677–681 (1989).
51. N. L. Ogihara, H. E. Parge, P. J. Hart, M. S. Weiss, J. J. Goto, B. R. Crane, J. Tsang, K. Slater, J. A. Roe, J. S. Valentine, D. Eisenberg, and J. A. Tainer, *Biochemistry*, *35*, 2316–2321 (1996).
52. W. R. Rypniewski, S. Mangani, B. Bruni, P. L. Orioli, M. Casati, and K. S. Wilson, *J. Mol. Biol.*, *251*, 282–296 (1995).
53. K. A. Thomas, B. H. Rubin, C. J. Bier, J. S. Richardson, and D. C. Richardson, *J. Biol. Chem.*, *249*, 5677–5683 (1974).
54. J. S. Richardson, K. A. Thomas, B. H. Rubin, and D. C. Richardson, *Proc. Natl. Acad. Sci. USA*, *72*, 1349–1353 (1975).
55. K. Djinovic, A. Coda, L. Antolini, G. Pelosi, A. Desideri, M. Falconi, G. Rotilio, and M. Bolognesi, *J. Mol. Biol.*, *226*, 227–238 (1992).
56. K. Djinovic-Carugo, F. Polticelli, A. Desideri, G. Rotilio, K. S. Wilson, and M. Bolognesi, *J. Mol. Biol.*, *240*, 179–183 (1994).
57. D. E. McRee, S. M. Redford, E. D. Getzoff, J. R. Lepock, R. A. Hallewell, and J. A. Tainer, *J. Biol. Chem.*, *265*, 14234–14241 (1990).
58. C. D. Smith, M. Carson, M. van der Woerd, J. Chen, H. Ischiropoulos, and J. S. Beckman, *Arch. Biochem. Biophys.*, *299*, 350–355 (1992).

59. K. Djinovic-Carugo, C. Collyer, A. Coda, M. T. Carri, A. Battistoni, G. Bottaro, F. Polticelli, A. Desideri, and M. Bolognesi, *Biochem. Biophys. Res. Commun.*, *194*, 1008–1011 (1993).
60. K. Djinovic-Carugo, A. Battistoni, M. T. Carri, F. Polticelli, A. Desideri, G. Rotilio, A. Coda, K. S. Wilson, and M. Bolognesi, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, *1*, 176–188 (1996).
61. Y. Kitagawa, N. Tanaka, Y. Hata, M. Kusunoki, G. Lee, Y. Katsube, K. Asada, S. Aibara, and Y. Morita, *J. Biochem.*, *109*, 477–485 (1991).
62. W. H. Bannister, J. V. Bannister, D. Barra, J. Bond, and F. Bossa, *Free Rad. Res. Commun.*, *12–13*, 349–361 (1991).
63. D. Bordo, K. Djinovic, and M. Bolognesi, *J. Mol. Biol.*, *238*, 366–386 (1994).
64. J. V. Bannister, A. Anastasi, and W. H. Bannister, *Biochem. Biophys. Res. Commun.*, *81*, 469–472 (1978).
65. F. Marmocchi, G. Venardi, F. Bossa, A. Rigo, and G. Rotilio, *FEBS Lett.*, *94*, 109–111 (1978).
66. A. Rigo, F. Marmocchi, D. Cocco, P. Viglino, and G. Rotilio, *Biochemistry*, *17*, 534–537 (1978).
67. F. Marmocchi, G. Venardi, G. Caulini, and G. Rotilio, *FEBS Lett.*, *44*, 337–339 (1974).
68. F. Marmocchi, G. Caulini, G. Venardi, D. Cocco, L. Calabrese, and G. Rotilio, *Physiol. Chem. Phys.*, *7*, 465–471 (1975).
69. G. Caulini, F. Marmocchi, G. Venardi, and G. Rotilio, *Boll. Soc. Ital. Biol. Sper.*, *50*, 1091–1094 (1974).
70. D. P. Malinkowski and I. Fridovich, *Biochemistry*, *18*, 5055–5060 (1979).
71. K. Inouye, A. Osaki, and B. Tonomura, *J. Biochem.*, *115*, 507–515 (1994).
72. G. Mei, N. Rosato, N. Silva, Jr., R. Rusch, E. Gratton, I. Savini, and A. Finazzi-Agro, *Biochemistry*, *31*, 7224–7230 (1992).
73. N. Silva, Jr., E. Gratton, G. Mei, N. Rosato, R. Rusch, and A. Finazzi-Agro, *Biophys. Chem.*, *48*, 171–182 (1993).
74. F. Marmocchi, I. Mavelli, A. Rigo, R. Stevanato, F. Bossa, and G. Rotilio, *Biochemistry*, *21*, 2853–2856 (1982).
75. S. Folcarelli, A. Battistoni, M. T. Carri, F. Polticelli, M. Falconi, L. Nicolini, L. Stella, N. Rosato, G. Rotilio, and A. Desideri, *Protein Eng.*, *9*, 323–325 (1996).

76. I. Bertini, M. Piccioli, M. S. Viezzoli, C. Y. Chiu, and G. T. Mullenbach, *Eur. Biophys. J.*, *23*, 167–176 (1994).
77. L. Banci, I. Bertini, C. Y. Chiu, G. T. Mullenbach, and M. S. Viezzoli, *Eur. J. Biochem.*, *234*, 855–860 (1995).
78. L. Banci, M. Benedetto, I. Bertini, R. Del Conte, M. Piccioli, T. Richert, and M. S. Viezzoli, *Magnet. Reson. Chem.*, *35*, 845–853 (1997).
79. L. Banci, L. Bertini, M. S. Viezzoli, E. Argese, E. F. Orsega, C. Y. Chiu, and G. T. Mullenbach, *J. Biol. Inorg. Chem.*, *2*, 295–301 (1997).
80. S. J. Lippard, A. R. Burger, K. Ugurbil, J. S. Valentine, and M. W. Pantoliano, in *Advances in Chemistry Series, No. 162, Bioinorganic Chemistry II* (K. Raymond, ed.), American Chemical Society, Washington, D.C., 1977, pp. 251–262.
81. J. A. Roe, A. Butler, D. M. Scholler, J. S. Valentine, L. Marky, and K. Breslauer, *Biochemistry*, *27*, 950–958 (1988).
82. E. M. Fielden, P. B. Roberts, R. C. Bray, D. J. Lowe, G. N. Mautner, G. Rotilio, and L. Calabrese, *Biochem. J.*, *139*, 49–60 (1974).
83. A. Rigo, P. Viglino, L. Calabrese, D. Cocco, and G. Rotilio, *Biochem. J.*, *161*, 27–30 (1977).
84. M. S. Viezzoli and Y. Wang, *Inorg. Chim. Acta*, *153*, 189–191 (1988).
85. J. Hirose, S. Toida, H. Kuno, S. Ozaki, and Y. Kidani, *Chem. Pharm. Bull. (Tokyo)*, *36*, 2103–2108 (1988).
86. M. Falconi, R. Gallimbeni, and E. Paci, *J. Comput.-Aided Mol. Des.*, *10*, 490–498 (1996).
87. G. Chillemi, M. Falconi, A. Amadei, G. Zimatore, A. Desideri, and A. DiNola, *Biophys. J.*, *73*, 1007–1018 (1997).
88. J. C. Dunbar, B. Holmquist, and J. T. Johansen, *Biochemistry*, *23*, 4330–4335 (1984).
89. T. Lyons, A. Nersissian, J. J. Goto, H. Zhu, E. B. Gralla, and J. S. Valentine, *J. Biol. Inorg. Chem.* (in press).
90. P. J. Hart, H. Liu, M. Pellegrini, A. M. Nersissian, E. B. Gralla, J. S. Valentine, and D. Eisenberg, *Prot. Sci.*, *7*, 545–555 (1998).

91. L. Benov, H. Sage, and I. Fridovich, *Arch. Biochem. Biophys.*, *340*, 305–310 (1997).
92. L. T. Benov and I. Fridovich, *J. Biol. Chem.*, *269*, 25310–25314 (1994).
93. A. Battistoni, S. Folcarelli, L. Cervoni, F. Polizio, A. Desideri, A. Giartosio, and G. Rotilio, *J. Biol. Chem.*, *273*, 5655–5661 (1998).
94. J. R. Lepock, L. D. Arnold, B. H. Torrie, B. Andrews, and J. Kruuv, *Arch. Biochem. Biophys.*, *241*, 243–251 (1985).
95. Y. Senoo, K. Katoh, Y. Nakai, Y. Hashimoto, K. Bando, and S. Teramoto, *Acta Medica Okayama*, *42*, 169–174 (1988).
96. J. Kajihara, M. Enomoto, K. Seya, Y. Sukenaga, and K. Katoh, *J. Biochem.*, *104*, 638–642 (1988).
97. W. H. Bannister and E. J. Wood, *Life Sci.*, *9*, 229–233 (1970).
98. N. Crosti, *Biochem. Genet.*, *16*, 739–742 (1978).
99. E. Wenisch, K. Vorauer, A. Jungbauer, H. Katinger, and P. G. Righetti, *Electrophoresis*, *15*, 647–653 (1994).
100. L. Calabrese, G. Federici, W. H. Bannister, J. V. Bannister, G. Rotilio, and A. Finazzi-Agro, *Eur. J. Biochem.*, *56*, 305–309 (1975).
101. B. P. Sharonov and I. V. Churilova, *Biochem. Biophys. Res. Commun.*, *189*, 1129–1135 (1992).
102. I. Mavelli, M. R. Ciriolo, and G. Rotilio, *Biochem. Biophys. Res. Commun.*, *117*, 677–681 (1983).
103. S. L. Jewett, *Inorg. Chim. Acta*, *79*, 144–145 (1983).
104. K. Arai, S. Iizuka, Y. Tada, K. Oikawa, and N. Taniguchi, *Biochim. Biophys. Acta*, *924*, 292–296 (1987).
105. N. Taniguchi, K. Arai, and N. Kinoshita, *Meth. Enzymol.*, *179*, 570–581 (1989).
106. N. Kawamura, T. Ookawara, K. Suzuki, K. Konishi, M. Mino, and N. Taniguchi, *J. Clin. Endocrinol. Metab.*, *74*, 1352–1354 (1992).
107. D. E. Bredesen, L. M. Ellerby, P. J. Hart, M. Wiedau-Pazos, and J. S. Valentine, *Ann. Neurol.*, *42*, 135–137 (1997).
108. L. Rossi, E. Marchese, A. De Martino, G. Rotilio, and M. R. Ciriolo, *Biometals*, *10*, 257–262 (1997).
109. S. L. Jewett, G. S. Latrenta, and C. M. Beck, *Arch. Biochem. Biophys.*, *215*, 116–128 (1982).

110. S. A. Goscin and I. Fridovich, *Biochim. Biophys. Acta*, *289*, 276–283 (1972).
111. S. A. Cockle and R. C. Bray, in *Superoxide and Superoxide Dismutases* (A. M. Michelson, J. M. McCord, and I. Fridovich, eds.), Academic Press, New York, 1977, pp. 215–216.
112. F. Polizio, A. R. Bizzarri, S. Cannistraro, and A. Desideri, *Eur. Biophys. J. Biophys. Lett.*, *26*, 291–297 (1997).
113. R. A. Hallewell, K. C. Imlay, P. Lee, N. M. Fong, C. Gallegos, E. D. Getzoff, J. A. Tainer, D. E. Cabelli, P. Tekamp-Olson, G. T. Mullenbach, and L. S. Cousens, *Biochem. Biophys. Res. Commun.*, *181*, 474–480 (1991).
114. J. R. Lepock, H. E. Frey, and R. A. Hallewell, *J. Biol. Chem.*, *265*, 21612–21618 (1990).
115. G. E. Schulz and R. H. Schirmer, *Principles of Protein Structure*, Springer-Verlag, New York, 1979.
116. L. Jia, C. Bonaventura, J. Bonaventura, and J. S. Stamler, *Nature*, *380*, 221–226 (1996).
117. L. Banci, I. Bertini, D. E. Cabelli, R. A. Hallewall, J. W. Tung, and M. S. Viezzoli, *Eur. J. Biochem.*, *196*, 123–128 (1991).
118. A. Merli, G. Rossi, K. Djinovic-Carugo, M. Bolognesi, A. Desideri, and G. Rotilio, *Biochem. Biophys. Res. Commun.*, *210*, 1040–1044 (1995).
119. I. Ascone, R. Castaner, C. Tarricone, M. Bolognesi, M. E. Stroppolo, and A. Desideri, *Biochem. Biophys. Res. Commun.*, *241*, 119–121 (1997).
120. I. Bertini, C. Luchinat, and R. Monnanni, *J. Am. Chem. Soc.*, *107*, 2178–2179 (1985).
121. S. Hashimoto, S. Ohsaka, H. Takeuchi, and I. Harada, *J. Am. Chem. Soc.*, *111*, 8926–8928 (1989).
122. L. M. Murphy, R. W. Strange, and S. S. Hasnain, *Structure*, *5*, 371–379 (1997).
123. M. E. McAdam, E. M. Fielden, F. Lavelle, L. Calabrese, D. Cocco, and G. Rotilio, *Biochem. J.*, *167*, 271–274 (1977).
124. P. O'Neill, E. M. Fielden, D. Cocco, G. Rotilio, and L. Calabrese, *Biochem. J.*, *205*, 181–187 (1982).
125. L. Calabrese, G. Rotilio, and B. Mondovi, *Biochim. Biophys. Acta*, *263*, 827–829 (1972).

126. L. Calabrese, D. Cocco, L. Morpurgo, B. Mondovi, and G. Rotilio, *Eur. J. Biochem., 64*, 465–470 (1976).
127. K. M. Beem, W. E. Rich, and K. V. Rajagopalan, *J. Biol. Chem., 249*, 7298–7305 (1974).
128. J. A. Fee and R. G. Briggs, *Biochim. Biophys. Acta, 400*, 439–450 (1975).
129. H. J. Forman and I. Fridovich, *J. Biol. Chem., 248*, 2645–2649 (1973).
130. L.-J. Ming and J. S. Valentine, *J. Am. Chem. Soc., 109*, 4426–4428 (1987).
131. J. Kajihara, M. Enomoto, K. Katoh, K. Mitsuta, and M. Kohno, *Agric. Biol. Chem., 54*, 495–499 (1990).
132. L. Calabrese, D. Cocco, and A. Desideri, *FEBS Lett., 106*, 142–144 (1979).
133. P. Kofod, R. Bauer, E. Danielsen, E. Larsen, and M. J. Bjerrum, *Eur. J. Biochem., 198*, 607–611 (1991).
134. L.-J. Ming and J. S. Valentine, *J. Am. Chem. Soc., 112*, 6374–6383 (1990).
135. K. M. Beem, D. C. Richardson, and K. V. Rajagopalan, *Biochemistry, 16*, 1930–1936 (1977).
136. M. R. Ciriolo, P. Civitareale, M. T. Carri, A. De Martino, F. Galiazzo, and G. Rotilio, *J. Biol. Chem., 269*, 25783–25787 (1994).
137. V. C. Culotta, H. D. Joh, S. J. Lin, K. H. Slekar, and J. Strain, *J. Biol. Chem., 270*, 29991–29997 (1995).
138. K. T. Tamai, E. B. Gralla, L. M. Ellerby, J. S. Valentine, and D. J. Thiele, *Proc. Natl. Acad. Sci. USA, 90*, 8013–8017 (1993).
139. C. T. Dameron and E. D. Harris, *Biochem. J., 248*, 663–668 (1987).
140. R. A. DiSilvestro, *Arch. Biochem. Biophys., 274*, 298–303 (1989).
141. A. Levieux, D. Levieux, and C. Lab, *Free Radic. Biol. Med., 11*, 589–595 (1991).
142. K. L. Olin, M. S. Golub, M. E. Gershwin, A. G. Hendrickx, B. Lonnerdal, and C. L. Keen, *Am. J. Clin. Nutr., 61*, 1263–1267 (1995).
143. F. Galiazzo, M. R. Ciriolo, M. T. Carri, P. Civitareale, L. Marcocci, F. Marmocchi, and G. Rotilio, *Eur. J. Biochem., 196*, 545–549 (1991).

144. C. Steinkuhler, M. T. Carri, G. Micheli, L. Knoepfel, U. Weser, and G. Rotilio, *Biochem. J., 302*, 687–694 (1994).
145. C. Steinkuhler, O. Sapora, M. T. Carri, W. Nagel, L. Marcocci, M. R. Ciriolo, U. Weser, and G. Rotilio, *J. Biol. Chem., 266*, 24580–24587 (1991).
146. N. Petrovic, A. Comi, and M. J. Ettinger, *J. Biol. Chem., 271*, 28331–28334 (1996).
147. V. C. Culotta, L. W. Klomp, J. Strain, R. L. Casareno, B. Krems, and J. D. Gitlin, *J. Biol. Chem., 272*, 23469–23472 (1997).
148. J. R. Ellis, *Science, 250*, 954–959 (1990).
149. G. Rotilio, R. C. Bray, and E. M. Fielden, *Biochim. Biophys. Acta, 268*, 605–609 (1972).
150. E. K. Hodgson and I. Fridovich, *Biochem. Biophys. Res. Commun., 54*, 270–274 (1973).
151. M. A. Symonyan and R. M. Nalbandyan, *FEBS Lett., 28*, 22–24 (1972).
152. J. A. Fee and P. E. DiCorleto, *Biochemistry, 12*, 4893–4899 (1973).
153. M. W. Pantoliano, J. S. Valentine, A. R. Burger, and S. J. Lippard, *J. Inorg. Biochem., 17*, 325–341 (1982).
154. J. P. Crow, Y. Z. Ye, M. Strong, M. Kirk, S. Barnes, and J. S. Beckman, *J. Neurochem., 69*, 1945–1953 (1997).
155. R. J. Carrico and H. F. Deutsch, *J. Biol. Chem., 245*, 723–727 (1970).
156. P. Viglino, M. Scarpa, F. Coin, G. Rotilio, and A. Rigo, *Biochem. J., 237*, 305–308 (1986).
157. P. Viglino, M. Scarpa, D. Cocco, and A. Rigo, *Biochem. J., 229*, 87–90 (1985).
158. A. Rigo, P. Viglino, M. Scarpa, and G. Rotilio, in *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine* (G. Rotilio, ed.), Elsevier, Amsterdam, 1986, pp. 184–188.
159. M. B. Yim, P. B. Chock, and E. R. Stadtman, *Proc. Natl. Acad. Sci. USA, 87*, 5006–5010 (1990).
160. D. E. Cabelli, D. Allen, B. J. Bielski, and J. Holcman, *J. Biol. Chem., 264*, 9967–9971 (1989).
161. E. K. Hodgson and I. Fridovich, *Biochemistry, 14*, 5294–5298 (1975).

162. K. Uchida and S. Kawakishi, *J. Biol. Chem.*, *269*, 2405–2410 (1994).

163. J. S. Beckman, H. Ischiropoulos, L. Zhu, M. van der Woerd, C. Smith, J. Chen, J. Harrison, J. C. Martin, and M. Tsai, *Arch. Biochem. Biophys.*, *298*, 438–445 (1992).

164. J. S. Beckman, J. Chen, J. P. Crow, and Y. Z. Ye, *Prog. Brain Res.*, *103*, 371–380 (1994).

165. D. G. Searcy, J. P. Whitehead, and M. J. Maroney, *Arch. Biochem. Biophys.*, *318*, 251–263 (1995).

166. D. G. Searcy, *Arch. Biochem. Biophys.*, *334*, 50–58 (1996).

167. D. Deters and U. Weser, *Biometals*, *8*, 25–29 (1995).

168. W. H. Koppenol, in *Oxygen and Oxy-Radicals in Chemistry and Biology* (M. A. J. Rogers and E. L. Powers, eds.), Academic Press, New York, 1981, pp. 671–674.

169. E. D. Getzoff, J. A. Tainer, P. K. Weiner, P. A. Kollman, J. S. Richardson, and D. C. Richardson, *Nature*, *306*, 287–290 (1983).

170. D. P. Malinkowski and I. Fridovich, in *Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase* (J. V. Bannister and H. A. O. Hill, eds.), Elsevier, New York, 1980, pp. 299–317.

171. A. Cudd and I. Fridovich, *J. Biol. Chem.*, *257*, 11443–11447 (1982).

172. W. F. Beyer, I. Fridovich, G. T. Mullenbach, and R. Hallewell, *J. Biol. Chem.*, *262*, 11182–11187 (1987).

173. E. D. Getzoff, D. E. Cabelli, C. L. Fisher, H. E. Parge, M. S. Viezzoli, L. Banci, and R. A. Hallewall, *Nature*, *358*, 347–351 (1992).

174. P. Viglino, M. Scarpa, G. Rotilio, and A. Rigo, *Biochim. Biophys. Acta*, *952*, 77–82 (1988).

175. J. A. Graden, L. M. Ellerby, J. A. Roe, and J. S. Valentine, *J. Am. Chem. Soc.*, *116*, 9743–9744 (1994).

176. L. M. Ellerby, D. E. Cabelli, J. A. Graden, and J. S. Valentine, *J. Am. Chem. Soc.*, *118*, 6556–6561 (1996).

177. D. T. Sawyer and J. S. Valentine, *Acc. Chem. Res.*, *14*, 393–400 (1981).

178. P. Saltman, *Semin. Hematol.*, *26*, 249–256 (1989).

179. D. Touati, *Free Rad. Res. Commun.*, *8*, 1–9 (1989).

180. A. Carlioz and D. Touati, *EMBO J.*, *5*, 623–630 (1986).

181. X. F. Liu, I. Elashvili, E. B. Gralla, J. S. Valentine, P. Lapinskas, and V. C. Culotta, *J. Biol. Chem.*, *267*, 18298–18302 (1992).

182. E. B. Gralla and J. S. Valentine, *J. Bacteriol.*, *173*, 5918–5920 (1991).

183. A. P. van Loon, B. Pesold-Hurt, and G. Schatz, *Proc. Natl. Acad. Sci. USA*, *83*, 3820–3824 (1986).

184. C. A. Marres, A. P. Van Loon, P. Oudshoorn, H. Van Steeg, L. A. Grivell, and E. C. Slater, *Eur. J. Biochem.*, *147*, 153–161 (1985).

185. T. Bilinski, Z. Krawiec, A. Liczmanski, and J. Litwinska, *Biochem. Biophys. Res. Commun.*, *130*, 533–539 (1985).

186. R. S. Sohal and R. Weindruch, *Science*, *273*, 59–63 (1996).

187. V. D. Longo, E. B. Gralla, and J. S. Valentine, *J. Biol. Chem.*, *271*, 12275–12280 (1996).

188. V. L. Longo, L. L. Liou, J. S. Valentine, and E. B. Gralla (submitted).

189. J. P. Phillips, S. D. Campbell, D. Michaud, M. Charbonneau, and A. J. Hilliker, *Proc. Natl. Acad. Sci. USA*, *86*, 2761–2765 (1989).

190. Y.-S. Ho, M. Gargano, J. Cao, R. T. Bronson, I. Heimler, and R. J. Hutz, *J. Biol. Chem.*, *273*, 7765–7769 (1998).

191. Y. Li, T. T. Huang, E. J. Carlson, S. Melov, P. C. Ursell, J. L. Olson, L. J. Noble, M. P. Yoshimura, C. Berger, P. H. Chan, D. C. Wallace, and C. J. Epstein, *Nature Genet.*, *11*, 376–381 (1995).

192. R. M. Lebovitz, H. Zhang, H. Vogel, J. Cartwright, Jr., L. Dionne, N. Lu, S. Huang, and M. M. Matzuk, *Proc. Natl. Acad. Sci. USA*, *93*, 9782–9787 (1996).

193. S. Melov, J. A. Schneider, B. J. Day, D. Hinerfeld, P. Coskun, S. S. Mirra, J. D. Crapo, and D. C. Wallace, *Nature Genet.*, *18*, 159–163 (1998).

194. L. M. Carlsson, F. Jonsson, T. Edlund, and S. L. Marklund, *Proc. Natl. Acad. Sci. USA*, *92*, 6264–6268 (1995).

195. T.-T. Huang, M. Yasunami, E. J. Carlson, A. M. Gillespie, A. G. Reaume, E. K. Hoffman, P. H. Chan, R. W. Scott, and C. J. Epstein, *Arch. Biochem. Biophys.*, *344*, 424–432 (1997).

196. K. Murakami, T. Kondo, M. Kawase, Y. Li, S. Sato, S. F. Chen, and P. H. Chan, *J. Neurosci.*, *18*, 205–213 (1998).

197. L. J. Greenlund, T. L. Deckwerth, and E. M. J. Johnson, *Neuron*, *14*, 303–315 (1995).

198. P. M. Sinet, F. Lavelle, A. M. Michelson, and H. Jerome, *Biochem. Biophys. Res. Commun.*, *67*, 904–909 (1975).

199. K. B. Avraham, M. Schickler, D. Sapoznikov, R. Yarom, and Y. Groner, *Cell*, *54*, 823–829 (1988).

200. M. Schickler, H. Knobler, K. B. Avraham, O. Elroy-Stein, and Y. Groner, *EMBO J.*, *8*, 1385–1392 (1989).

201. B. Nabarra, M. Casanova, D. Paris, E. Paly, K. Toyoma, I. Ceballos, and J. London, *Mech. Ageing Dev.*, *96*, 59–73 (1997).

202. R. De La Torre, A. Casado, E. Lopez-Fernandez, D. Carrascosa, V. Ramirez, and J. Saez, *Experientia*, *52*, 871–873 (1996).

203. K. B. Beckman and B. N. Ames, in *Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (J. Scandalios, ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997, pp. 201– 246.

204. K. Z. Guyton, M. Gorospe, and N. J. Holbrook, in *Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (J. G. Scandalios, ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997, pp. 247–272.

205. I. Reveillaud, A. Niedzwiecki, K. G. Bensch, and J. E. Fleming, *Mol. Cell. Biol.*, *11*, 632–640 (1991).

206. W. C. Orr and R. S. Sohal, *Science*, *263*, 1128–1130 (1994).

207. J. Sun and J. Tower (in preparation).

208. H. C. Yen, T. D. Oberley, S. Vichitbandha, Y. S. Ho, and D. K. St. Clair, *J. Clin. Invest.*, *98*, 1253–1260 (1996).

209. M. D. Scott, S. R. Meshnick, and J. W. Eaton, *J. Biol. Chem.*, *262*, 3640–3645 (1987).

210. A. S. Gupta, J. L. Heinen, A. S. Holaday, J. J. Burke, and R. D. Allen, *Proc. Natl. Acad. Sci. USA*, *90*, 1629–1633 (1993).

211. A. S. Gupta, R. P. Webb, A. S. Holaday, and R. D. Allen, *Plant Physiol.*, *103*, 1067–1073 (1993).

212. P. Amstad, R. Moret, and P. Cerutti, *J. Biol. Chem.*, *269*, 1606–1609 (1994).

213. M. J. Kelner, R. Bagnell, M. Montoya, L. Estes, S. F. Uglik, and P. Cerutti, *Free Rad. Biol. Med.*, *18*, 497–506 (1995).

214. T.-T. Huang, E. J. Carlson, S. A. Leadon, and C. J. Epstein, *FASEB J.*, *6*, 903–910 (1992).

215. D. M. Glerum and A. Tzagoloff, personal communication.

216. K. H. Slekar, D. J. Kosman, and V. C. Culotta, *J. Biol. Chem.*, *271*, 28831–28836 (1996).

217. X. F. Liu, F. Supek, N. Nelson, and V. C. Culotta, *J. Biol. Chem.*, *272*, 11763–11769 (1997).

218. S. J. Lin, R. A. Pufahl, A. Dancis, T. V. O'Halloran, and V. C. Culotta, *J. Biol. Chem.*, *272*, 9215–9220 (1997).

219. S. J. Lin and V. C. Culotta, *Mol. Cell. Biol.*, *16*, 6303–6312 (1996).

220. P. J. Lapinskas, K. W. Cunningham, X. F. Liu, G. R. Fink, and V. C. Culotta, *Mol. Cell. Biol.*, *15*, 1382–1388 (1995).

221. J. S. Valentine and E. B. Gralla, *Science*, *278*, 817–818 (1997).

222. P. R. Gardner, *Biosci. Rep.*, *17*, 33–42 (1997).

223. D. H. Flint and R. M. Allen, *Chem. Rev.*, *96*, 2315–2334 (1996).

224. P. R. Gardner, I. Raineri, L. B. Epstein, and C. W. White, *J. Biol. Chem.*, *270*, 13399–13405 (1995).

225. K. Keyer and J. A. Imlay, *Proc. Natl. Acad. Sci. USA*, *93*, 13635–13640 (1996).

226. R. Meneghini, *Free Rad. Biol. Med.*, *23*, 783–792 (1997).

227. E. S. Henle and S. Linn, *J. Biol. Chem.*, *272*, 19095–19098 (1997).

228. S. Oikawa and S. Kawanishi, *Biochemistry*, *35*, 4584–4590 (1996).

229. M. Babcock, D. de Silva, R. Oaks, S. Davis-Kaplan, S. Jiralerspong, L. Montermini, M. Pandolfo, and J. Kaplan, *Science*, *276*, 1709–1712 (1997).

230. S. I. Liochev and I. Fridovich, *Free Rad. Biol. Med.*, *16*, 29–33 (1994).

231. S. W. Hetts, *JAMA*, *279*, 300–307 (1998).

232. M. E. Peter, A. E. Heufelder, and M. O. Hengartner, *Proc. Natl. Acad. Sci. USA*, *94*, 12736–12737 (1997).

233. J. L. Rinkenberger and S. J. Korsmeyer, *Curr. Opin. Genet. Dev.*, *7*, 589–596 (1997).

234. S. Rabizadeh, E. B. Gralla, D. R. Borchelt, R. Gwinn, J. S. Valentine, S. Sisodia, P. Wong, M. Lee, H. Hahn, and D. E. Bredesen, *Proc. Natl. Acad. Sci. USA*, *92*, 3024–3028 (1995).

235. S. Papa and V. P. Skulachev, *Mol. Cell. Biochem.*, *174*, 305–319 (1997).

236. N. Zamzami, T. Hirsch, B. Dallaporta, P. X. Petit, and G. Kroemer, *J. Bioenerg. Biomembr.*, *29*, 185–193 (1997).

237. J. C. Reed, *Semin. Hematol.*, *34*, 9–19 (1997).
238. V. D. Longo, L. M. Ellerby, D. E. Bredesen, J. S. Valentine, and E. B. Gralla, *J. Cell Biol.*, *137*, 1581–1588 (1997).
239. F. Madeo, E. Frohlich, and K. U. Frohlich, *J. Cell Biol.*, *139*, 729–734 (1997).
240. B. Ink, M. Zornig, B. Baum, N. Hajibagheri, C. James, T. Chittenden, and G. Evan, *Mol. Cell. Biol.*, *17*, 2468–2474 (1997).
241. S. Manon, B. Chaudhuri, and M. Guerin, *FEBS Lett.*, *415*, 29–32 (1997).
242. R. H. Brown, Jr., *Arch. Neurol.*, *54*, 1246–1250 (1997).
243. L. A. Bristol and J. D. Rothstein, *Ann. Neurol.*, *39*, 676–678 (1996).
244. M. E. Cudkowicz and R. H. Brown, Jr., *J. Neurol. Sci.*, *139 Suppl.*, 10–15 (1996).
245. M. Jackson, A. Al-Chalabi, Z. E. Enayat, B. Chioza, P. N. Leigh, and K. E. Morrison, *Ann. Neurol.*, *42*, 803–807 (1997).
246. P. M. Andersen, L. Forsgren, M. Binzer, P. Nilsson, V. Ala-Hurula, M.-L. Karenen, L. Bergmark, A. Saarinen, T. Haltia, I. Tarvainen, E. Kinnunen, B. Udd, and S. L. Marklund, *Brain*, *119*, 1153–1172 (1996).
247. C. Hayward, D. J. H. Brock, R. A. Minns, and R. J. Swingler, *J. Med. Genet.*, *35*, 174 (1998).
248. C. Nishida, E. B. Gralla, and J. S. Valentine, *Proc. Natl. Acad. Sci. USA*, *91*, 9906–9910 (1994).
249. M. T. Carri, A. Ferri, A. Battistoni, L. Famhy, R. Gabbianelli, F. Poccia, and G. Rotilio, *FEBS Lett.*, *414*, 365–368 (1997).
250. M. C. Dal Canto and M. E. Gurney, *Acta Neuropathol. (Berl)*, *93*, 537–550 (1997).
251. L. I. Bruijn, M. W. Becher, M. K. Lee, K. L. Anderson, N. A. Jenkins, N. G. Copeland, S. S. Sisodia, J. D. Rothstein, D. R. Borchelt, D. L. Price, and D. W. Cleveland, *Neuron*, *18*, 327–338 (1997).
252. M. E. Gurney, H. Pu, A. Y. Chiu, M. C. Dal Canto, C. Y. Polchow, D. D. Alexander, J. Caliendo, A. Hentati, Y. W. Kwon, H. X. Deng, W. Chen, P. Zhai, R. L. Sufit, and T. Siddique, *Science*, *264*, 1772–1775 (1994).
253. M. E. Gurney, *J. Neurol. Sci.*, *152 Suppl. 1*, S67–S73 (1997).

254. T. Siddique, *Cold Spring Harbor Symp. Quant. Biol.*, *61*, 699–708 (1996).
255. R. W. Orrell, J. J. Habgood, D. I. Shepherd, D. Donnai, and J. de Belleroche, *Eur. J. Neurol.*, *4*, 48–51 (1997).
256. M. Kostrzewa, M. S. Damian, and U. Mueller, *Hum. Genet.*, *98*, 48–50 (1996).
257. J. S. Valentine, P. J. Hart, and E. B. Gralla, in *Copper Transport and Its Disorders: Molecular and Cellular Aspects* (A. Leone and J. F. B. Mercer, eds.), Plenum Press (in press).
258. D. W. Cleveland, L. I. Bruijn, P. C. Wong, J. R. Marszalek, J. D. Vechio, M. K. Lee, X. S. Xu, D. R. Borchelt, S. S. Sisodia, and D. L. Price, *Neurology*, *47 Suppl. 2*, S54–S62 (1996).
259. D. O. Natvig, K. Imlay, D. Touati, and R. A. Hallewell, *J. Biol. Chem.*, *262*, 14697–14701 (1987).
260. Y. Ogawa, H. Kosaka, T. Nakanishi, A. Shimizu, N. Ohoi, H. Shouji, T. Yanagihara, and S. Sakoda, *Biochem. Biophys. Res. Commun.*, *241*, 251–257 (1997).
261. Y. Watanabe, N. Kuno, Y. Kono, E. Nanba, E. Ohama, K. Nakashima, and K. Takahashi, *Acta Neurol. Scand.*, *95*, 167–172 (1997).
262. Y. Watanabe, Y. Kono, E. Nanba, E. Ohama, and K. Nakashima, *FEBS Lett.*, *400*, 108–112 (1997).
263. H. D. Durham, J. Roy, L. Dong, and D. A. Figlewicz, *J. Neuropathol. Exptl. Neurol.*, *56*, 523–530 (1997).
264. C. B. Kunst, E. Mezey, M. J. Brownstein, and D. Patterson, *Nature Genet.*, *15*, 91–94 (1997).
265. T. L. Williamson, J. R. Marszalek, J. D. Vechio, L. I. Bruijn, M. K. Lee, Z. Xu, R. H. Brown, Jr., and D. W. Cleveland, *Cold Spring Harbor Symp. Quant. Biol.*, *61*, 709–723 (1996).
266. P. H. Tu, M. E. Gurney, J. P. Julien, V. M. Lee, and J. Q. Trojanowski, *Lab. Invest.*, *76*, 441–456 (1997).
267. L. I. Bruijn and D. W. Cleveland, *Neuropathol. Appl. Neurobiol.*, *22*, 373–387 (1996).
268. B. Zhang, P. Tu, F. Abtahian, J. Q. Trojanowski, and V. M. Lee, *J. Cell Biol.*, *139*, 1307–1315 (1997).
269. J. Y. Koh, S. W. Suh, B. J. Gwag, Y. Y. He, C. Y. Hsu, and D. W. Choi, *Science*, *272*, 1013–1016 (1996).

270. Z. L. Harris and J. D. Gitlin, *Am. J. Clin. Nutr.*, *63*, 836S–841S (1996).

271. T. J. Lyons, H. Liu, J. J. Goto, A. M. Nersissian, J. A. Roe, J. A. Graden, C. Café, L. M. Ellerby, D. E. Bredesen, E. B. Gralla, and J. S. Valentine, *Proc. Natl. Acad. Sci. USA*, *93*, 12240–12244 (1996).

272. J. P. Crow, J. B. Sampson, Y. Zhuang, J. A. Thompson, and J. S. Beckman, *J. Neurochem.*, *69*, 1936–1944 (1997).

273. M. T. Carri, A. Battistoni, F. Polizio, A. Desideri, and G. Rotilio, *FEBS Lett.*, *356*, 314–316 (1994).

274. S. L. Marklund, P. M. Andersen, L. Forsgren, P. Nilsson, P. I. Ohlsson, G. Wikander, and A. Öberg, *J. Neurochem.*, *69*, 675–681 (1997).

275. A. Radunovic, H. T. Delves, W. Robberecht, P. Tilkin, Z. E. Enayat, C. E. Shaw, Z. Stevic, S. Apostolski, J. F. Powell, and P. N. Leigh, *Ann. Neurol.*, *42*, 130–131 (1997).

276. A. F. Hottinger, E. G. Fine, M. E. Gurney, A. D. Zurn, and P. Aebischer, *Eur. J. Neurosci.*, *9*, 1548–1551 (1997).

277. M. B. Yim, P. B. Chock, and E. R. Stadtman, *J. Biol. Chem.*, *268*, 4099–4105 (1993).

278. H. S. Yim, J. H. Kang, P. B. Chock, E. R. Stadtman, and M. B. Yim, *J. Biol. Chem.*, *272*, 8861–8863 (1997).

279. M. B. Yim, J. H. Kang, H. S. Yim, H. S. Kwak, P. B. Chock, and E. R. Stadtman, *Proc. Natl. Acad. Sci. USA*, *93*, 5709–5714 (1996).

280. S. I. Liochev, L. L. Chen, R. A. Hallewell, and I. Fridovich, *Arch. Biochem. Biophys.*, *346*, 263–268 (1997).

281. R. J. Ferrante, S. E. Browne, L. A. Shinobu, A. C. Bowling, M. J. Baik, U. MacGarvey, N. W. Kowall, R. H. Brown, Jr., and M. F. Beal, *J. Neurochem.*, *69*, 2064–2074 (1997).

282. M. E. Gurney, F. B. Cutting, P. Zhai, A. Doble, C. P. Taylor, P. K. Andrus, and E. D. Hall, *Ann. Neurol.*, *39*, 147–157 (1996).

283. J. S. Beckman, M. Carson, C. D. Smith, and W. H. Koppenol, *Nature*, *364*, 584 (1993).

284. L. I. Bruijn, M. F. Beal, M. W. Becher, J. B. Schulz, P. C. Wong, D. L. Price, and D. W. Cleveland, *Proc. Natl. Acad. Sci. USA*, *94*, 7606–7611 (1997).

285. M. F. Beal, R. J. Ferrante, S. E. Browne, R. T. Matthews, N. W. Kowall, and R. H. Brown, Jr., *Ann. Neurol.*, *42*, 644–654 (1997).

286. R. J. Ferrante, L. A. Shinobu, J. B. Schulz, R. T. Matthews, C. E. Thomas, N. W. Kowall, M. E. Gurney, and M. F. Beal, *Ann. Neurol.*, *42*, 326–334 (1997).

287. B. Moulard, A. Sefiani, A. Laamri, A. Malafosse, and W. Camu, *J. Neurol. Sci.*, *139 Suppl.*, 34–37 (1996).