

Forum Review

Posttranslational Modifications in Cu,Zn-Superoxide Dismutase and Mutations Associated with Amyotrophic Lateral Sclerosis

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ABSTRACT

Activation of the enzyme Cu,Zn-superoxide dismutase (SOD1) involves several posttranslational modifications including copper and zinc binding, as well as formation of the intramolecular disulfide bond. The copper chaperone for SOD1, CCS, is responsible for intracellular copper loading in SOD1 under most physiological conditions. Recent *in vitro* and *in vivo* assays reveal that CCS not only delivers copper to SOD1 under stringent copper limitation, but it also facilitates the stepwise conversion of the disulfide-reduced immature SOD1 to the active disulfide-containing enzyme. The two new functions attributed to CCS, (i.e., O₂-dependent sulfhydryl oxidase- and disulfide isomerase-like activities) indicate that this protein has attributes of the larger class of molecular chaperones. The CCS-dependent activation of SOD1 is dependent upon oxygen availability, suggesting that the cell only loads copper and activates this enzyme when O₂-based oxidative stress is present. Thiol/disulfide status as well as metallation state of SOD1 significantly affects its structure and protein aggregation, which are relevant in pathologies of a neurodegenerative disease, amyotrophic lateral sclerosis (ALS). The authors review here a mechanism for posttranslational activation of SOD1 and discuss models for ALS in which the most immature forms of the SOD1 polypeptide exhibits propensity to form toxic aggregates. *Antioxid. Redox Signal.* 8, 847–867.

PHYSIOLOGICAL ROLES OF CU,ZN-SUPEROXIDE DISMUTASE

REACTIVE OXYGEN SPECIES are frequently proposed to exert a toxic effect on cells via oxidation of a wide range of biomolecules including DNA, proteins, and lipids. Oxidative modifications of these biomolecules often lower enzymatic activities and/or induce mutations in DNA, and can lead to cell death through necrosis or apoptosis (39). Hydrogen peroxide (H₂O₂) and hydroxyl radical exhibit high redox potential and have been shown to damage biomolecules *in vitro* and *in vivo*. While superoxide anion (O₂⁻) is not a strong oxidant and readily acts as a reductant, it can attack

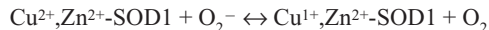
and disassemble the iron-sulfur cluster in the enzymes such as dehydratases and release Fe²⁺ ion, which produces hydroxyl radical *via* the Fenton reaction (102). Furthermore, reaction of O₂⁻ with nitric oxide rapidly forms the potent and versatile oxidant peroxynitrite (78). The toxic effects of superoxide anion are employed in host-pathogen interactions: O₂⁻ is generated by NADPH oxidase in phagocytes to kill infectious bacteria and fungi (37). Although the mechanisms underlying reactive oxygen species toxicity need further investigation, it is clear that uncontrolled oxidative stress damages many essential components in the cell.

One of the cellular defense systems for oxidative insults is an antioxidant enzyme, Cu,Zn-superoxide dismutase (SOD1)

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(111). SOD1 is a cuproenzyme (Fig. 1) and catalyzes the disproportionation reaction of superoxide anion to oxygen and hydrogen peroxide at a bound copper ion (111);



SOD1 is a very robust protein with respect to physical and chemical denaturation; catalytic activity can be observed in the presence of 10 M urea or 4% SDS and at 80°C (51). While nonenzymatic dismutation of O_2^- to O_2 and H_2O_2 occurs with a rate constant, $2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, at pH 7.4, SOD1-catalyzed dismutation is significantly accelerated to an extent that it is diffusion-controlled ($2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$) (53). Interestingly, this enzymatic rate of disproportionation is very similar to that of copper salts; however, given that intracellular free copper concentrations are quite low under normal aerobic growth (133), there has been a strong selection for organisms to elaborate a more efficient defense. Given negative charge of the substrate, O_2^- , positive characters of the charge distribution near the SOD1 active site (Arg 143 and Lys 136) enhance the dismutation reaction through the electrostatic steering mechanism (19, 170) (Fig. 1). The intracellular concentration of SOD1 is high (ranging from 10 to 100 μM (92, 101)), and apparently sufficient to consume physiological levels of superoxide radical.

Primary sequence as well as three-dimensional structure of SOD1 is highly conserved from prokaryotes to eukaryotes (21). SOD1 forms a tight dimer with a dissociation constant, $\sim 1.0 \times 10^{-10} \text{ M}^{-1}$ (90), and each subunit has an immunoglobulin-like β -sandwich fold with an intrasubunit disulfide bond (Fig. 1). A copper and zinc ion are embedded in each subunit (Fig. 1); Cu^{2+} ion is ligated with four histidines (His 46, 48, 63, 120), forming a distorted square plane, and binds water as a fifth ligand. One of the histidines, His 63, is also a ligand for binding a Zn^{2+} ion with the other three amino acid residues, His 71, 80, and Asp 83, in a distorted tetrahedral arrangement. Binding of Zn^{2+} ion is not essential for the dismutation reaction but confers high protein stability to SOD1. Several structural variations have been found in bacterial SOD1 proteins: a monomeric form in *Escherichia coli* (12), a heme binding site in *Haemophilus ducreyi* (126), no Zn binding site in *Mycobacterium tuberculosis* (160).

Eukaryotic SOD1 is mainly localized in cytosol with a smaller fraction at the intermembrane space of mitochondria (49, 101, 122, 166) and has been also found in nuclei, lysosomes, and peroxisomes, using immunocytochemical methods (30). While superoxide is produced in cytosol by several enzymes such as xanthine oxidase (114), respiration process in mitochondria is a major source of O_2^- generation (27). In fact, >95% of our daily oxygen consumption is reduced to water in the respiratory chain. It has been estimated that ca 0.1% of the O_2 consumed is converted to O_2^- in the electron transport chain, corresponding to production of ca. 30 mmol O_2^- in a 60–80 kg person on a daily basis (27). A good deal of mitochondrial O_2^- is disproportionated in the mitochondrial matrix by Mn-superoxide dismutase (SOD2) which is present there in high levels (ca. $1 \times 10^{-5} \text{ M}$) (181). While the possible presence of copper, zinc enzyme (SOD1) in the mitochondrial matrix is still debated (184), its localization in the intermembrane space of mitochondria (IMS) is now well established (49, 166). Thus, superoxide produced via adventitious reactions of O_2 with components of the respiratory chain can be disproportionated by SOD1 in the intermembrane space (71). Interestingly, in gram-negative bacteria, SOD1 is localized in a compartment that is distantly related to the IMS, namely the periplasmic space (91). SOD1 is important for survival of prokaryotes in relatively late/stationary phase and contributes to the ability to grow aerobically (161). The periplasmic sources of O_2^- have not yet been characterized (16), but it is likely that periplasmic SOD1 protects against both endogenous and exogenous sources of O_2^- , for instance those arising from host-parasite responses.

At a tissue level, SOD1 is widely expressed with high in liver and kidney (7). It is also abundant in motor neurons (127). This correlates with high expression of xanthine oxidase in liver and with high susceptibility of neuronal cells to oxidative stress. Knockout studies indicate that elimination of the SOD1 gene results in the widespread oxidative damage and has been shown to induce various symptoms/phenotypes in rodent models including alcohol-induced liver injury (89, 129), hepatocarcinogenesis (43), infertility in female mice (110), age-related hearing loss through cochlear hair cell degeneration (112), vulnerability to motor neuron loss after axonal injury (140), and the life span decrease (43). Also in humans, the level of glycosylated SOD1 is in-

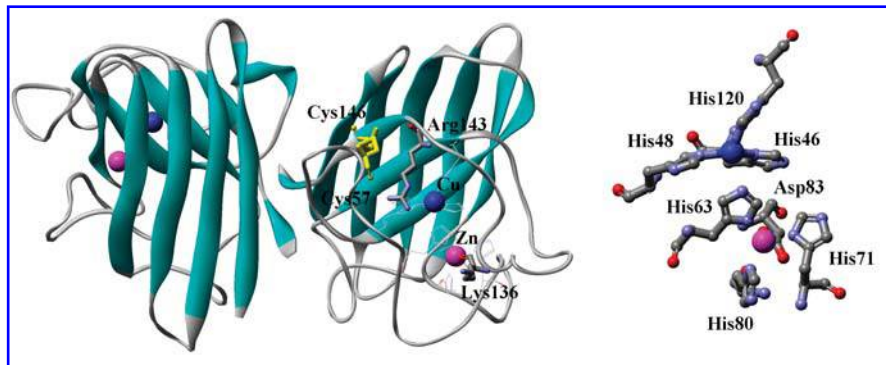


FIG. 1. Crystal structure of human SOD1 shown in a ribbon model (PDB ID: 1HL5). Cu, Zn ion and intramolecular disulfide bond are shown on ball and stick, respectively. Coordination structure around Cu and Zn ion is also shown in the right panel.

creased in the erythrocytes of patients with diabetes mellitus (5). Given that glycation of SOD1 causes fragmentation of the enzyme (124), reduction of SOD1 activity may lead to the altered redox status in diabetic patients (72). Elevated levels of SOD1 activity have been discussed in relation to Down's syndrome, which results from triplication of human chromosome 21 (45). SOD1 is encoded by a gene localized to 21q22.1, and the expressed levels of this protein are ca. 1.5-fold increased in Down's patients. Although there is no clear evidence for the pathogenic role of SOD1 in Down's syndrome, elevated SOD1 activity has been reported in some adult patients (45).

As we will discuss later in detail, over 100 types of point mutations in SOD1 have been reported to cause familial form of amyotrophic lateral sclerosis (ALS) (26). Many of the mutant proteins exhibit a normal level of the SOD1 activity, which leads to the idea that some aberrant 'gain-of-function' is associated with the neurodegeneration observed in ALS patients.

CELLULAR CONTROL OF SOD1 ACTIVITY

SOD1 needs a copper ion for its enzymatic activity; in fact, copper deficiency in diet results in reduction of both SOD1 protein and activity levels in a rat model (131), and this loss in activity is proportional to the degree of copper deficiency (130). Interestingly, the converse is true for the copper chaperone for SOD1: CCS levels are significantly elevated under conditions of copper deficiency in rodents (17, 188). In response to elevated copper ion, SOD1 transcription is induced to a small extent by a Cu-binding protein transcriptional factor, ACE1, in yeast (63). To maintain the copper ion homeostasis in the face of excess copper (133), many eukaryotic organisms express the metal-binding protein, metallothionein (87). Interestingly, overexpression of SOD1 can suppress copper toxicity in yeast cells lacking metallothionein (35). While physiological connections between these various pools of cellular copper are not well understood, these studies reveal the delicate interplay between antioxidant defense and metal homeostasis machinery.

Consistent with the fact that SOD1 is involved in the oxygen metabolism, SOD1 activity is also augmented upon increase of O₂ concentration in yeast (66) and *E. coli* (65). Anaerobiosis of yeast cells is reported to result in ca. 4- and 10-fold decrease in SOD1 protein and activity level, respectively (56, 57). Oxygen-dependent control of SOD1 activity has been also suggested in animal models; uptake of O₂ in active muscles is markedly facilitated during exercise, leading to an increased generation of reactive oxygen species. SOD1 activity is upregulated in rat diaphragm muscle during endurance training at the post-transcriptional level (121). Chronic exercise training in pig also increases SOD1 mRNA, protein, and activity levels (148, 149). Physiological regulation of SOD1 activity thus appears to involve transcriptional, post-transcriptional, and posttranslational mechanisms. In this review article, we will overview posttranslational regulation of SOD1 activation and introduce several recent findings in our lab.

COPPER CHAPERONE: POSTTRANSLATIONAL ACTIVATION OF SOD1

While copper and zinc insertion into the apo-SOD1 polypeptide can be achieved by addition of the corresponding metal salts *in vitro* (18), the situation inside the cell is more complex. Many but not all organisms employ one or more accessory proteins for copper insertion. The copper chaperone protein for SOD1, CCS, was first identified in the yeast strain *Saccharomyces cerevisiae* using genetic approaches (36) and has emerged as an important posttranslational regulator of SOD1 structure, function, and physiology. Yeast cells lacking the CCS gene, formerly known as LYS7, exhibit the same phenotypes as *sod1Δ* null mutant, which is auxotrophic for methionine and lysine in aerobic conditions. In yeast *ccsΔ* null mutant, any SOD1 activity is not observed, whereas SOD1 expression remains unchanged relative to wild type. SOD1 activation in yeast cells is thus strictly dependent upon the existence of CCS. CCS is highly specific for SOD1 and cannot deliver a copper ion to other proteins, such as cytochrome *c* oxidase or copper transport ATPase, that have different metallochaperones (120). CCS proteins have been also identified in various species including humans (29), rodents (193), insects (159), and plants (191). While CCS is 15- to 30-fold and fivefold less abundant than SOD1 in mammalian (147) and yeast cells (133), respectively, cellular distribution and expression of CCS appear to parallel those of SOD1. Recently, however, CCS-knockout mouse and *CCS*^{-/-} fibroblast mouse cells have been shown to retain a certain degree of SOD1 activity, suggesting the CCS-independent pathway of SOD1 activation in mammalian cells (28, 167). Reduced glutathione appears involved in CCS-independent activation of SOD1 in mammalian cells (28). It is interesting to note that the nematode *Caenorhabditis elegans* does not have a CCS homologue and that this organism uses some type of CCS-independent pathway for activation of intracellular Cu,Zn-SOD proteins (83). The apo-form of mammalian SOD1 has also been proposed to acquire a copper ion from metallothionein (MT) and ceruloplasmin *in vitro* (38, 105, 168, 169); however, the latter protein is extracellular and is unlikely to donate copper to SOD1 *in vivo*. Further experiments will be required to delineate *in vivo* roles of MT and other proteins in SOD1 activation.

We have recently found that posttranslational activation of apo-SOD1 by CCS requires oxygen (23). Air-exposure of anaerobically grown yeast triggers the protein expression and activation of SOD1 within an hour. Interestingly, even after inhibiting the protein synthesis using cycloheximide, the pre-existing pool of apo-SOD1 in anaerobically grown yeast is spontaneously (within 5 min) activated upon exposure of the yeast cells to air. Furthermore, *in vitro* experiments have shown that CCS can activate apo-SOD1 under aerobic conditions but not in the absence of oxygen (23). The cellular protection against oxidative stress is thus multitiered; existing pools of apo-SOD1 are activated by CCS in the early response to a sudden elevation of oxygen availability, followed

by increasing expression of SOD1 protein with persistent posttranslational regulation of SOD1 activity.

MECHANISTIC FUNCTIONS OF CCS IN SOD1 ACTIVATION

To better understand the posttranslational mechanism of SOD1 activation, several groups, including us, have characterized the copper chaperone protein, CCS. CCS is a 28 kDa protein and comprised of three domains, while a fourth domain at C-terminus showing homology with metallothionein has been reported in *Schizosaccharomyces pombe* CCS (93). Crystal structures are available for human (97) and *S. cerevisiae* (96) CCS, although some parts of the protein are disordered or truncated during crystallization. The N-terminus region (~8 kDa) has a similar structure to a secretory pathway copper chaperone, ATX1, and contains the "MXCXXC" Cu-binding motif (Fig. 2A). Domain I alone of human CCS retains a copper ion when overexpressed in *E. coli* with supplement of CuSO_4 (134). While EXAFS studies on human CCS have also suggested that two Cys residues in the MXCXXC motif are involved in copper binding (41), Domain I is not essential to SOD1 activation in the cell under normal conditions; CCS mutant protein, in which Domain I is truncated, can still activate SOD1, albeit at much reduction in the activation (151). Besides, the Cys to Ser mutations in the MXCXXC motif still exhibit 70% metallochaperone activity compared to the wild-type protein *in vitro* (162). Domain I-truncated CCS, however, cannot complement the aerobic ly-

sine auxotrophy of *ccsΔ* null yeast strain in the presence of a copper(I) chelator, bathocuproine sulfonate, implying that Domain I plays some roles in acquiring a copper ion when intracellular availability of a copper ion is limited (151). Interestingly, however, two cysteine residues of Domain I Cu-binding motif is not conserved in *D. melanogaster* CCS, which further complicates exact roles of Domain I in SOD1 activation.

Domain II of CCS (~16 kDa) is highly homologous to SOD1 (Fig. 2B); 47% identity between human SOD1 and human CCS Domain II. While no metal binding sites are found in yeast CCS Domain II, all of the SOD1 zinc binding ligands are conserved in the human counterpart. In fact, an equimolar amount of zinc ion is bound in human CCS Domain II when overexpressed in *E. coli* (134), and Zn binding is essential to human CCS function possibly through protein stabilization (44). Three of four histidine residues in the copper binding site are also conserved in Domain II of human CCS, and the fourth histidine residue is replaced by an aspartate (Asp 200). Copper ion is not held at this site, and SOD1 activation by CCS is still observed after mutations of these His residues to Ala (151). Potential copper binding site in Domain II would thus be not required for copper activation of SOD1.

Given the similarity of the SOD1 and CCS dimer interfaces, CCS has been proposed to interact with SOD1 through SOD1-like Domain II by mimicking SOD1 dimerization. In fact, when dimerization of yeast SOD1 is retarded by mutations at the subunit interface, F50E/G51E, this mutant SOD1 is not activated by yeast CCS (150). Similarly, when the corresponding amino acid residues in yeast CCS Domain II are

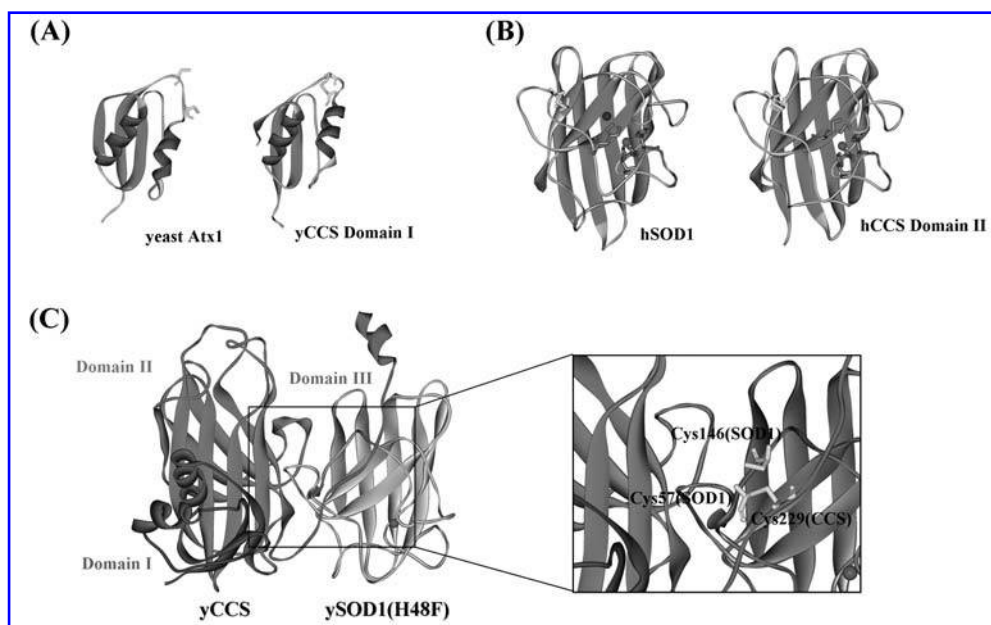


FIG. 2. Crystal structures of CCS. (A) Domain I of CCS (PDB ID: 1QUP) has a homologous structure with another copper chaperone, Atx1 (PDB ID: 1FES). (B) Domain II of human CCS (PDB ID: 1DO5) is similar to human SOD1 (PDB ID: 1HL5). (C) Heterodimer structure between yeast SOD1 with the mutation, H48F, and yeast CCS (PDB ID: 1JK9). All three domains in CCS are identified in this crystal structure. Magnified image of the interfacial region containing intermolecular disulfide bond between SOD1 and CCS is also shown.

mutated (F136E/G137E), *in vivo* activation of yeast SOD1 is not observed (150). These findings have supported the idea that CCS Domain II interacts with the SOD1 subunit interface. On the other hand, SOD1 forms a tightly associating dimer, and monomeric forms of the WT holo protein have not been observed. Based upon high dimer stability of either SOD1 (K_d , $1 \times 10^{-10} M^{-1}$) (90) or CCS (K_d , $3.0 \times 10^{-6} M^{-1}$ in yeast) (69), the hetero-tetramer model, in which both proteins do not need to dissociate into monomers, has been proposed (69). We have recently shown that the SOD1 quaternary structure is dependent on the degree of posttranslational modifications and that the most immature form (i.e., the disulfide reduced apo-SOD1 polypeptide) favors the monomeric state (6, 55). In fact, the Cu-bound yeast CCS can only activate the disulfide-reduced apo-yeast SOD1 monomeric form: a dimeric apo-form of SOD1 that already possesses an intramolecular disulfide bond cannot accept copper from the chaperone and cannot support the heterodimeric interaction between SOD1 and CCS (55).

Domain III of CCS is a short polypeptide (30–40 amino acids) without any preference to form the secondary structures but is essential to the CCS function; deletion of Domain III cannot complement the lysine auxotrophy in *ccsΔ* null yeast cells (151). Domain III shows a weak homology with a part of prolyl *cis-trans* isomerase, and the CXC motif found in this domain is highly conserved among all species. Domain III polypeptide alone is sufficient to bind Cu^{1+} ion, and the Cys to Ser mutations in CXC motif dramatically reduces the metallochaperone activity *in vivo* and *in vitro* (151). Although it is possible for Cu^{1+} ion to be bound at the CXC site with an exogenous ligand (137), recent EXAFS studies have suggested that the trigonal copper-thiolate cluster is formed between two Domain III CXC sites within a human CCS dimer (162). Depending upon the experimental conditions, more than one copper ion can be bound in both yeast and human CCS proteins (94, 134). Electronic absorption spectra of cobalt-substituted CCS have also implied formation of copper-thiolate cluster between CXXC of Domain I and CXC of Domain III (196). These data have shown that domain III plays essential roles in Cu binding and SOD1 activation, but the copper coordination chemistry in CCS has still been controversial.

The crystal structure of the yeast SOD1–yeast CCS heterodimeric complex (Fig. 2C) has strengthened several conclusions from biochemical studies on copper transfer from CCS to SOD1 (95). SOD1 dimer-like interaction between SOD1 monomer and CCS Domain II is observed, and functionally essential Domain III is well positioned to interact with SOD1. Given that crystallization is performed using a mutant SOD1 (H48F), which cannot bind a copper ion, it is still speculative how a copper ion changes its coordination structure when transferred from CCS to SOD1. In another metallochaperone system, Atx1 and Ccc2, a Cu^{1+} ion rapidly partitions between the surface-exposed metal-binding sites of these two proteins *via* formation and decay of two- and three-coordinate Cu^{1+} -thiolate intermediates (132). The thermodynamic gradient for metal exchange between Atx1 and Ccc2 is quite shallow with a Cu^{1+} exchange equilibrium constant of 1.4, where the driving force for the copper transfer is considered to be an ATP hydrolysis to remove Cu^{1+} from the cytosol

to the Golgi compartment. In the SOD1–CCS system, in contrast, the copper binding site in SOD1 is relatively buried in the protein interior, and there is no cellular compartmentalization of the transferred copper ion. Compared to Atx1/Ccc2 partnership, therefore, a more complex mechanism of copper transfer by CCS is expected to be exerted. In fact, one of the important aspects in the SOD1–CCS copper transfer has been implicated in the heterodimer structure (Fig. 2C): introduction of a conserved intramolecular disulfide bond in SOD1. In the SOD1–CCS heterodimer, a disulfide bond forms between Cys 57 (SOD1) and Cys 229 (CCS) (Fig. 2C). Instead, the active SOD1 has the intramolecular disulfide bond between Cys 57 and 146 (Fig. 1). This finding has implied the involvement of CCS in the disulfide formation in SOD1 and recently highlighted essential roles of this disulfide in the SOD1 activation mechanism.

CCS-DEPENDENT FORMATION OF ESSENTIAL DISULFIDE IN SOD1

The intramolecular disulfide bond of SOD1 was characterized in 1974 (1) and is highly conserved in SOD1 proteins from all species found so far. Disulfide formation generally stabilizes protein structure by lowering entropy of the unfolded polypeptide chain (174), and we have recently found essential characters of the intramolecular disulfide bond in enzymatic activity of yeast SOD1 (55). A structural role of the disulfide between Cys 57 and Cys 146 has been implied as formation of a hydrogen bond between the backbone amide of Cys 57 and Arg 143 (50), by which the side-chain of Arg 143 is optimally oriented for uptake of the superoxide anion (Fig. 1). It is, however, enigmatic how this essential disulfide bond in SOD1 forms and survives under reducing environment of the cytosol.

An important feature of the SOD1 disulfide bond is its high stability in the cytosolic environment; however, we have found that redox potential of thiol/disulfide pair in SOD1 is about -0.23 eV (55). This value is comparable to the ones of other disulfide-containing proteins and the cytosol (59). Maintenance of the SOD1 disulfide bond in cytosol is thus not due to its thermodynamic stability but is possibly kinetically controlled. Kinetic stabilization of a disulfide bond has been proposed in immunoglobulin light chain, a disulfide bond of which is completely buried in the protein interior and is unreactive toward reductants (60–62). In the holo-form of human SOD1, solvent accessible area of the disulfide-bonding cysteines, Cys 57 and 146, is small (2), implying that reducing agents are not accessible to the disulfide bond. In addition, location of the disulfide bond near the dimerization interface (Fig. 1) would also play an important role in stabilizing the disulfide bond. We have suggested that dimerization sterically limits access of cellular reductants to the disulfide bond and thereby contributes to kinetic stabilization of the disulfide bond. To examine effects of dimerization on the disulfide stability, the kinetic parameters of the disulfide reduction need to be compared between SOD1 monomeric and dimeric states in future studies.

In addition to its high stability, the disulfide formation in SOD1 needs to be explained under the reducing environment

of cytosol. Transient disulfide formation in cytoplasm is possible as seen in redox cycles of Cys pairs of thioredoxins and glutaredoxins (75); however, stable and persistent disulfide bonds are usually found in extracellular proteins, and the presence of cytoplasmic proteins with stable disulfide bonds is rare except several viral proteins (106, 152). It has been reported that disulfide bond in prokaryotic SOD1 is introduced by the oxidative folding machinery called Dsb proteins (11), which is consistent with the fact that prokaryotic SOD1 localizes at an oxidizing cellular compartment, periplasm (91). In eukaryotes, disulfide bonds are generally formed at endoplasmic reticulum (ER), which involves a set of enzymes for disulfide formation, Ero1 and PDI (179). Compared to the reducing environment of the cytosol, ER is maintained in a significantly more oxidized state by preferential transport of the oxidized glutathione over its reduced counterpart from cytosol (79). Interestingly, there is little evidence that the ER-based thiol-oxidases play a role in SOD1 disulfide formation. For instance, immunocytochemistry studies show that ER does not contain significant amount of SOD1, which is otherwise abundant in the cytosol (30). Other compartments may have specialized mechanisms for SOD1 maturation. It has recently been shown that the disulfide-reduced apo-form of SOD1 is imported from cytosol into the intermembrane space (IMS) of mitochondria (49). The discovery that this form is unfolded (54, 55) and has a monomeric structure (6) compared to the dimeric holo-SOD1 places it well within paradigms for the mechanism of import of other immature polypeptides into the IMS. Very recently, it has been shown that Mia40 and Erv1 catalyze disulfide formation in proteins in the IMS of mitochondria (113). The disulfide bond may thus be introduced in the reduced SOD1 protein imported into the IMS via Mia40 and Erv1. Given that the SOD1-CCS heterodimer contains the intermolecular disulfide bond (Fig. 2C) and that SOD1 is colocalized with CCS at the cytosol (29), Cu-CCS is a good candidate to introduce the disulfide bond in cytosolic SOD1. In fact, our *in vitro* and *in vivo* ex-

periments have recently shown that Cu-CCS regulates the SOD1 disulfide formation in the cytosol (55).

The disulfide formation in SOD1 also begins to explain the requirement of O_2 for SOD1 activation by CCS (Fig. 3). Under anaerobic conditions, the cysteine residues in SOD1 remain reduced even after incubation with Cu-CCS, and the protein remains inactive. Oxygen itself can function as an oxidant to form a disulfide bond, but it is kinetically sluggish for SOD1 disulfide formation (> 6 h). Rather, oxygen will be the ultimate source of oxidizing equivalents for disulfide formation in SOD1 and would first oxidize the thiol groups in CCS that ligate Cu^{1+} ion. The bound copper ion may then be released from CCS to apo-SOD1 upon oxidation of the thiol groups into a disulfide (Fig. 3). Metal release upon disulfide formation has been proposed in the Hsp33 activation mechanism; the thiol groups binding a Zn ion are oxidized to form the disulfide bond, leading to release of Zn ion from those thiol groups (64, 82). The disulfide formed in CCS is then transferred to SOD1 through formation of inter-molecular disulfide between SOD1 and CCS (Fig. 3). Alternatively, Cu^{1+} in CCS would be first attacked by oxygen to form Cu^{2+} . In the copper transfer from CCS to SOD1, the coordination environment around copper ion is changed from Cys-rich (CCS) to His-rich (SOD1). According to the empirical hard-soft chemistry, oxidation of Cu^{1+} ion may be required to break an extremely favorable interaction between Cys ligands and Cu^{1+} ion (soft-soft interactions). Detailed investigation in the chemical mechanism of SOD1 activation by CCS is now underway in our laboratory.

There are several precedents for O_2 -dependent disulfide formation in proteins; one of those is sulfhydryl oxidase (SOX). SOX is a flavin-containing enzyme and is comprised of two distinct domains intervened by a long amino acid stretch (175). N-terminal domain has a thioredoxin-like structure with CXXC motif, and C-terminal domain is related to Erv1 protein (*vide infra*), where another CXXC motif and a flavin-binding site are found. Oxidizing equivalents for disul-

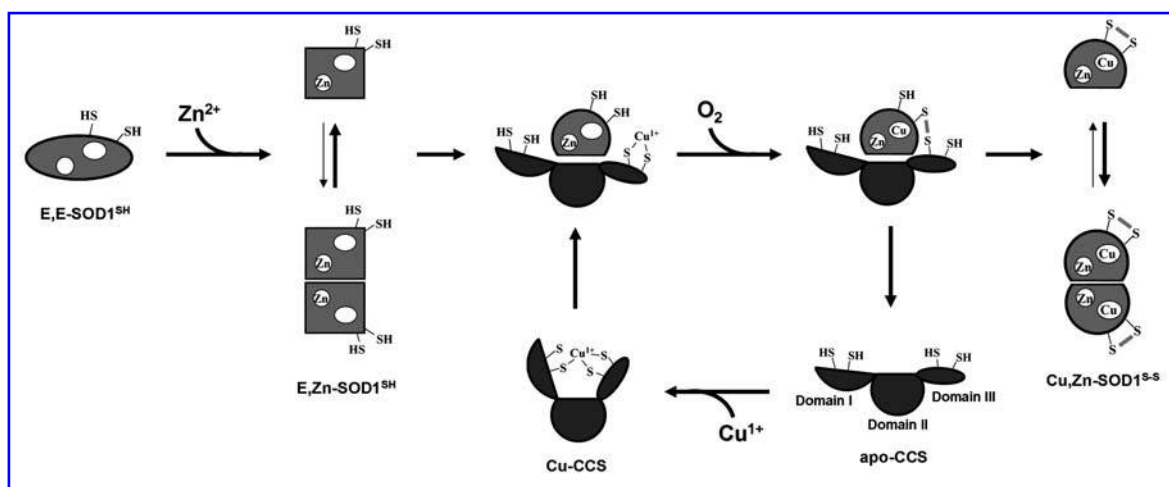


FIG. 3. Our proposed model for the SOD1 activation cycle by Cu-CCS in yeast. Cu-CCS will interact with the Zn-bound and disulfide-reduced form of SOD1, E,Zn-SOD1^{SH}. Yeast SOD1 favors monomeric state when the disulfide bond is reduced. Oxygen attacks the SOD1-CCS heterodimer to trigger the copper transfer, resulting in the formation of the intermolecular disulfide bond. Then the disulfide isomerization results in the intramolecular disulfide bond in SOD1 to form the dimeric holo-SOD1.

fide formation are relayed from O_2 via FAD, CXXC in C-terminal domain, CXXC in N-terminal domain and finally to substrate proteins (175). A relaying mechanism of oxidizing equivalents in disulfide formation is also found in pathways for protein oxidative folding. In eukaryotes, Ero1p transfers oxidizing equivalents directly to PDI protein, which oxidizes the substrate proteins through a disulfide-exchange mechanism (52). Ero1p, a single domain protein, contains two disulfide bonds and FAD (67). Erv protein, another protein responsible for PDI oxidation, also contains FAD, CXXC and CXC motifs at its C-terminus, where oxidizing equivalents from O_2 are transferred to form a disulfide bond in the substrate (68). Given that CCS has a copper ion, CXXC and CXC motifs, CCS can be paralleled with these enzymes for O_2 -dependent disulfide formation to the extent that a redox active cofactor with disulfide bond(s) seems a general architecture for disulfide formation. CCS is nonetheless unique in that not only oxidizing equivalents but also the redox active cofactor itself (i.e., copper ion) are transferred to the substrate, SOD1. While CCS has been defined as one of metallochaperone proteins, its assist of SOD1 folding through the disulfide formation suggests its role as a real "chaperone" protein.

In one proposed mechanism of CCS-dependent SOD1 activation (Fig. 3), the first step is incorporation of a Zn^{2+} ion into the disulfide-reduced protein, given that fully demetallated SOD1 was not activated with Cu-CCS (94). Our gel filtration studies also reveal that the apo and disulfide-reduced form of yeast SOD1 tends to unfold/aggregate much more readily than more mature forms, but incorporation of a Zn^{2+} ion in the disulfide-reduced SOD1 can lead to the protein folding (55). In the next step, the reduced (but not the oxidized) state of E,Zn -SOD1 forms a heterodimeric complex with the copper chaperone, which would be consistent with the fact that disulfide-reduced yeast SOD1 favors monomeric state (55). The subsequent reaction of CCS-SOD1 heterodimer with dissolved oxygen forms an intermolecular disulfide bond between CCS and SOD1. Following the copper insertion in the heterodimeric complex, Cu binding in SOD1 could induce the conformational changes especially around the Cys residues, promoting the protein disulfide isomerase-like reaction from intermolecular to intramolecular SOD1 disulfide (*vide infra*). Disulfide exchange within the heterodimeric intermediate further drives dissociation of the docked complex to the individual proteins, Cu,Zn -SOD1^{S-S} and apo-CCS, due to the thermodynamic preference of the disulfide-bonded SOD1 protein for the dimeric state. This reaction cycle predicts that CCS will be able to rapidly catalyze formation of the active SOD1 with uptake of the substrates: Cu^{1+} , E,Zn -SOD1^{SH}, and O_2 .

As reviewed above, copper insertion and disulfide formation in apo-SOD1 are performed by CCS; however, despite important roles of $Zn(II)$ ion in the SOD1 folding and structure, little is known on how this protein acquires a $Zn(II)$ ion in the cell. Given that human CCS can bind a $Zn(II)$ ion in Domain II and possibly Atx1-like Domain I, CCS may also function as a "Zn chaperone" protein. Interestingly, our recent studies have shown that apo and disulfide-reduced human SOD1 becomes dimeric upon binding of $Zn(II)$ ion, which is in apparent contrast to the observations in yeast

SOD1. Human CCS may thus favorably interact with a monomeric state of human SOD1 (i.e., E,E -hSOD1^{SH}); further *in vitro* and *in vivo* studies will prove how and when Zn ion is incorporated in SOD1 during the activation process. Posttranslational activation of SOD1 including Cu/Zn binding and disulfide formation is regulated in a well-concerted manner, but if there are deficits in the posttranslational activation pathway, SOD1 protein itself turns to be toxic to the cell.

TOXICITY INDUCED BY MUTATIONS IN SOD1—RELEVANCE TO ALS

Aberrant copper chemistry

In 1993, several point mutations in SOD1 were reported as a cause of the familial form of amyotrophic lateral sclerosis (ALS), which has shed light on a dark side of this antioxidant enzyme (146). Given that significant decline of SOD1 activity is found in several mutant proteins (e.g., A4V), it had been initially considered that inability to remove superoxide anion would lead to increase of intracellular oxidative stress and damage to neuronal cells (142, 178). The other ALS-associated mutant SOD1 proteins, however, retain partial or even full enzymatic activity (e.g., G37R, G93A) (22, 192). Point mutations in SOD1 thus do not seem to induce neurodegeneration based on the "loss-of-function" mechanism, which has been further supported by the following animal models. Overexpression of wild-type SOD1 does not reduce or even deteriorates ALS symptoms in the transgenic mice expressing mutant SOD1 proteins (25, 81), and an SOD1-knockout mouse exhibits no overt signs of the ALS pathology in spinal cord (140). A consensus has thus been established that SOD1 protein gains new toxic properties upon introduction of ALS-associated mutations.

One of the proposed functions acquired by mutations is the increased peroxidase activity; H_2O_2 , normally a product of the superoxide disproportionation, is catalytically converted into highly reactive and toxic hydroxyl radical at Cu^{1+} ion bound in SOD1 mutant proteins (189). In fact, increased levels of hydroxyl radical have been reported in the transgenic mouse expressing human SOD1 with G93A mutation (20). Another aberrant copper chemistry proposed in SOD1 mutant proteins involves a strong oxidant, peroxynitrite ($ONOO^-$). $ONOO^-$ forms with reaction between nitric oxide (NO) and superoxide anion at a diffusion-controlled rate. When SOD1 has no Zn ion bound, a Cu^{1+} ion in SOD1 is proposed to undergo one-electron reduction of molecular oxygen (O_2) to form superoxide anion, which further reacts with NO and leads to the peroxynitrite production (47). Given that many ALS-associated mutant SOD1 proteins have reduced affinity for Zn ion (34), an acquired toxic function of SOD1 by mutations may arise from the peroxynitrite chemistry including the tyrosine nitration (34). This is supported by the result that amounts of free 3-nitrotyrosine are increased in the transgenic mice and the ALS patients (14, 24). Mishandling of a copper ion may thus lead to oxidative damage in neuronal cells; consistent with this, administration of copper chelators such as trientine (116) and *D*-penicillamine (76) exhibits pro-

tective effects on the disease onset and progression in mouse models.

Recently, however, aberrant copper chemistry has been questioned as a cause of the SOD1-linked familial ALS; symptoms have been found in the transgenic mouse overexpressing the copper-binding-site-null mutant SOD1, in which all four copper ligands (His 46, 48, 63, and 120) are mutated (185). Human SOD1 protein with a mutation in one of these copper binding residues, H46R, has been shown to bind a copper ion at a site where copper is not typically seen (i.e., at a surface Cys residue, Cys 111) (103). This has led to the proposal that binding of copper ion at region(s) other than a canonical binding site may be associated with the toxicity of the ALS-variants. This proposal does not, however, explain the fact that the presence of ALS-associated mutations in mouse SOD1 can cause ALS-like symptoms (141), even though mouse SOD1 has no Cys residue at position 111. Besides, there is no *in vivo* evidence that copper ions bind at the SOD1 protein surface with significant affinity under physiological conditions. While increased oxidative stress is a major pathology found in the ALS-model mice and ALS patients, the bulk of the evidence to date does not strongly support the idea that aberrant copper chemistry originating with mutant SOD1 proteins is responsible for the disease.

Aggregation of SOD1: defects in posttranslational modifications

As an alternative mechanism of this disease, protein aggregation has been proposed on the basis of the fact that Bunina body and Lewy body-like hyaline inclusion are found in motor neurons of sporadic and familial ALS patients, respectively (153). Accumulation of protein aggregates in motor neuronal cells will interfere with axonal transport, protein degradation and mitochondrial function, resulting in neuronal cell death (26). The protein aggregates found in SOD1-related familial ALS patients and transgenic mouse models are strongly immunoreactive to SOD1, whereas involvement of SOD1 in protein aggregates has been controversial in sporadic cases (154). Aggregation generally occurs through unfolding/misfolding of protein molecules; therefore, the intracellular regulation of SOD1 stability will be an important factor in the molecular mechanism of familial ALS pathology.

Holo-form of SOD1 (Cu,Zn-SOD1) is a very stable protein to the extent that the melting temperature, T_m , is around 90°C (51); however, removing a copper ion from holo-SOD1 (E,Zn-SOD1) decreases T_m to ~70°C, and further removal of Zn ion from E,Zn-SOD1 (E,E-SOD1) results in ~60°C of T_m (145). Destabilization of SOD1 protein upon demetallation has also been confirmed in experiments using a chemical denaturant, guanidium hydrochloride (Gdn-HCl) (100). The metal-binding process thus maintains structural integrity as a folded SOD1 molecule and seems to protect the protein from aggregation; in fact, a Cu-bound but Zn-deficient SOD1 molecule tends to aggregate through oxidation of the histidine residues into 2-oxohistidine (136), although there is no *in vivo* evidence of this type of SOD1 aggregates.

Roles of the metal binding processes in keeping the protein integrity are further supported by the crystal structure of metal-deficient human SOD1 protein, in which an extended

arrangement of β -barrel shaped apo-SOD1 is reminiscent of the common structure associated with amyloid-like fibrils (165). Besides, demetallation-induced conformational changes of the SOD1 proteins with pathogenic mutations (H46R, S134N) can create a new protein-protein interaction site (gain-of-interaction), which tolerates formation of higher-order amyloid-like assemblies of SOD1 molecules (42). While the pathogenic SOD1 aggregates do not seem to have an amyloid structure (based on Congo Red staining) (123), mutant SOD1 in the absence of bound metal ions can adopt alternative conformations that facilitate protein aggregate formation. X-ray scattering data on the mutant SOD1 (A4V, I113T) have indeed predicted significant alterations of the subunit orientation in solution; structural destabilization of the dimer interface would trigger the protein aggregation (77). An aberrant increase in the hydrophobicity of the disulfide-reduced metal-deficient SOD1 mutant proteins has been reported in the presence of SDS, and is consistent with conformational differences in these forms of mutant and WT SOD1 (177). Monomerization induced by demetallation has also been proposed as a step leading to misfolded intermediates in the SOD1 aggregation pathway (135). Given that many ALS-associated mutations in SOD1 are found to provoke significant destabilization and conformational changes, especially in the metal-deficient state (100, 144), instability of apo mutant SOD1 may be a "common denominator" for the increased aggregation propensity among over 100 ALS-linked mutant proteins (100).

The stability of the apo-state of SOD1 is increased by several types of mutations [H46R and D124V (143), which incidentally are associated with relatively milder symptoms (4, 86)], but most of the mutant apo-proteins (21 out of 25 mutant proteins) are still significantly destabilized (143). Besides, stability of apo-SOD1 mutant proteins exhibits apparent inverse correlation with the mean survival time after disease onset (100), indicating that the protein stability plays an important role in the SOD1-linked ALS pathogenesis. We have, nonetheless, noted that metal-deficient form of mutant SOD1 still has a higher/comparable melting temperature than physiological body temperature, ~37°C; for example, apo-form of A4V, G93A, and E100G hSOD1 has 50.7, 49.1, and 51.8°C, respectively (163). Significant fractions of mutant holo-forms of SOD1 are thus expected to be folded under the physiological conditions; these may not be predisposed to the protein aggregation mechanism. This suggests that other states in the posttranslational SOD1 activation pathway may be more important in the aggregation process.

As described in a previous section, our proposed mechanism of the SOD1 activation consists of three major posttranslational modifications; copper/zinc binding, and disulfide formation (Fig. 3). Depending upon these modifications plus monomer-dimer equilibrium, 44 canonical microstates are possible in SOD1 protein (Fig. 4). This only takes into account SOD1 proteins where the zinc is bound in the zinc site and copper in its site. Given that these modifications generally increase the protein structural stability, it is well expected that the most immature form [i.e., apo- and disulfide-reduced SOD1 (E,E-SOD1^{SH}), is susceptible to aggregation. In fact, we have confirmed that disulfide reduction in wild-type apo-SOD1 drops the melting temperature to

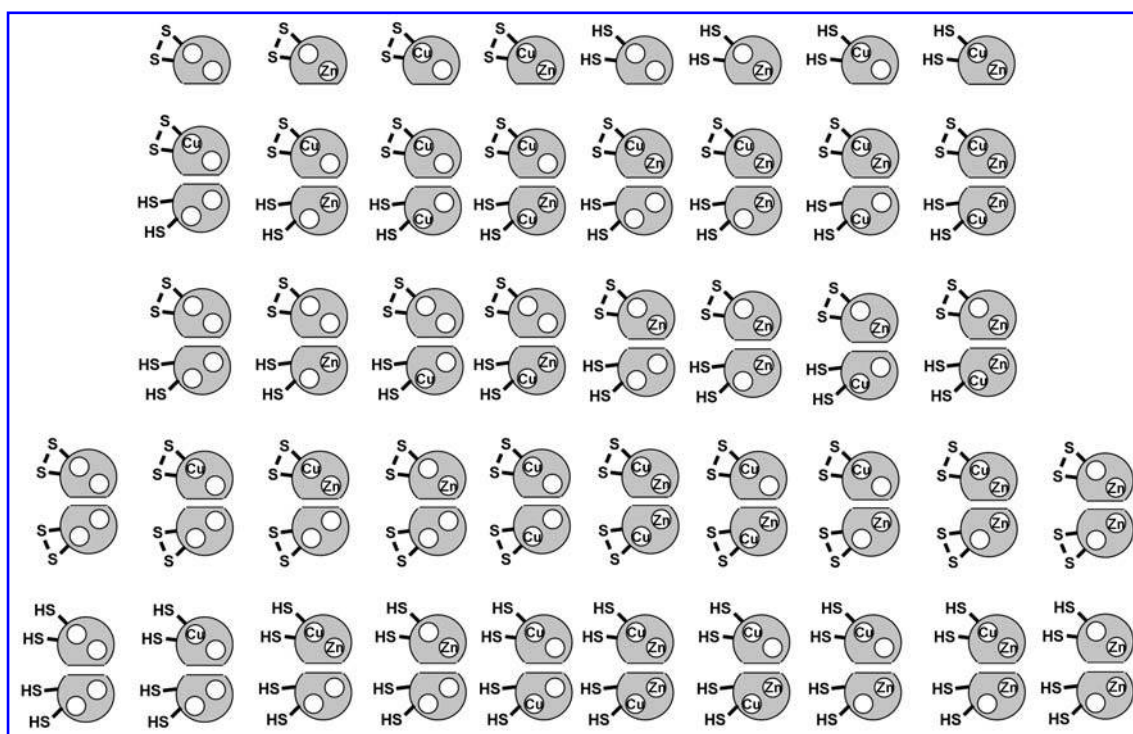


FIG. 4. 44 canonical microstates in SOD1. SOD1 microstates are dependent upon copper and zinc binding, intramolecular disulfide bond, and dimerization.

46°C (54). This melting temperature is about 45°C lower than that of holo-SOD1 and close to body temperature (~37°C), implying that a small fraction of even wild-type protein may be unfolded when any posttranslational modifications are absent. Structural instability of unmodified wild-type SOD1 may relate to the fact that spinal cord tissues of some sporadic ALS cases contain protein aggregates immunoreactive toward SOD1 (154). When SOD1 has an ALS-associated mutation, the melting temperature of the E,E-SOD1^{SH} state further drops to 35 (A4V) or 31 (G93A)°C even in the presence of the stabilizing reagent, glycerol (54). These values are >10°C lower than that of the wild-type protein and also lower than body temperature (~37°C); apo and disulfide-reduced SOD1 with ALS-associated mutations would be unfolded in physiological conditions. Interestingly, in the absence of glycerol, E,E-hSOD1^{SH} of A4V and G93A exhibit no endothermic transition between 5° and 100°C, implying the protein unfolding/aggregation even below 5°C. This is consistent with the observation that, using gel filtration chromatography at 4°C, an oligomeric state larger than dimer has been detected in the apo and disulfide-reduced SOD1 with ALS-associated mutations (54). Our recent findings have thus suggested that E,E-hSOD1^{SH} state is most prone to protein aggregation at physiological temperature.

Furthermore, ALS-associated mutations increase a chance to restore SOD1 protein to the most immature state by decreasing both disulfide stability and metal affinity. While the intramolecular disulfide bond in wild-type SOD1 is kinetically robust against reducing agents (55), the disulfide in ALS-mutant SOD1 would not persist in reducing environment of the cytosol (176). SOD1 has extremely high Cu/Zn

affinity to the extent that it functions as an intracellular Cu/Zn sink (35, 187), but significantly reduced affinity and/or altered binding geometry of Zn ion in some mutant SOD1 proteins has been shown by spectrophotometric techniques (34, 107). We have confirmed that the disulfide-reduced form of mutant SOD1 also exhibits decreased association with zinc ion by using a cobalt substitution method (Furukawa Y. and O'Halloran T.V., unpublished) (108). Addition of an equimolar amount of CoCl₂ to E,E-hSOD1^{SH} gives the absorption peaks at 535, 570, and 585 nm (Fig. 5A). This spectral shape is virtually the same with that previously reported in E,Co-hSOD1^{S-S}(WT) (107), suggesting little perturbation of Zn binding upon disulfide reduction. Using apo form of mutant SOD1, in which all four Cys residues (Cys 6, 57, 111, and 146) are mutated to Ser, a similar spectrum is obtained after addition of equimolar CoCl₂, excluding possibility of the Co(Zn) ion ligation through Cys residues (data not shown). Addition of a strong metal chelator, EDTA ($K_a^{\text{Co(II)}} = 10^{13} \text{ M}^{-1}$ at pH 7), to E,Co-hSOD1^{SH}(WT) does not change the spectrum (Fig. 5A) at least within an hour. Co²⁺ ion at the Zn-binding site is hence kinetically inert or thermodynamically stable in the reduced wild-type protein. By comparison, the disulfide-reduced mutant proteins (A4V, G93A) with equimolar CoCl₂ show relatively broadened absorption between 500 and 600 nm with its reduced intensity (Figs. 5B and C), although the detailed binding geometries around Zn ion are still elusive in mutant proteins. Addition of 100 μM EDTA to the Co-substituted mutant proteins immediately decreases the absorption intensity (Figs. 5B and C), consistent with a lower affinity for zinc ion in the disulfide-reduced state. Given high capacity for chelation of zinc ion in reduc-

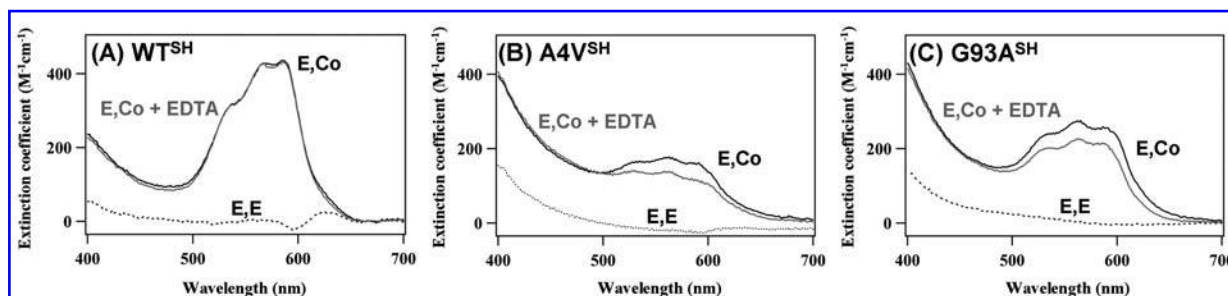


FIG. 5. ALS-associated mutations in SOD1 cause the reduction of affinity for a Zn ion. Electronic absorption spectra of E,E-hSOD1^{SH} (A: wild-type, B: A4V, C: G93A) in 50 mM HEPES, 1 mM tris-(2-carboxyethyl)phosphine, pH 7.4, with an equimolar CoCl₂ at 20°C (solid black curve). 0.1 mM EDTA was further added to the E,Co-hSOD1^{SH} samples, and the spectrum was obtained after 1 h incubation at 20°C (solid gray curve). Spectra of E,E-hSOD1^{SH} alone were also shown as dotted curves. Molar extinction coefficient shown in the figure was calculated based upon the absorption at 280 nm, and the following protein concentration was used for the measurements: 67.3 μ M wild-type, 74.5 μ M A4V, 78.2 μ M G93A hSOD1.

ing environment of the cytosol (125), we suggest that the intracellular milieu facilitates formation of the aggregation-prone state, E,E-hSOD1^{SH}, in the ALS-associated mutant SOD1 proteins.

Oxidative damage on

SOD1—disulfide-linked multimers

Reduction of the disulfide bond not only decreases protein stability but also affords vulnerability toward oxidative stress to SOD1 protein; oxidative injuries in the cell have been reported to augment in both sporadic and familial ALS patients (48). A thiol group (-SH) of a Cys residue is susceptible to oxidation under physiological conditions, resulting in the formation of sulfenic (-SOH), sulfinic (-SO₂H) and sulfonic acid (-SO₃H). As found in the brains of patients with Alzheimer and Parkinson diseases, Cys 146 in SOD1 can be irreversibly oxidized to cysteine sulfonic acid (31). Given that Cys 146 constitutes one of the thiol groups for formation of the intramolecular disulfide bond (Fig. 1), stability of the SOD1 protein oxidized at Cys 146 would be as much decreased as that of the disulfide-reduced form, which may explain the SOD1-immunoreactive senile plaques and neurofibrillary tangles in Alzheimer disease patients. As an alternative oxidized state of SOD1, we have found that E,E-hSOD1^{SH} molecules are cross-linked with each other by forming intermolecular disulfide bonds upon addition of either hydrogen peroxide or even milder oxidant, oxidized glutathione, resulting in the insoluble SOD1 multimers (54). While the covalent aggregation of SOD1 has been reported to occur in the presence of carbonate radical anion through the tryptophan oxidation *in vitro* (194), involvement of disulfide formation in the protein aggregation has been recently noticed in several protein conformation diseases such as transmissible spongiform encephalopathy (prion) (99) and familial amyloid polyneuropathy (transthyretin) (195). Random crosslinks between Cys residues sometimes lead to formation of insoluble protein inclusions during purification of recombinant proteins in *E. coli*, but disulfide crosslinks of SOD1 is not completely random; the conserved Cys residues (Cys 57 and 146) exhibit higher reactivity for formation of the disulfide-linked multimers than the nonconserved Cys

residues (Cys 6 and 111). Covalent addition of persulfide at the thiol group of Cys 111 makes SOD1 more resistant to the oxidation-induced aggregation (40), suggesting that selective quench of the thiol reactivity by chemical modifications and/or genetic engineering reduce the disulfide-linked multimerization and is a potential therapeutics for SOD1-related familial ALS disease. *In vivo* evidence of the disulfide-linked multimerization of SOD1 would be invoked in the observations using the transgenic *C. elegans* co-expressing the mutant SOD1 proteins (A4V, G37R, and G93A) (118); mutant SOD1 is degraded more rapidly than the wild-type protein, but oxidative stress by paraquat retards the degradation of mutant SOD1 and induces formation of discrete aggregates. It needs to be established whether the disulfide-linked multimer is a toxic species causing neurodegeneration or an end product of the oxidative modifications of SOD1 molecules. It is also well expected that SOD1 exhibits intrinsically high propensity for aggregate formation, since significant truncations from the C-terminus of SOD1 (as much as 117 amino acids) still tolerate the protein aggregation (58). Our recent findings have, nonetheless, suggested that oxidative crosslinks at Cys residues play an important role in the physiological SOD1 aggregate formation.

Cytotoxicity of ALS-SOD1 aggregates?

The toxicity of ALS-SOD1 aggregates in cell culture studies has been controversial. Lee and coworkers concluded that aggregate formation does not correlate with neuronal death in differentiated PC12 cells (98); however, a very recent time-lapse study following the fate of individual differentiated PC12 cells reveals that cell death correlates with formation of the aggregates and occurs within 6–24 h after aggregate appearance (109). While neither study addresses the status of the intrinsic disulfide, we note that inappropriate disulfide formation in the mutant SOD1 would promote and stabilize aggregate formation. These new results from Morimoto and coworkers are thus consistent with mechanisms that invoke SOD1 protein aggregation as a trigger for neurodegeneration (26). One of those is that the essential chaperone proteins are entangled in the SOD1 aggregates (180). Increased interaction between SOD1 and Hsp70 has been re-

ported upon treatment of SOD1 with DTT (182), which corroborates reported susceptibility to the disulfide reduction in mutant SOD1 (176). Hsp70, Hsp40, and $\alpha\beta$ -crystallin are co-immunoprecipitated with mutant SOD1 (156), suggesting deactivation of protein chaperones in the aggregate formation. Another proposed mechanism involves inhibition of the ubiquitin-proteasome protein degradation system by protein aggregates (182, 183). The aggregates containing SOD1 are intensely immunoreactive with antibodies to ubiquitin (155), and mutant SOD1 is selectively ubiquitinated by the RING finger-type E3 ubiquitin ligase, dorf1 (117). Addition of proteasomal inhibitors facilitates the aggregation of mutant SOD1 in cultured cells (84). Given that intermolecular disulfide crosslinks inhibit complete unfolding of SOD1 into the polypeptide thread state, it is interesting to speculate that the disulfide-linked SOD1 aggregates are stuck in the proteasome and lead to its dysfunction. Due to its insolubility and bulkiness, SOD1 aggregates have been also proposed to obstruct the axonal transport of essential components including neurotrophic factors (26). In fact, intracerebroventricular delivery of vascular endothelial growth factor, VEGF, has been recently found to significantly reduce the ALS-like symptoms in the rat model (164). SOD1 itself is delivered to the nerve termini in a slow axonal transport system (190), the rate of which is on the order of 1–2 mm/day (74). For the meter-long axon, it will take one to two years for SOD1 to arrive at the nerve termini (32). Such a long time will lead to an increased chance of oxidative damage for SOD1, and, therefore, neuronal cell may provide an oxidative environment facilitating the formation of disulfide-linked SOD1 aggregates.

With regards to oxidative stress, SOD1 protein will experience significant oxidative environment when incorporated into mitochondrial IMS, where reactive oxygen species are found as byproducts of the consumption of molecular oxygen in the respiratory chain. We have found that SOD1 must be in a disulfide-reduced state for its import into the intermembrane space of mitochondria (Fig. 7, inset) (49). Reduction of the conserved disulfide leads to the protein monomerization (6), which downsizes SOD1 protein and possibly facilitates its crossing over the outer membrane of mitochondria. Disulfide-linkage in a polypeptide may also be a three-dimensional obstruct for crossing the membrane. Upon mitochondrial import of SOD1, therefore, the reduced Cys residues will be exposed to a relatively high oxidative environment of mitochondrial IMS. Although CCS and/or the disulfide relay system, Mia40/Erv1 (113), in mitochondrial IMS correctly introduce the intramolecular disulfide in SOD1, oxidative modifications of thiol groups including the disulfide-linked multimerization would occur in the IMS, which may further lead to the apoptotic cell death cascade. Interestingly, high molecular-weight SDS-resistant SOD1 aggregates are associated with the spinal cord mitochondria of transgenic mice expressing ALS-mutant SOD1 (104) and can interact with antiapoptotic protein, Bcl-2 (128).

Possible therapeutics—facilitating posttranslational modifications on SOD1

Based on our proposed aggregation mechanism, investigation of several therapeutic avenues are warranted, including a

search for agents that stimulate maturation of the reduced and apo-forms of the mutant proteins, as well as agents that inhibit SOD1 oligomerization and disulfide-linked multimerization. Protein stabilization has been shown as one of treatment options for other aggregate-associated neurodegenerative diseases (171, 172) and amyloidosis (70); for example, in a transgenic mouse model for Huntington disease, where a protein with polyglutamine expansion is overexpressed, oral administration of trehalose decreases protein aggregates and improves motor dysfunction through stabilization of partially unfolded polyglutamine-containing protein (171). In the transgenic ALS-model mouse overexpressing G93A human SOD1, administration of trehalose (5,000 mg/kg/day) resulted in little effect on the disease onset and duration (<http://www.als.net/>). Our experiments have, nonetheless, shown that glycerol is another candidate to stabilize mutant SOD1, given that addition of glycerol increases the protein thermal stability and inhibits the oligomerization of E,E-hSOD1^{SH} state of the ALS-associated mutant proteins (A4V and G93A) (54). Dietary zinc administration also seems to be a promising treatment because of its role in increasing the protein thermal stability; in fact, moderate supplementation of zinc (12 mg/kg/day) delayed death in the G93A SOD1 transgenic mice by 11 days compared to mice on a zinc-deficient diet (46). Dietary copper supplementation should be also evaluated in light of the recent progress of Multhaup and co-workers in mouse models of other neurodegenerative diseases (13). Stabilization of the dimeric state has been attempted as an effective approach to reduce/inhibit SOD1 aggregation; insertion of an engineered intersubunit disulfide bond into mutant SOD1 (A4V) (139), although this approach does not seem potent *in vivo* (58). Using *in silico* screening, small molecules have been searched that can bind at the subunit interface and inhibit the monomerization/aggregation (138). Some reducing reagents as well as the physiological antioxidant systems are another effective choice to lessen the intracellular oxidative stress (3, 85, 119) and possibly inhibit formation of the disulfide-linked SOD1 aggregates. In the light of the aggregate suppression by stabilizing a protein molecule, we have shown that CCS is responsible for at least two posttranslational modifications of SOD1, copper insertion and disulfide formation; therefore, stimulation of CCS activity or protein levels is expected to be another good therapeutic approach for SOD1-associated familial ALS.

Effects of CCS on SOD1 aggregation pathway

CCS has been excluded as a candidate gene for ALS (157), but protein aggregation occurs when Domain II of CCS has a mutation homologous to an ALS-associated SOD1 mutation (158). Using immunohistochemistry, CCS has been reported to co-aggregate with SOD1 in neuronal hyaline inclusions found in familial ALS patients with an SOD1 mutation of either A4V or a frame-shift mutation at 126 (88) and in transgenic mice expressing G85R or G93A mutant SOD1 (186); however, possible involvement of CCS in the SOD1 aggregation pathway is still unclear. To prove roles of CCS in SOD1-linked familial ALS disease, the *ccs* gene knock-out has been examined in transgenic mice overexpressing human SOD1 mutant proteins (G37R, G85R, and G93A); both onset

and progression of the ALS-like symptoms have been little affected (167), implying that CCS does not play a primary role in causing the ALS disease. Small fractions of SOD1 are, however, active even in the CCS-knockout mice (15), which has left some ambiguities on the involvement of CCS in the ALS disease pathology. Indeed, it has been later shown that, unlike yeast, mammalian cells are equipped with CCS-independent pathway(s) for SOD1 activation, in which reduced glutathione plays an important role (28). CCS-dependent activation pathway can function in the ALS-associated mutant SOD1 (33), which has led us to the expectation that changes in the CCS expression in transgenic ALS-model mice will perturb the disulfide formation and/or the copper insertion in SOD1 and reduce the cellular fractions of the aggregation-prone E,E-hSOD1^{SH} state.

As reported in the yeast SOD1-yeast CCS system (55), Cu,Zn-hCCS interacts with E,Zn-hSOD1^{SH}(WT) and introduces the disulfide bond in hSOD1 within 10 min under aerobic conditions (Furukawa Y and O'Halloran TV, unpublished). Likewise, the intramolecular disulfide bond is introduced in the ALS-mutant proteins by aerobic mixing with an equimolar Cu,Zn-hCCS. Notable in mutant SOD1 proteins is formation of a high molecular-weight (MW) species, which is more significant in H46R and G85R compared to A4V and G93A mutant proteins (Furukawa Y and O'Halloran TV, unpublished). The higher MW species is also found in the reaction between Cu,Zn-hCCS and the hSOD1 protein with Cys → Ser mutations at positions 6, 111, and 146. Analysis using MALDI-TOF mass spectrometry has confirmed this higher MW species as a disulfide-linked SOD1-CCS heterodimer. Stabilization of the disulfide-linked heterodimer may relate to reduced binding of a copper ion, because it is one of shared properties between H46R and G85R (73). This is also supported by the fact that the disulfide-linked heterodimer has been successfully characterized in yeast SOD1/CCS by a mutation at a copper-binding ligand of SOD1 (H48F) (94, 95). In a subset of the SOD1 mutant proteins, therefore, it is expected that CCS cannot exercise its role in facilitating the posttranslational maturation of SOD1, given that metal binding process influences the CCS-dependent pathway of SOD1 activation.

Binding of a copper ion affects the SOD1 structure (9); the arrangement of Loops IV and VII is significantly altered with a minimal change in the overall folding of SOD1 protein (Fig. 6) (8–10). This is because Loops IV and VII contain the ligands for copper and zinc ions. Given that one of the con-

served Cys residues (Cys 57) is also a component of Loop IV, it is plausible that binding of metal ions facilitates the intramolecular disulfide formation by exerting conformational changes of Loop IV. Comparison of the structures among E,E-,E,Zn- and Cu,Zn-hSOD1^{S-S} indeed shows orientational changes of the disulfide bond (Fig. 6), which may present a mechanistic implication of how the intramolecular disulfide bond is favored upon binding of metal ions over the intermolecular disulfide bond with CCS. In the copper-binding mutant SOD1 such as H46R and G85R, therefore, the structural changes required to drive the disulfide isomerization step may not be exerted, resulting in stabilization of the disulfide-linked heterodimer.

The disulfide-linked heterodimer is on the physiological pathway to activate SOD1 by CCS (Fig. 3) (55). Given that CCS is about 30-fold less abundant than SOD1 in mammals (147), stabilization of the disulfide-linked heterodimer in H46R and G85R SOD1 would result in sequestration of available CCS proteins. Interestingly, SOD1 homologues in poxviruses, which do not encode a cysteine at position 146, act as the catalytically inert “decoy” by forming the disulfide-linked heterodimer with CCS of the host cell and interfering in the proper metallation of SOD1 of the host cell (173). The metal-binding mutant SOD1 can thus be also a catalytically inert decoy by forming and stabilizing the disulfide-linked heterodimer with CCS, possibly causing significant shortage of intracellular CCS and increase of the cytosolic fractions of aggregation-prone E,E-hSOD1^{SH} state. In this respect, it is interesting to note that the familial ALS is linked to the C146R mutation in SOD1 (80), which can stabilize the disulfide-linked heterodimer with CCS (Furukawa Y and O'Halloran TV, unpublished observations). Besides, stabilization of SOD1 through CCS-independent activation pathway will not be operated in metal-binding mutant SOD1. In this respect, it is interesting to note that the metal-binding ALS mutant SOD1 leads to different pathologies relative to other mutants; more protein inclusions are found in the transgenic mice with the Cu binding mutants, H46R and G85R, relative to the mutants with normal Cu binding such as G37R and G93A (115, 186).

Interestingly, the latter mutations are associated with more aggressive forms of the disease, thus propensity for aggregation may not correlate with disease severity. Although Subramaniam *et al.* have concluded that knockout of *ccs* gene exhibits no effects on the disease onset/progression (167), it is interesting to note that G85R but not G37R and G93A transgenic mice exhibit slightly longer life span in the absence of

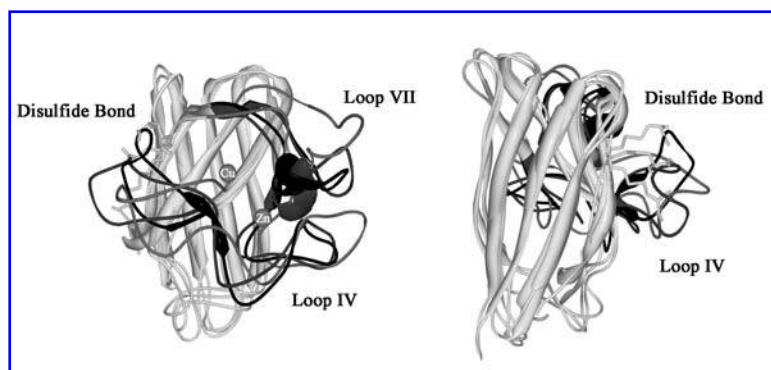


FIG. 6. Superimposed images of E,E-, E,Zn- and Cu,Zn-hSOD1^{S-S} structures (PDB ID: 1RK7, 1KMG, and 1BA9, respectively). An image from a different angle is also shown in the right panel. The conserved disulfide bond is shown in stick, and the Loops IV and VII are also highlighted by color; light gray, dark gray, and black in E,E-, E,Zn-, and Cu,Zn-hSOD1^{S-S}, respectively.

ccs gene (see Fig. 5 in ref. 167). This result, if supported by further studies, suggests that effects of CCS on the ALS pathology are dependent upon the type of mutations in SOD1; CCS-dependent/independent posttranslational modifications of SOD1 may help to inhibit protein aggregation in those mutant SOD1s that can bind a copper ion, while overexpression of CCS may be incompetent or even toxic in the familial ALS with the SOD1 mutations showing reduced affinity for a copper ion. To explore roles of CCS in the pathology of SOD1-linked familial ALS disease and pursue a possibility of CCS as a therapeutic target, *in vivo* characterization of the CCS-dependent pathway of SOD1 posttranslational activation is now underway in our laboratory.

CONCLUSION

As summarized in Fig. 7, we depict the intracellular fate of SOD1 protein from peptide synthesis at mRNA-ribosome complex, through folding, zinc and copper ion acquisition, and formation of the essential intrasubunit disulfide with the latter two steps mediated by CCS (also see Fig. 3). As long as this activation pathway functions correctly, SOD1 will protect the cell from various oxidative injuries as an enzymatic antioxidant. Mutations in SOD1, however, increase the lifetime of incorrectly folded states, and, depending on the concentration of apoSOD1, allows for reversible formation of SOD1 oligomers. If these oligomers are exposed to even mild oxida-

tive stress, incorrect disulfide links form and stabilize larger aggregates that may be resistant to the degradation by the quality control machinery of the cell. Thus, two events may be necessary to explain how mutations in SOD1 can lead to toxic aggregates: first the mutations must destabilize the folded state of immature SOD1 polypeptide or significantly slow down the rate at which the peptide folds into the proper conformation; secondly, an oxidative stress event can lead to crosslinking of and thus stabilization of the aggregated state. As some of the mutant SOD1 is sequestered in inactive aggregates, some cell may respond to further oxidative stress by induction of SOD1 transcription and translation. This would lead to a snow-ball effect in the stressed cells, perhaps explaining why aggregates are not observed in all cell types. As discussed above, aggregates have been observed in the mitochondria (104), which we note is an oxidative compartment. This then is a possible site for adventitious disulfide-oxidation and formation of incorrect disulfides that would further stabilize mutant SOD1 aggregates (Fig. 7, Inset). The disulfide-linked SOD1 aggregate at this site would be particularly dangerous if it compromised mitochondrial membrane functions: this could trigger apoptosis and may be involved in the neuronal cell death (Fig. 7). The SOD1 protein can be described as a “double-edged sword” to the extent that it can function as either a beneficial antioxidant or, upon mutation of a single residue, form an intractable protein aggregate that, depending upon the quality control machinery and the amount of excess apoSOD1 protein produced, may prove

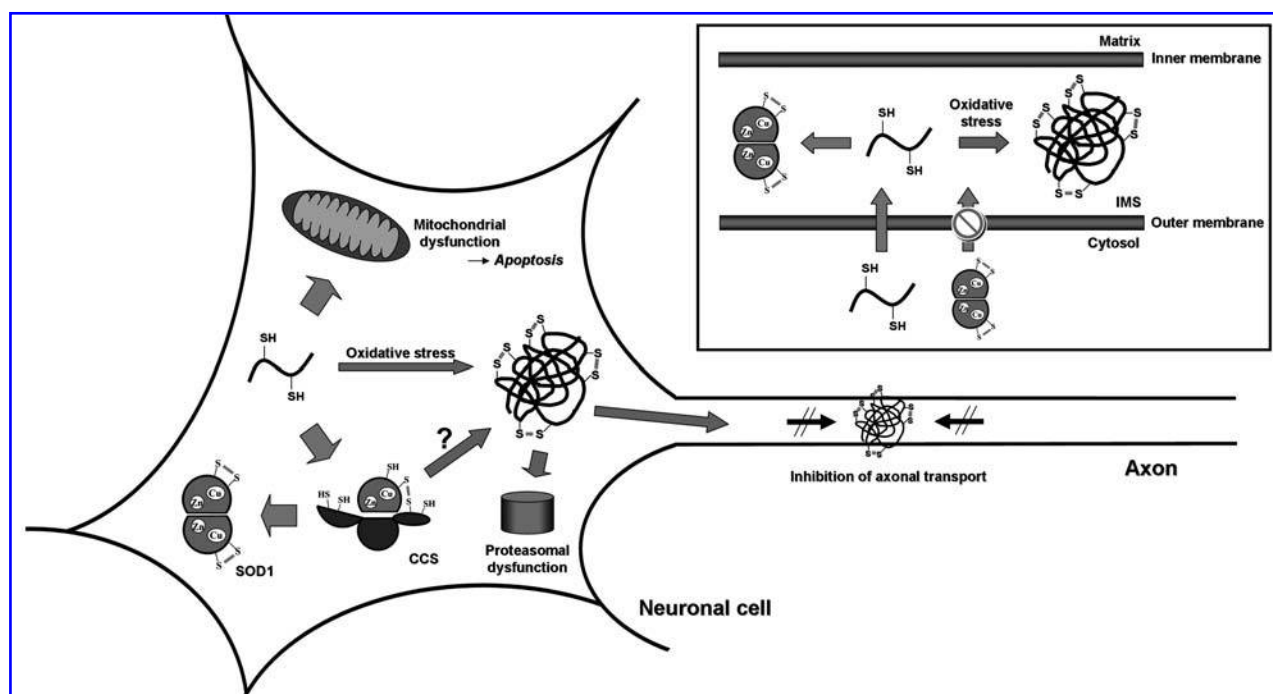


FIG. 7. Proposed processing of SOD1 protein in neuronal cells. Unmodified SOD1 polypeptide acquires a copper ion and a disulfide bond by forming a heterodimer with CCS, resulting in an enzymatic activation of SOD1. The free thiol group on the SOD1 polypeptide can also be oxidized to form the disulfide-linked protein aggregates, which would lead to dysfunctions of proteasome and/or axonal transport. (Inset) The disulfide-reduced form of SOD1 can cross the outer membrane of mitochondria, but the disulfide form cannot. Due to the highly oxidative environment in the mitochondrial IMS, the imported SOD1 polypeptide is susceptible to disulfide crosslinks and aggregation in the absence of any posttranslational modifications.

toxic to the cell. If this model is born out by additional studies, it would place SOD1-linked ALS squarely as a disease of protein folding. Although exact path from mutations in SOD1 to neurodegeneration remains unclear, agents that stimulate productive posttranslational modifications in SOD1 may be one of effective therapeutic approaches to cure ALS disease.

NOTE ADDED IN PROOF

We have recently found the disulfide-linked SOD1 aggregates in the spinal cord of the transgenic ALS model mice (*Proc Natl Acad Sci USA* 103: 7148–7153, 2006). Involvement of the wild-type protein in the disulfide-linked aggregates also explains aggravation of ALS phenotype in mutant SOD1 transgenic mice with overexpressed wild-type protein (*Proc Natl Acad Sci USA* 103: 7142–7147, 2006).

ABBREVIATIONS

ALS, Amyotrophic lateral sclerosis; CCS, copper chaperone for SOD1; IMS, intermembrane space of mitochondria; SOD1, Cu,Zn-superoxide dismutase.

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