*Serial Review*

## Copper Biology in Health and Disease
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# A pathological link between dysregulated copper binding in Cu/Zn-superoxide dismutase and amyotrophic lateral sclerosis

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**Mutations in the gene coding Cu/Zn-superoxide dismutase (SOD1) are linked to a familial form of amyotrophic lateral sclerosis (ALS), and its pathological hallmark includes abnormal accumulation of mutant SOD1 proteins in spinal motorneurons. Mutant SOD1 proteins are considered to be susceptible to misfolding, resulting in the accumulation as oligomers/aggregates. While it remains obscure how and why SOD1 becomes misfolded under pathological conditions *in vivo*, the failure to bind a copper and zinc ion in SOD1 *in vitro* leads to the significant destabilization of its natively folded structure. Therefore, genetic and pharmacological attempts to promote the metal binding in mutant SOD1 could serve as an effective treatment of ALS. Here, I briefly review the copper and zinc binding process of SOD1 *in vivo* and discuss a copper chaperone for SOD1 as a potential target for developing ALS therapeutics.**

***Key Words*: superoxide dismutase, amyotrophic lateral sclerosis, copper chaperone, protein misfolding**

Cu/Zn-superoxide dismutase (SOD1) is a copper and zinc-binding, homodimeric enzyme with an intra-subunit disulfide bond and converts superoxide into hydrogen peroxide and molecular oxygen at the bound copper ion (Fig. 1).[1] Equipped with SOD1, organisms are supposed to combat against oxidative insult under aerobic environment.[2] Indeed, deletion of the gene coding SOD1 leads to deleterious phenotypes such as suppression of aerobic growth in budding yeast,[3] shortened lifespan in fruit flies,[4] and slowly progressive motor deficits in mice.[5] Also, human patients with a homozygous truncating variant c.335dupG (p.C112Wfs*11) in the *SOD1* gene that leads to total absence of the enzymatic activity were reported, and the resulting phenotype was marked by progressive loss of motor abilities.[6,7]

More notably, a genetic link of *SOD1* mutations to a familial form of amyotrophic lateral sclerosis (ALS) was reported in 1993;[8] since then, many researchers have attempted to reveal the maturation mechanism of SOD1 proteins in cells. It had been initially expected that the pathogenic mutations would cause the disease by retarding the activity of SOD1;[9,10] however, the disease severity appears not to be correlated with the activity of SOD1.[11] A pathological contribution of wild-type SOD1 to sporadic ALS cases have been also proposed albeit controversial.[12] Besides, transgenic mice expressing human SOD1 with pathogenic mutations exhibit the ALS-like symptoms,[13] but SOD1-knockout mice do not.[14] Therefore, SOD1 has been considered to gain a toxicity with the pathogenic mutations, while a mutation-dependent degree of SOD1 activity might be involved in the pathological variability of ALS.[5]

As a pathological hallmark of familial ALS with *SOD1* mutations, SOD1 proteins are abnormally accumulated in affected motoneurons and are known to be aggregated.[15] The protein folding into altered, non-native conformations (i.e., misfolding) is thus suggested to be one of the properties of SOD1 gained by pathogenic mutations. While mature SOD1 equipped with the copper and zinc ion and the disulfide bond is extremely stable ($T_m$ ~90°C) and is resistant to misfolding,[16] the dissociation of those metal ions and/or the breakage of the intra-subunit disulfide bond significantly destabilize the native structure of SOD1.[17] Given that some of ALS-causing mutant SOD1 proteins exhibit comparable thermostability to that of wild-type SOD1 in their mature state,[18] the pathogenic mutations would disturb the metal-binding status and/or the thiol-disulfide status in SOD1 proteins and thereby facilitate the misfolding to abnormal oligomers/aggregates.[19]

## Pathological Modulation of *SOD1*-related ALS by a Copper Chaperone CCS

Because SOD1 is known to readily bind a copper and zinc ion when added *in vitro*,[20] less attention had been paid upon the acquisition of those metal ions by SOD1 inside cells. Also in many other metalloproteins than SOD1, it was not clear how they specifically recognized and bound their own metal ions. Nonetheless, taking into account potential cytotoxicity of heavy metal ions, intracellular dynamics of the metal ions should be strictly regulated. Then, the discovery of a new class of proteins called "metallochaperone" has created a new concept on how metalloproteins get their metal ions in cells.[21–24] Among those, a copper chaperone for SOD1 (CCS) was shown to specifically recognize SOD1 in the apo form and deliver a copper ion to SOD1 possibly by shuttling between a copper importer CTR1 and SOD1 (Fig. 2).[25] Besides, CCS is responsible for the introduction of the conserved intra-subunit disulfide bond in SOD1;[26] therefore, deletion of CCS led to complete absence (in yeast) or significant reduction (in mouse) of SOD1 activity.[25,27] While the

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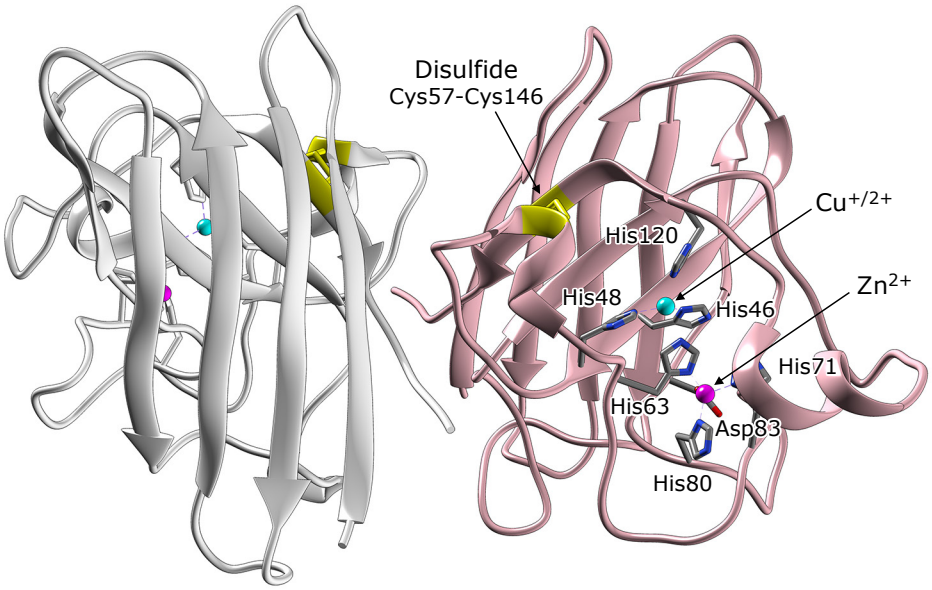


**Fig. 1.** Crystal structure of human SOD1 (PDB ID: 1HL5). SOD1 forms a homodimer, and each of the subunits is colored differently (gray and pink). The binding site of a copper ion (cyan) is composed of four histidine residues (His46, His48, His63, and His120), while a zinc ion (magenta) is bound by four residues (His63, His71, His80, and Asp83). His63 functions as a ligand for both a cuprous and zinc ion and called a "bridging ligand". The conserved intra-subunit disulfide bond (yellow) forms between Cys57 and Cys146. See color figure in the on-line version.

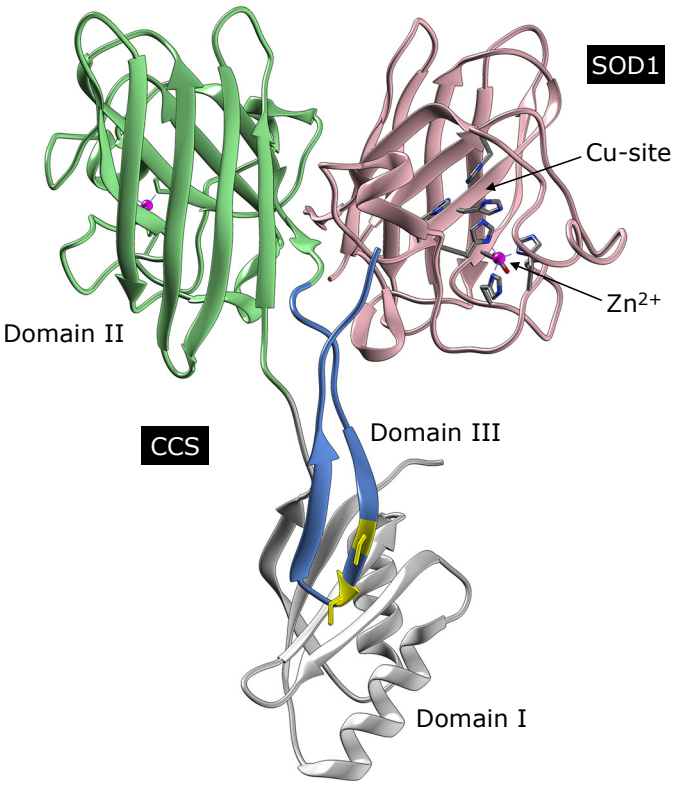


**Fig. 2.** Crystal structure of a heterodimer between human SOD1 and human CCS (PDB ID: 6FON). CCS is a multidomain protein, and each of the domains is colored differently (gray, green, and blue for domain I, II, and III, respectively). CCS forms a heterodimer with human SOD1 through the SOD1-like domain II, which mimics the homodimerization of SOD1 (also see Fig. 1). Zinc ions bound in SOD1 and also the CCS domain II are colored in magenta, and the Cys residues of CCS involved in the binding of a copper ion (Cys244 and Cys246) are colored yellow. See color figure in the on-line version.

*CCS* gene has not been linked to familial ALS cases, a patient with clinical phenotypes presumably due to the homozygous mutation in *SLC33A1* has been reported to have the R163W substitution in CCS;[28] the mutant CCS was characterized by its impaired interaction with SOD1, resulting in the reduced SOD1 activity.[28,29] CCS hence plays a primary role in the activation of SOD1 *in vivo*.

Given that metal-deficient SOD1 is susceptible to misfolding, it was first expected that knockout of the *CCS* gene would lead to the accumulation of misfolded SOD1 and thereby aggravate ALS-like symptoms in model mice. In contrast to such expectation, however, deletion of CCS did not affect the disease course of transgenic mice overexpressing human SOD1 with ALS-causing mutations.[27] In those ALS-model mice lacking CCS, SOD1 activity was significantly decreased but not completely diminished. The CCS-independent maturation of SOD1 might hence prevent further accumulation of metal-deficient, misfolded SOD1s, but it should be also noted that the expression level of endogenous CCS is much (12~30-fold) less than that of SOD1 in the model mice.[30] In other words, the overexpression level of mutant SOD1 in the model mice will be so high that endogenous CCS could not keep up with the metallation of those mutant SOD1 proteins;[13] therefore, the CCS deletion would have minimal impact on pathological changes in the ALS-model mice.

An alternative expectation thus follows that overexpression of CCS would facilitate the metallation and prevent the misfolding of mutant SOD1. Again, in contrast to such expectation, the overexpression of CCS was found to result in dramatically reduced lifespan (from 242 days to 36 days) of transgenic mice overexpressing human SOD1 with an ALS-causing mutation G93A.[31] This is partly because an overexpressed pair of mutant SOD1 and CCS would consume most of intracellular copper ions; as a result, the other essential cuproproteins appeared not to be sufficiently metallated.[32] Indeed, little effects of the CCS overexpression on the lifespan have been reported in the mice expressing human SOD1 with another mutation L126Z, which has no capability to bind a copper ion.[33,34] Nonetheless, transgenic mice overexpressing both CCS and wild-type SOD1 have normal


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lifespan without any abnormal neurological phenotypes,[31] which raises another but not mutually exclusive possibility that the interaction of CCS with pathogenic mutant SOD1 is non-productive and hence contributes to the aggravation of ALS-like phenotypes. Indeed, CCS overexpression increases the proportion of the G93A-mutant SOD1 in the disulfide-reduced form, but virtually all of the wild-type SOD1 are equipped with the disulfide bond in the CCS/WT SOD1 double-transgenic mice.[34,35]

CCS is also suggested to have a chaperoning activity to suppress the misfolding of immature SOD1, and the activity appears not to require the copper transfer from CCS to SOD1.[35] CCS is composed of three distinct domains (I, II, and III),[36] among which the domain III binds a copper ion at Cys residues (Cys244, 246) and transfers it to SOD1 (Fig. 2).[37] CCS with C244S/C246S mutations is hence not able to supply a copper ion to SOD1 but is reported to suppress the formation of insoluble mutant SOD1 aggregates in cultured cell models.[35] This suggests a role of CCS in the structural stabilization of immature SOD1, and such a chaperoning role is considered to be played by the domain II, which structurally resembles SOD1.[36] The domain II of CCS forms a heterodimer with the most immature (i.e., apo and disulfide-reduced) SOD1 and would thereby prevent the accumulation of misfolded SOD1.[38] Further studies on specific interactions of the domain II with SOD1 will hence contribute to the development of *SOD1*-ALS therapeutics through effective prevention of the SOD1 misfolding.[39] Collectively, those previous studies using the ALS-model mice suggest that the quantitative balance among SOD1, CCS, and copper in cells is critical for the understanding of the ALS pathology.

In that sense, it is important to note that the central nervous system (CNS) is characterized by a distinct copper metabolism from that of peripheral tissues. To be delivered to SOD1 proteins in motor neurons, copper ions need to cross the blood-brain or blood-spinal cord barrier. Given that the capillaries in the CNS are largely covered by the foot of astrocytes, copper ions are considered to be transferred first to the astrocytes, from which only a requisite amount of copper ions would be distributed to the neurons.[40,41] Indeed, astrocytes are known to highly express a metal-storage protein metallothionein and also contain high levels of reduced glutathione, suggesting a metal buffering role of the astrocytes in the CNS.[40] Moreover, copper ions are known to be more toxic to neurons than to astrocytes;[42] therefore, a basal level of intracellular copper ions in neurons would need to be kept low. In contrast, the expression level of SOD1 in neurons is higher than that of astrocytes,[30] and CCS is also expressed mostly in neurons and much less in astrocytes,[30] which possibly reflects a lower level of copper in neurons than in astrocytes.[43–45] Even so, SOD1 in neurons could be unsatiated with copper ions, which might be a trigger to cause the protein misfolding and eventually the neurodegeneration.

## Copper Supplementation as a Possible Treatment of *SOD1*-related ALS

Probably due to the blood-brain or blood-spinal cord barrier, oral administration of simple copper salts was not effective to ameliorate the disease phenotypes of the ALS-model mice expressing mutant human SOD1.[46] A review has been published on therapeutic interventions focusing on pharmacological as well as genetic regulations of copper homeostasis to modify the pathological process in *SOD1*-related ALS.[47] Among those interventions, it is quite notable that a copper complex, diacetylbis [*N*(4)-methylthiosemicarbazonato] copper(II) [$Cu^{II}$(atsm)], delays the disease onset and extends the lifespan of the ALS-model mice when orally administered.[48–50] Furthermore, the administration of $Cu^{II}$(atsm) has been found to increase activity of mutant SOD1 overexpressed in the ALS-model mice.[48–50] The complex $Cu^{II}$(atsm) is known to readily cross the blood-brain barrier with high membrane-permeability,[51] and its oral intake is indeed shown to result in a significantly elevated level of copper ions in the spinal cord of the ALS-model mice.[49] More strikingly, oral administration of $Cu^{II}$(atsm) dramatically extended the lifespan of the ALS-model mice co-expressing human CCS (from 36 days to 18 months).[52] These results thus suggest that satiation of neuronal SOD1 proteins with copper ions could be effective for the treatment of ALS. Indeed, $Cu^{II}$(atsm) is now in phase 2/3 testing for treatment of ALS (NCT04082832, NCT04313166); while therapeutic effects of the copper complex awaits more vigorous analysis, $Cu^{II}$(atsm) appears to ameliorate some of the disease symptoms of ALS patients (NCT02870634, NCT03136809).

## Abnormal Zinc Binding in SOD1 as a Pathological Signature of *SOD1*-ALS

In contrast to the acquisition of a copper ion, it remains totally unknown how SOD1 finds and binds a zinc ion in cells. A "zinc chaperone" for SOD1 might exist but has not been discovered. Binding of a zinc ion is well known to reduce the structural fluctuation and also increase the thermal stability of SOD1.[17,53] Pathological SOD1 proteins are thus supposed to fail to bind/maintain the zinc ion at its canonical binding site and become prone to misfolding.[19] Despite this, wild-type as well as mutant human SOD1 in the ALS-model mice has been found to bind zinc ions albeit with an abnormal binding stoichiometry: for example, one copper and three zinc ions per homodimer.[54] It thus remains to be investigated whether the pathological misfolding of SOD1 *in vivo* is triggered by losing the bound metal ions. Alternatively, it is also possible that the metal selectivity at each of the metal-binding sites in SOD1 plays a key role in the folding into the native conformation.

The native SOD1 binds a copper and zinc ion at the copper- and zinc-binding site, respectively (Fig. 1), but a zinc ion has been known to bind also at the copper-site *in vitro*.[55] It is thus enigmatic how SOD1 binds a zinc ion exclusively at the zinc-site and not at the copper-site *in vivo*. Recently, the zinc binding in human SOD1 was investigated by native mass spectrometry,[56] which revealed a critical role of the conserved intra-subunit disulfide bond in the selective binding of a zinc ion at the zinc-site. More precisely, a zinc ion was considered to bind at the copper-site as well as the zinc-site when added to SOD1 equipped with the disulfide bond; in contrast, only one zinc ion per subunit was detected in the disulfide-reduced SOD1 even when present in excess.[56] This is probably because the copper-site is disordered in the disulfide-reduced SOD1 even after the binding of a zinc ion at the zinc-site.[57,58] In contrast, the copper-site is well organized in apo-SOD1 with the disulfide bond and is almost indistinguishable from that of the native protein.[59] Therefore, the disulfide bond would need to be reduced in order to prevent the aberrant binding of a zinc ion at the copper-site. This is also consistent with the fact that CCS recognizes and then activates only a disulfide-reduced but not disulfide-bonded SOD1.[26]

Given that human SOD1 in the ALS-model mice binds more than one zinc ion per subunit, the disulfide bond would be introduced into SOD1 prior to the metal binding under pathological conditions. In other words, such a pathological SOD1 with abnormal zinc stoichiometry would indicate uncontrolled introduction of the disulfide bond most probably due to augmented oxidative stress in affected tissues of ALS.[60] It is also notable that the disulfide-bonded SOD1 exhibits an increased propensity to aggregate upon the binding of an over-stoichiometric amount of zinc ions.[61] It is, therefore, tempting to speculate that reduction of the disulfide bond in metal-deficient SOD1 *in vivo* is an efficient strategy to suppress the protein misfolding and also to start over correct binding of the right metal ion in the right place of SOD1.

## Future Perspectives

As reviewed above, many studies have implied a pathomechanism in which losing the intracellular balance among copper, zinc, CCS, and SOD1 would contribute to ALS. While a sufficient amount of zinc is supposed to be available to mutant as well as wild-type SOD1, pathological SOD1 appears to be unsatiated for copper. To correct the imbalance, oral intake of $Cu^{II}$(atsm) is a promising strategy but may be associated with potential risk to health, because accumulation of excess copper can cause damage the liver, kidneys, and brain. Copper poisoning appears to be rare, but it should be noted that patients with Wilson's disease caused by a loss-of-function mutation in a copper exporter ATP7B suffer from liver disease and neuropsychiatric symptoms.[62,63] Moreover, based upon the studies using model mice, $Cu^{II}$(atsm) is probably not effective for the treatment of ALS cases with mutant SOD1 proteins that cannot bind a copper ion. Nonetheless, even those mutant SOD1s with little copper-binding ability could be specifically recognized by CCS to form the heterodimer; therefore, more investigation on the interaction between SOD1 and CCS will definitely give us a hint to circumvent the pathological aggregation of SOD1 and thereby develop ALS therapeutics.


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## Conflict of Interest

No potential conflicts of interest were disclosed.

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