1. Amyotrophic Lateral Sclerosis (ALS) and mutant Superoxide Dismutase (SOD)

Amyotrophic lateral sclerosis (ALS) is a progressive paralytic disease characterized by selective degeneration of the upper and lower motor neurons.\textsuperscript{1,2} Although ALS is predominantly a sporadic diseases, \textasciitilde10\% cases are inherited in an autosomal dominant manner (familial ALS (fALS)) and a subset of the fALS cases are caused by mutations in the SOD1 gene.\textsuperscript{3,4} The gene product of SOD1, cytoplasmic Cu,Zn-superoxide dismutase (SOD1), is a ubiquitously expressed enzyme that catalyzes the disproportionation reaction of superoxide radicals.\textsuperscript{5}

\[
\begin{align*}
O_2^- + Cu(II) &\rightarrow O_2 + Cu(I) \quad (1) \\
Cu(I) + O_2^- &\rightarrow O_2H_2 + Cu(II) \quad (2)
\end{align*}
\]

The crystal structure of the SOD(Cu/Zn) has been determined,\textsuperscript{8} its dimeric structure being illustrated in Fig. 1 (PDB, 1spd_x). The copper and zinc ions are bridged by anionic form of imidazole ring of histidine.

There are several lines of evidence that suggests that SOD1 mutations result in a gain, rather than loss of function that causes ALS. For instance, some fALS-associated mutant SOD1s retain full enzymatic activity.\textsuperscript{6} In addition, SOD1 knock-out mice lack ALS symptoms, whereas transgenic mice expressing the fALS-associated mutant G93A SOD1 develop ALS-like symptoms despite expression of endogenous mouse SOD1.\textsuperscript{7} Lastly, overexpression of human wild type SOD1 fails to alleviate symptoms in this transgenic mouse model for ALS.\textsuperscript{7}

One hypothesis explaining the gain of function of SOD1 is that misfolding of the mutant alters the catalytic mechanism to allow production of oxidants such as peroxynitrite\textsuperscript{9} and possibly hydrogen peroxide.\textsuperscript{10} Another major hypothesis suggests that toxicity caused by intracellular aggregation of SOD1. SOD1 inclusion bodies, which also react with anti-ubiquitous antibodies, are a common pathological finding in motor neurons and neighboring astrocytes of ALS patients.\textsuperscript{11}

These two hypotheses, however, are not mutually exclusive when considering that oxidative modification of proteins may contribute to aggregation and protease resistance. Protein aggregation is a common pathological features of many neurodegenerative disorders\textsuperscript{12}, including Huntington’s, Alzheimer’s, and Parkinson’s diseases. In each case, misfolding and aggregation propensity of mutant SOD1s may be the mechanism by which over 100 disparate mutations cause a common ALS phenotype. Although SOD1 aggregates may be inherently toxic or cause motor neuron toxicity by sequestering chaperons and blocking proper functioning of the proteasome, origin of toxicity by SOD1 aggregates has not been elucidated.

To understand ALS pathogenesis, we must clarify how altering SOD molecule can induce cell injury. To carry out such an investigation, we have started to clarify the origin of the gain-of-function, and mechanism of SOD aggregation in solution.

The reaction mechanism of SOD1 enzyme has been investigated by many authors. Very recently Nishida et al. have a postulated new mechanism for this enzyme based
on the results used by the model compounds. We have pointed out the importance of formation of a copper(II)-OOH species as an intermediate (see Scheme-I) in the second step (2) above, and this hydrogen peroxide produced is immediately removed from the wild-type enzyme because of the negligible interaction between hydrogen peroxide and the copper(II) ion and the surrounding organic groups.

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2. Origin of “Gain-of Function” in Mutant SOD Enzyme and the Unique Reactivity of Copper(II)-hydroperoxide Adduct

In order to obtain a comprehensive solution for the correlation between the structural change in mutant SOD and pathogenesis of fALS, we have studied the reactivity of a copper(II)-OOH, proposed as an important intermediate in the SOD reaction. For this purpose, we have synthesized many copper(II) compounds with ligands that contain N,N-bis(2-picolylmethyl)amine moiety, as illustrated in Figure 3. The structural features of all the copper(II) compounds are very similar to each other (as an example, crystal structure of [Cu(bdpg)Cl]+ is illustrated in Fig. 4). In the presence of hydrogen peroxide, formation of a adduct formation as shown at the right side of Fig. 4 is anticipated, and this was consistent with the results on the reaction with cyclohexane, etc. and we have found that the reactivity of a peroxide adduct of the copper(II) compounds is highly dependent on the R of the ligand system, which should be due to the slight structural change around the copper(II) ion due to the different R in the ligand system.

We have measured the ESR spectra of the solution containing a copper (II) complex and spin-trapping reagent, such as PBN (α-phenyl- N- tert-butynitrone) and TMPN (N,N,N',N'- tetramethyl-4-piperidinol), specific reagents for OH• radical and singlet oxygen (Δg) (Scheme-II), respectively.

Fig. 2. ESR spectra of DMPO-OH radical adducts formed in solutions containing H2O2 and the human SOD enzymes. They reported that the free-radical generating activity of the mutant, as measured by the spin-trapping method at low H2O2 concentration, is enhanced relative to that of the wild-type and G93A, wild-type < G93A < A4V, but the reason for the above observation has not been clarified.
No ESR signal due to the formation of radical of PBN was detected when the copper (II) complexes with (tpa) (=tris(2-pyridylmethyl)amine) or (bdpg), was mixed with H₂O₂ and PBN. However, strong peaks due to nitron radical formation of the corresponding TMPN was detected in the solution with Cu(tpa) complex, but not with the Cu(bdpg) complex. Especially comparison between the Cu(pipy)Cl⁺ and the Cu(mopy)Cl⁺ is very interesting.¹⁵ Structural features of the two compounds are very closely related, apart from the difference of the oxygen atom on the morphorin ring of Cu(mopy)Cl⁺ complex is replaced by the -CH₂ in the Cu(pipy)Cl⁺ complex. (see the figure below)

In the case of Cu(pipy)Cl⁺, no formation of the nitron radical was observed in the presence of hydrogen peroxide. In contrast, high activity for the radical formation by the Cu(mopy)Cl⁺ complex was detected as illustrated in Figure 5. The similar high activity for radical formation of TMPN was also observed for the copper(II) complex, [Cu(Hphpy)Cl⁺]. In this case, similar to the Cu(mopy)Cl⁺ complex, the addition of the H₂O₂ to the copper(II) solution does not induce the change in ESR spectrum due to the copper(II) ion; but the addition of TMPN leads to the dramatic change in the ESR signals attributed to the copper(II) species (i.e., the change of hyperfine structure values due to copper atom). These are all comprehensively

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**Scheme-II**

**Fig. 3.** Chemical structures of the ligands used in our study.

**Fig. 4.** Left side: crystal structure of [Cu(bdpg)Cl⁺]; Right side: assumed structure of a peroxide adduct [Cu(bdpg)(OOH)]⁺.
elucidated on the assumption that the complex formation of copper (II), hydrogen peroxide, and TMPN occurs only when three reagents are present in the solution, (see the Fig. 6), and unique reactivity of the hydrogen peroxide observed is detected only when the intermediate is formed in the solution.

Above facts are indicating that the reactivity of the Cu(II)-OOH is determined by the structural properties of the intermediate (see Fig. 6) i.e., by the chemical interactions among copper(II)-OOH species, peripheral groups and substrate. It should be noted here that although hydrogen peroxide has been believed to be relatively inert and not toxic to cells, our present results clearly show that some copper (II) chelates can activate the hydrogen peroxide to exhibit high reactivity similar to that of the singlet oxygen ($^1\Delta_g$).

We also found that some copper(II) complexes exhibit high activity in the oxygenation of the methionine residue of amyloid beta-peptide(1-40) at sulfur atom, and the decomposition of several proteins in the presence of hydrogen peroxide. All these facts may suggest that the “gain-of-function” of the mutant SOD is due to formation of a long-lived highly reactive copper(II)-OOH as an intermediate in the process of SOD reaction. The chemical structures around the copper (II) in the mutant SOD is slightly changed, and this gives an unexpected effect on the reactivity of a copper(II)-OOH as observed in our papers. In the mutant SOD C-N bond cleavage by the Cu(II)-OOH may give great changes in the surface of SOD, leading to destabilizing of the dimer contact of the SOD enzyme. Thus, it is quite likely that formation and existence of a highly reactive Cu(II)-OOH species is an intrinsic origin for oxidative stress in the pathogenesis of ALS, which may be consistent with the recent studies on the destabilizing of the dimer contact of the SOD enzyme.

3. Dissociation of Dimeric SOD molecule into Monomers

As stated before, it is widely recognized that protein aggregation are a common pathological features of many neurological disorders, including Huntington’s, Alzheimer’s, and Parkinson’s diseases and that SOD1 aggregates may be inherently toxic or cause motor neuron toxicity by sequestering chaperons and blocking proper functioning of the proteasome.

In 2004, Rakhit et al. reported that SOD1, normally a dimeric enzyme, dissociates to monomers prior to aggregation for both wild type and mutant proteins. They used the “Dynamic Light Scattering (DLS)” method to detect the dissociation of dimeric SOD to monomers. Very recently we have reported that the capillary electrophoresis method (CE) is very suitable to investigate the conformational change of the proteins and aggregation states of the proteins in solution. As an example, two CE profiles of SOD and transferrin are illustrated in Fig. 6. Although the concentrations of the two enzymes are the same, the peak intensities are quite different from each other, and this has been rationalized on the fact that SOD has a rigid dimeric structure in solution, but dimeric structure of apo-transferrin is more flexible.
We have observed that the drastic decrease of the peak strength due to the dimeric SOD molecule occurs when the copper(II)/ascorbic acid solution was added to the SOD molecule as shown in Fig. 7.; our experimental system was same as that reported by Rakhit et al. This clearly shows that the dissociation of the dimeric SOD molecule can be readily detected by the CE method.

We also have found that the presence of excess hydrogen peroxide induces the loosening or dissociation of dimeric structure of SOD molecule.\(^{26}\) As the origin of the dissociation of the dimeric SOD in the presence of hydrogen peroxide is clear,\(^{26}\) it seems quite likely that the oxidant in the system used by Kakhit et al. should be hydrogen peroxide, and that sporadic ALS may be related with the presence of hydrogen peroxide, and the same discussion may be applied to the elucidation of sporadic prion diseases (see alter).

![Figure 6.](image1) CE profiles of the solutions (protein 2 mg/1ml). A: SOD; B: Apo-transferrin, and C; Fe(ida) complex was added to solution B.

![Figure 7.](image2) CE profiles of the solutions containing SOD (SOD 3 mg/1ml). red: SOD only. Green and blue: copper(II)/ascorbate solution was added to SOD solution. (green, measured immediately after addition, and blue, at after 60 min.)
By using antibody methods to rapidly purified SOD1 and coupling this with mass spectrometry, Sato et al. have measured the relative accumulated levels of wild-type and mutant SOD1 in erythrocytes of 29 SOD1-mutated ALS patients. They observed that the patients with undetectable SOD1 mutant had the shortest disease durations. Although age at disease onset was found to be uncorrelated with the amount of mutant SOD1, the evidence convincingly shows a strong inverse correlation between disease duration and mutant accumulation. In other words, an accelerated disease course is found for mutants that are less stable. This surprising discovery implies that it is the misfolded unstable forms of SOD1 mutants that contribute to toxicity underlying disease progression, and that despite its apparent importance for progression, SOD1 mutant stability is not correlated with disease onset. Thus dissociation of the dimeric SOD1 molecule to misfolded monomers should be an essential important process for ALS pathogenesis. As it has become apparent that hydrogen peroxide plays an important role in the formation of SOD1 monomers, we should pay attention to the formation of excess hydrogen peroxide in the human body, especially due to the reaction between a dimeric iron(III) species and glutathione cycle and other related systems.

4. Copper(II)-OOH in Sporadic Prion Diseases

Between 1980 and roughly 1996, about 750,000 cattle infected with BSE (bovine spongiform encephalopathy, one of TSEs) were slaughtered for human consumption in Great Britain, and at present it is accepted that the central event in TSEs is the post-translational conversion of the normal cellular prion protein (PrP<sup>C</sup>) into an abnormal isoform of called scrapie PrP (PrP<sup>Sc</sup>) that has a high-β-sheet content and is associated with transmissible disease. It is generally recognized that PrP<sup>C</sup> is a copper-containing protein (at most 4 copper ions are present within the octarepeat region located in the unstructured N-terminus).

Analysis of recombinant mouse and chicken PrP<sup>C</sup> has lead to the discovery of an important “gain-of-function” following the formation of the PrP<sup>C</sup> copper complex; PrP<sup>C</sup> has been shown to contribute directly to cellular SOD activity.

The misfolded prions (PrP<sup>Sc</sup>) ultimately kills neurons and leaves the brain riddled with holes, like a sponge. In addition to PrP<sup>Sc</sup>, another protease-resistant PrP of 27-30 kDa, which is called as PrP27-30 was extracted from affected brains. It should be noted here that PrP27-30 is derived from only PrP<sup>Sc</sup> (not from PrP<sup>C</sup>), and no difference in amino acid sequence between PrP<sup>C</sup> and PrP<sup>Sc</sup> have been identified. Based on these facts we may assume that the chemical environment around the copper ion in the PrP<sup>Sc</sup> should be different from those in the PrP<sup>C</sup>; this situation is similar to the difference observed between the those around copper(II) ions in the wild-type and mutant SOD enzyme. Thus, it is most likely that the “gain-of-function” in the PrP<sup>Sc</sup> due to a “highly reactive” Cu(II)-OOH formation may occur as described for the mutant SOD molecule, which leads to the cleavage of the peptide bonds around the copper ion (near at about 90 site), giving dangerous PrP27-30; the latter protein may behave as like the misfolded SOD monomer. In addition to this, it seems quite likely that the copper(II) ions in PrP<sup>C</sup> and also PrP<sup>Sc</sup> may react with hydrogen peroxide to yield a Cu(II)-OOH species, which may give serious effects toward the PrP<sup>C</sup> such as oxygenation at methionine residue, conformational change (i.e., formation of PrP<sup>Sc</sup>), and degradation of protein in the presence of hydrogen peroxide (see Scheme-III). Several experimental facts observed for the native prion proteins seem to be consistent with our discussions. All these findings support our proposal that hydrogen peroxide, which may derive from the SOD function of PrP<sup>Sc</sup> and abnormal metabolism of iron ions is likely to be the real origin of oxidative stress in sporadic prion diseases.

![Scheme-III](image-url)
References


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Introduction of authors

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