Complete Loss of Post-translational Modifications Triggers Fibrillar Aggregation of SOD1 in the Familial Form of Amyotrophic Lateral Sclerosis*\

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Dominant mutations in Cu,Zn-superoxide dismutase (SOD1) cause a familial form of amyotrophic lateral sclerosis (fALS), and aggregation of mutant SOD1 has been proposed to play a role in neurodegeneration. A growing body of evidence suggests that fALS-causing mutations destabilize the native structure of SOD1, leading to aberrant protein interactions for aggregation. SOD1 becomes stabilized and enzymatically active after copper and zinc binding and intramolecular disulfide formation, but it remains unknown which step(s) in the SOD1 maturation process is important in the pathological aggregation. In this study we have shown that apoSOD1 without disulfide is the most facile state for formation of amyloid-like fibrillar aggregates. fALS mutations impair either zinc binding, disulfide formation, or both, leading to accumulation of the aggregation-prone, apo, and disulfide-reduced SOD1. Moreover, we have found that the copper chaperone for SOD1 (CCS) facilitates maturation of SOD1 and that CCS overexpression ameliorates intracellular aggregation of mutant SOD1 in vivo. Based on our in vivo and in vitro results, we propose that facilitation of post-translational modifications is a promising strategy to reduce SOD1 aggregation in the cell.

Amyotrophic lateral sclerosis (ALS) is a devastating disease causing degeneration of motor neurons, leading to paralysis and death. Approximately 20% of familial cases of ALS (fALS) are defined by mutations in the Cu,Zn-superoxide dismutase (SOD1) gene (1, 2), and more than 100 types of mutations have been identified as a cause of this disease. SOD1 is an antioxidant enzyme that disproportionates $O_2^-$ into $O_2$ and $H_2O_2$ at the bound copper ion (3), but a subset of fALS-causing mutant SOD1 fully retains enzymatic dismutase activity (4); therefore, toxicity arises from a gain of toxic function. Although the precise mechanism of how mutations in SOD1 cause neurodegeneration is still open to question, protein aggregates containing mutant SOD1 are found in motor neurons of symptomatic patients and rodent models (5). Accumulation of insoluble SOD1 aggregates has been proposed to inhibit various cellular activities such as axonal transport and mitochondrial function, contributing to neuronal cell death (6).

fALS-causing mutations in SOD1 are considered to trigger non-native interactions and self-aggregation by exerting structural destabilization of an SOD1 molecule. In particular, decreased thermostability of the metal-deficient (apo) form is evident in SOD1 with several types of fALS mutations (7, 8). Recently, however, Rodriguez et al. (9) claimed that the apo form of two fALS-causing mutant SOD1 (H48Q and D101N) exhibits comparable thermostability to that of the wild-type (WT) protein. Even in the absence of fALS-causing mutations, a fraction of SOD1 polypeptide is unfolded at body temperature ($T_m \sim 42 \degree C$) (7); indeed, WT SOD1 has been found in protein inclusions in a small subset of sporadic ALS patients (10). Thermostability itself, therefore, may not be a reliable indicator of SOD1 aggregation, and it is unclear how fALS-causing mutations promote the formation of SOD1 aggregates.

SOD1 is stabilized and matured after post-translational modifications including copper and zinc binding and disulfide formation (11). The copper chaperone for SOD1 (CCS) is responsible for copper loading and disulfide formation in SOD1 (12, 13). In mammalian cells, a CCS-independent pathway of SOD1 maturation has also been reported (14). Given combinations of status of copper and zinc binding, disulfide formation, and dimerization, SOD1 is theoretically able to adopt 44 canonical states (11). Among those, the most immature form is prone to unfolding at a physiological temperature and is susceptible to oligomerization particularly in mutant SOD1 (7). Furthermore, SOD1 aggregation has been observed even after being stabilized by several post-translational modifications; apoSOD1 with intact disulfide forms aggregates after prolonged incubation under oxidative conditions (15). Holo-SOD1 can also form Trp-linked covalent aggregates through its $HCO_3^-$-dependent peroxidase activity (16). Thus, the form(s) of SOD1 that is the most responsible for protein aggregation has yet to be specified.
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In addition to the post-translational modifications for SOD1 maturation, oxidative modifications on cysteine residues are recently described as a key factor in the aggregation of mutant SOD1. SOD1 aggregates cross-linked via intermolecular disulfide(s) have been found in protein aggregates purified from transgenic mice expressing human SOD1 with fALS mutations (17). In addition, the non-conserved cysteine residue, Cys-111, is susceptible to oxidation to cysteine sulfonic acid, which accumulates in the protein inclusions of FALS-modeled transgenic mice (18). Mutations in each of the four cysteine residues are, however, causative for fALS (C6F, C6G, C57R, C111Y, and C146R), implying that oxidative modification at a specific cysteine residue(s) is not essential for SOD1 aggregation.

In this study we have shown that apoSOD1, which lacks the intramolecular disulfide, is the most facile state for forming amyloid-like fibrillar aggregates in the reducing environment typical of the cytosol. An effect of fALS-causing mutations on protein aggregation is to increase intracellular fractions of the apo and disulfide-reduced state by impeding at least one of the SOD1 maturation steps. Given that either metal binding or formation of canonical intramolecular disulfide protects SOD1 from aggregation, we propose that aggregate formation of SOD1 can be ameliorated by facilitating the post-translational modifications of SOD1.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant SOD1 Proteins—Escherichia coli (Rosetta) harboring pET15b, which contains a human SOD1 cDNA fused with a His6 tag at its N terminus, was used for overexpression of SOD1 proteins after induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside (GdnHCl) was performed using Proteus Midi IMAC (ProChem Inc.). SOD1 is unfolded in the elute buffer (50 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, 6 M GdnHCl, pH 8.0), and further addition of 10 mM EDTA chelates copper and zinc ions in the sample solution. Then, the apo-form of SOD1 in 6 M GdnHCl with 10 mM EDTA was refolded in 50 mM Tris, 100 mM NaCl, pH 7.0. The addition of EDTA to the protein solution inhibits binding of metal ions present as trace contaminants in buffers and reagents. Refolded protein samples were concentrated, ultracentrifuged at 110,000 × g for 30 min at 4 °C to remove any insoluble material, and then stored at −80 °C. For experiments using Zn2+ ions, EDTA was further removed by buffer exchange to 50 mM Tris, 100 mM NaCl, pH 7.0. SOD1 with an intramolecular disulfide bond was prepared by incubating the corresponding disulfide-reduced form with 100 μM H2O2 at 37 °C for 1 h and precipitating the protein with 20% trichloroacetic acid. After washing with acetone, disulfide-bonded SOD1 was resolubilized in 50 mM Tris, 100 mM NaCl, 10 mM EDTA, pH 7.0. No other modifications such as tryptophan oxidation were observed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The theoretical averaged m/z of E.E-SOD1(C6S/C111S)S-S is 17,935, and a fALS mutation in which glycine is changed to arginine increases the m/z to 18,034. Observed m/z values were as follows: 17,928 in E.E-SOD1(C6S/C111S)S-S, 18,027 in E.E-SOD1(G37R/C6S/C111S)S-S, 18,030 in E.E-SOD1(G85R/C6S/C111S)S-S, 18,031 in E.E-SOD1(G93R/C6S/C111S)S-S.

For Co2+ titration experiments, SOD1 was bound to HIS-Select Nickel Affinity Gel (Sigma-Aldrich) in the presence of 6 M GdnHCl, washed with 50 mM Tris, 100 mM NaCl, 10 mM imidazole, pH 7.0, and incubated with thrombin-agarose (Sigma-Aldrich) at 37 °C for 1 h to cleave the His tag. SOD1 without a His tag was eluted from the spin column by centrifugation and treated with 20% trichloroacetic acid to remove the bound metal ions. After washing with acetone, SOD1 precipitates were re-solubilized in Chelex-treated 50 mM HEPES, pH 7.4. Treatment of buffers with Chelex resin (Bio-Rad) removes metal ions present as trace contaminants.

Before performing all in vitro experiments using purified SOD1 samples, we removed any insoluble material by ultracentrifugation at 110,000 × g for 30 min at 4 °C. Concentration of SOD1 was spectroscopically determined from the absorbance at 280 nm by using 5500 cm−1M−1 as an extinction coefficient; 1 μl of the refolded sample was 100-fold diluted in 6 M GdnHCl, and the absorption at 280 nm was measured.

Biochemical/Biophysical Analysis of SOD1 Aggregates—Electronic absorption and circular dichroism spectra were recorded using Shimazu UV-2400PC and Jasco J-720, respectively. Images of atomic force microscopy were acquired on a Digital Instruments Multimode Nanoscope IIIa scanning microscope using a J-Scanner. Solution containing SOD1 aggregates (10 μl) was deposited on freshly cleaved highly oriented pyrolytic graphite substrate and incubated for 1 min. Unbound proteins and salt in the buffer solution were washed away with 50 μl of H2O. Imaging was performed in tapping mode with RTESP (Veeco) cantilevers and acquired at a scan rate of 1.0 Hz with 512 lines per image.

Kinetics of SOD1 fibrillation using thioflavin T fluorescence was examined using SpectraMax M2 ( Molecular Devices). In a 96-well plate, 150 μl of the sample solution containing 10 μM SOD1 in 100 mM NaPO4, 100 mM NaCl, 5 mM EDTA, 16.7 μM thioflavin T, pH 7.0 was set per well. Fluorescence signal was monitored at 37 °C at intervals of 2 min with 442 and 485 nm of excitation and emission wavelength, respectively. The plate was shaken for 5 s before each fluorescence reading.

Transgenic Mice, Cell Culture, and Transfection—Details of the transgenic mice expressing SOD1G37R have been published elsewhere (19). Staining of lumenal spinal cords with thioflavin S was performed as described (20). Human SOD1 or SOD1 fused with GFP was cloned into pIRESneo3 (Clontech). For experiments of CCS co-expression, neomycin phosphotransferase coding sequence in pIRESneo3 vector was replaced by the CCS cDNA. Neuro2a cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, penicillin, and streptomycin. After transfection using Lipofectamine 2000 (Invitrogen) in DMEM, 10% fetal bovine serum for 20 h, the culture medium was replaced with DMEM containing either 50 μM CuSO4 or 50 μM ZnSO4 after transfection for 20 h, and cells were then incubated for 20 h at 37 °C.

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Western Blotting Analysis of SOD1 Thiol-disulfide Status—Cells were collected in 20% trichloroacetic acid, and the pellet was obtained by centrifugation. After washing with acetone, the pellet was re-dissolved in 50 mM Tris, 8 M urea, 2% SDS, 100 mM iodoacetamide, 1 mM EDTA, pH 7.4, with sonication and incubated at 37 °C for 1 h. Insoluble material was then removed by centrifugation, and the protein concentration of the supernatant fraction was determined by BCA assay using bovine serum albumin as a standard. Ten μg of total protein was boiled in SDS-PAGE sample buffer in the presence of 150 rpm at 37 °C. When the protein forms insoluble aggregates in the presence of a reducing agent, dithiothreitol (DTT), after overnight agitation by shaking soaks in SDS-PAGE running buffer containing 1% dithiothreitol (17, 21). By this procedure, protein bands corresponding to SOD1 with and without disulfide bond are equally transferred from the gel to the membrane. The protein was electroblotted on the polyvinylidene difluoride membrane and analyzed by Western blotting using sheep anti-human SOD1 antibody (1:5000 dilution; EMD Biosciences) and peroxidase AffiniPure donkey anti-sheep IgG (H+L) (1:5000 dilution; Jackson ImmunoResearch). Blots were developed with SuperSignal West Femto (Pierce), and the image was obtained using LAS 1000 (FUJI FILM).

High-throughput Analysis of SOD1 Aggregates—Neuro2a cells in a 12-well plate were transfected in DMEM, 10% fetal bovine serum with 1.6 μg of pIREsneo3 harboring an SOD1 gene fused with GFP. After overnight transfection, the cells were incubated in DMEM at 37 °C for 24 h and fixed with 4% paraformaldehyde, and the nucleus was stained with Hoechst 33342. Images were captured using the Cellomics ArrayScan V from a total of 150 fields per well at 20X objective magnification. Channel 1 (365 nm, 50-nm bandwidth filter) excites Hoechst 33342, whereas channel 2 (475 nm, 40-nm bandwidth filter) excites GFP. GFP-positive aggregates and GFP-positive cells were separately counted, and fractions of aggregate-containing cells out of transfected cells were calculated. The number of GFP-positive cells counted in each well was ~30,000 on average, and 3–5 independent experiments were done to estimate error bars.

RESULTS

SOD1 Becomes Insoluble in the Absence of Any Post-translational Modifications in Vivo and in Vitro—For specification of the metallation and thiol-disulfide status of SOD1, we designate the totally demetallated and reduced state as E,E-SOD1SH, the superscript of which indicates the status of the disulfide-forming Cys residues (Cys-57 and -146), and E represents “empty” at the copper or zinc binding sites. We first used pseudo-WT SOD1, in which two free cysteine residues, Cys-6 and -111, were mutated to serine, preventing possible Cys-linked oxidation. Therefore, after electrophoresis, the gel was soaked in SDS-PAGE running buffer containing 1% β-mercaptoethanol to reduce the disulfide bond, incubated for 30 min, and washed with SDS-PAGE running buffer without β-mercaptoethanol (17, 21). By this procedure, protein bands corresponding to SOD1 with and without disulfide bond are equally transferred from the gel to the membrane. The protein was electroblotted on the polyvinylidene difluoride membrane and analyzed by Western blotting using...
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are mutated to serine, and the disulfide bond cannot form, whereas the addition of excess Zn$^{2+}$ ion totally suppresses insolubility of SOD1(C$^{4S}$) (Fig. 1A). Apo- and disulfide-reduced form of the WT protein also precipitates after overnight shaking in the presence of both EDTA and DTT (data not shown), suggesting that removal of both bound metal ions and intramolecular disulfide is required for SOD1 aggregation.

Human SOD1 forms a tight dimer, but monomers form only when all post-translational modifications are absent (23). We have, thus, examined the role of monomerization in the decreased solubility using SOD1 with double mutations, F50E/G51E (24). This mutant SOD1 remains monomeric in the presence of an intramolecular disulfide bond and/or bound metal ions. The thioldisulfide status of the protein is confirmed by the mobility difference on an SDS-PAGE gel with and without β-mercaptoethanol in the samples (supplemental Fig. 1). As shown in Fig. 1B, monomeric E,E-SOD1(C6S/C111S/F50E/G51E)$^{5-5}$ does not form an insoluble precipitate after overnight agitation at 37 °C, whereas the addition of DTT results in increased fractions of insoluble SOD1. Monomerization alone, therefore, does not cause aggregation of SOD1 protein.

We further examined the aggregation propensity of SOD1 in the cellular environment using a mice neuroblastoma cell, Neuro2a. When fused with GFP, WT SOD1 diffuses throughout the cell (Fig. 1C). Transient expression of SOD1(C$^{4S}$)-GFP, which is a model of the disulfide-reduced form, does not show any protein inclusions in the cell (Fig. 1D). Because SOD1(C$^{4S}$) becomes insoluble only in the absence of metal ions in vitro (Fig. 1A), binding of intracellular metal ions would keep SOD1(C$^{4S}$)-GFP soluble. It is, however, difficult to control the intracellular concentration of Zn$^{2+}$ ions; therefore, we have prepared mutant SOD1 in which the ligands for Zn$^{2+}$ binding, when the cells were lysed with phosphate-buffered saline containing 1% Nonidet P-40 (supplemental Fig. 2). Taken together, SOD1 exhibits a tendency to become insoluble when all modifications are removed in both in vitro and intracellular conditions.

**SOD1 Aggregates Exhibit Amyloid-like Fibrillar Properties**—In some neurodegenerative diseases, pathogenetic proteins form insoluble material, which is a fibrous protein aggregate called “amyloid” (25). We have, thus, tested whether the insoluble form of SOD1 contains an amyloid-like structure using the amyloid-diagnostic dye, Congo red (26). Insoluble SOD1 was prepared in physiological buffer conditions (pH 7) by agitation of 10 μM E,E-SOD1(C$^{4S}$) at 37 °C overnight. Although the soluble form of E,E-SOD1(C$^{4S}$) does not significantly change the Congo red absorption spectrum, insoluble E,E-SOD1(C$^{4S}$) shifts the absorption peak from 485 to 530 nm (Fig. 2A). Insoluble aggregates of E,E-SOD1(WT)$^{3-3}$ prepared under reducing conditions also red-shifts the Congo red absorption spectrum (data not shown). These data are indicative of the presence of an amyloid-like structure in SOD1 aggregates. Additionally, fibrous morphology with height of ~10 nm was confirmed in insoluble E,E-SOD1(C$^{4S}$) using atomic force microscopy (Fig. 2B). SOD1 polypeptide is, thus, considered to become insoluble by adopting amyloid-like structures.

SOD1 fibrillar aggregates are also characterized using circular dichroism (CD) spectroscopy. One of the typical features of amyloid is a β-sheet structure (25), which is indicated by a negative CD peak around 220 nm observed in insoluble SOD1; soluble E,E-SOD1(C$^{4S}$) is also composed of β-sheets but shows a negative CD peak around 200 nm (Fig. 2C). Different CD spectra between soluble and insoluble SOD1 imply structural...
changes and/or rearrangement of β-sheets upon formation of SOD1 fibrillar aggregates.

Fibril formation is also monitored by a fluorescence increase of thioflavin T, which fluoresces upon specific binding with amyloid-like structures (27). SOD1 fibrillation at 37 °C with plate shaking (see “Experimental Procedures”) exhibits a lag time before an increase of thioflavin T fluorescence. In the absence of plate shaking, no changes in thioflavin T fluorescence were observed for at least 3 days. Experiments at 20 °C did not also result in an increase of thioflavin T. The lag time is reduced when pre-formed SOD1 aggregates are added to the soluble E,E-SOD1(C4S) solution (Fig. 2D). This “seeding” phenomenon is a typical feature observed in amyloid formation (25). We have further confirmed the amyloid-like pathology in a transgenic mouse expressing human SOD1 with a fALS-causing mutation, G37R, using another amyloid-staining dye, thioflavin S; thioflavin T fluorescence were observed for at least 3 days. Experiments at 20 °C did not also result in an increase of thioflavin T. The lag time is reduced when pre-formed SOD1 aggregates are added to the soluble E,E-SOD1(C4S) solution (Fig. 2D). This “seeding” phenomenon is a typical feature observed in amyloid formation (25).

Effects of fALS Mutations on Intracellular SOD1 SH/S-S States—To test how fALS mutations affect the intracellular population of post-translationally modified SOD1 species, the intracellular thiol-disulfide status of SOD1 was examined. Neuro2a cells expressing SOD1 were lysed in 20% trichloroacetic acid to freeze the thiol-disulfide exchange reactions followed by protection of free thiols with iodoacetamide modification. Acidic conditions using trichloroacetic acid also suppressed metal-catalyzed nonspecific formation of disulfide bonds during cell lysis. In cells transiently transfected with WT SOD1, densitometric analysis of the Western blot (Fig. 4) shows that
~60% of WT SOD1 remains reduced. The addition of 50 μM CuSO₄ to the culture medium significantly increases the disulfide form, whereas supplementation of 50 μM ZnSO₄ alone does not affect the SH/S-S ratio of SOD1 (Fig. 4). It is plausible that both CCS-dependent and CCS-independent pathways of SOD1 disulfide formation are enhanced by supplementing the culture media with CuSO₄.

In fALS-mutant SOD1, the band intensity is weaker than that of WT, particularly in its disulfide-reduced form even though equal amounts of total protein were loaded on the gel (Fig. 4A). This supports previous results showing that fALS mutations reduce the intracellular half-life of SOD1 (4). Furthermore, the addition of metal ions to the culture medium could affect the intracellular concentration of SOD1 by modulating its expression and/or degradation. Indeed, the addition of CuSO₄ significantly increases the amount of SOD1S-S (WT, G37R, and G93R mutant SOD1), which may reflect different susceptibilities toward intracellular degradation between SOD1SH and SOD1S-S. Despite this, the thiol-disulfide ratio of SOD1 in the cell is little-affected by either the G37R or G93R mutation; 60–70% of total SOD1 is populated to the reduced state in normal culture medium, whereas the ratio of reduced SOD1 drops to 10–20% in the presence of 50 μM CuSO₄ (Fig. 4B). In contrast, the relative amount of the reduced form of G85R is ~10% higher than that of WT, and 60% of the total G85R protein is in the reduced state even in the presence of 50 μM CuSO₄ (Fig. 4B). This mirrors previous reports showing that no disulfide formation is observed in G85R/C4S and G93R/C4S, but its stoichiometry is less than that of C4S (Fig. 5A) (35). Titration of Co²⁺ against E,E-SOD1(C⁴S) produces absorption peaks at 560 and 580 nm with a shoulder at 530 nm, consistent with Co²⁺ binding at the Zn²⁺ site in SOD1 (supplemental Fig. 3A) (35). Titration of Co²⁺ against E,E-SOD1(C⁴S) linearly increases the absorption at 560 nm, which is saturated at equimolar amounts of Co²⁺ (Fig. 5A) and suggests 1:1 tight binding between SOD1 and Co²⁺.

In G85R/C⁴S, the addition of Co²⁺ ion results in a single absorption peak at 555 nm (supplemental Fig. 3C), suggesting a different coordination of Co²⁺ ion from that in the WT protein. Furthermore, Co²⁺ titration of G85R/C⁴S does not show saturation behavior (Fig. 5A); Co²⁺ and presumably Zn²⁺ affinity of SOD1 significantly decreases with G85R mutation. In contrast, absorption spectra of G37R/C⁴S and G93R/C⁴S are similar to those of C⁴S until the added Co²⁺/protein ratio reaches 0.2 and 0.4, respectively (supplemental Figs. 3, B and D). As more Co²⁺ ion is added, the absorption at 560 nm increases but is less than that of C⁴S (Fig. 5A). This titration result implies “WT-like” tight binding of Co²⁺ at the Zn²⁺-binding site of G37R and G93R SOD1 followed by a weaker metal-protein interaction. It is interesting to note a recent study (36) showing that Zn²⁺ binding of WT SOD1 with an intact disulfide occurs at pH 5.5 with a stoichiometry of one Zn²⁺ (and also Co²⁺) per homodimer. Co²⁺ (Zn²⁺) ion can, therefore, be tightly bound in G37R/C⁴S and G93R/C⁴S, but its stoichiometry is less than one metal ion per monomer.

**Decreased Stoichiometry of Zn²⁺ Binding in fALS-causing Mutant SOD1**—Next, we investigated the effects of fALS mutations on Zn²⁺ binding by Co²⁺ titration under physiological conditions (pH ~ 7.0); Co²⁺ is a reliable substitute for spectroscopically silent Zn²⁺ ion (34). The addition of Co²⁺ in E,E-SOD1(C⁴S) produces absorption peaks at 560 and 580 nm with a shoulder at 530 nm, consistent with Co²⁺ binding at the Zn²⁺ site in SOD1 (supplemental Fig. 3A) (35). Titration of Co²⁺ against E,E-SOD1(C⁴S) linearly increases the absorption at 560 nm, which is saturated at equimolar amounts of Co²⁺ (Fig. 5A) and suggests 1:1 tight binding between SOD1 and Co²⁺.

**Fibrillar Aggregation of Disulfide-reduced SOD1**

**A**

WT G37R G93R G85R/C⁴S G85R/C⁴S G93R/C⁴S

50 μM Cu²⁺ 50 μM Zn²⁺

SH S-S SH S-S SH S-S

**B**

No metal ions added + 50 μM CuSO₄ + 50 μM ZnSO₄

Fraction of SH form (%)

WT G37R G85R G93R

FIGURE 4. A, Western blotting analysis of the intracellular thiol-disulfide status of SOD1, WT, G37R, G85R and G93R, as indicated on the top of each figure. Experimental details are described under “Experimental Procedures.” 100 ng of purified recombinant proteins, C⁴S and C⁴S/C⁹¹S-S (and those with corresponding fALS mutations), were loaded as controls of SOD1SH and SOD1S-S, respectively. B, quantification of intracellular fractions of disulfide-reduced SOD1 by densitometric analysis of the intensity of each band shown in A. Three independent experiments were performed to estimate error bars.
In the presence of substoichiometric Zn\(^{2+}\)/H\(^{11001}\) ion, the SOD1 aggregation propensity is inversely correlated with the affinity for Zn\(^{2+}\)/H\(^{11001}\) ion. The addition of 0.5 mol eq of Zn\(^{2+}\)/H\(^{11001}\) can suppress the fibrillation of C4S but still allows fibrillation of the C4S protein with G37R, G85R, or G93R mutation (Fig. 5 B). Particularly, in G85R/C4S, which exhibits the least affinity for Co\(^{2+}\)/H\(^{11001}\), fibrillation occurs even with an equimolar amount of Zn\(^{2+}\)/H\(^{11001}\) (Fig. 5 C). Considering that the metal-chelating capacity is extremely high in the cytosol (37, 38), reduced stoichiometry of Zn\(^{2+}\)/H\(^{11001}\) ion for its tight binding would contribute to the accumulation of apo and disulfide-reduced SOD1.

In the presence of substoichiometric Zn\(^{2+}\) ion, the SOD1 aggregation propensity is inversely correlated with the affinity for Zn\(^{2+}\) ion. The addition of 0.5 mol eq of Zn\(^{2+}\) can suppress the fibrillation of C\(^{110}\)S but still allows fibrillation of the C\(^{110}\)S protein with G37R, G85R, or G93R mutation (Fig. 5B). Particularly, in G85R/C\(^{110}\)S, which exhibits the least affinity for Co\(^{2+}\), fibrillation occurs even with an equimolar amount of Zn\(^{2+}\) (Fig. 5C). Considering that the metal-chelating capacity is extremely high in the cytosol (37, 38), reduced stoichiometry of Zn\(^{2+}\) ion for its tight binding would contribute to the accumulation of apo and disulfide-reduced SOD1.

Post-translational Modifications Rescue fALS-SOD1 from Intracellular Aggregation—Based on our in vitro and in vivo results, we suppose that intracellular aggregation of SOD1 can be suppressed by increasing fractions of mature SOD1. Because the CCS plays a major role in the loading of a catalytic copper ion as well as formation of an intramolecular disulfide in SOD1 (12, 13), we overexpressed CCS with GFP-fused SOD1 in Neuro2a cells. Using recombinant SOD1(WT)-GFP and CCS proteins, we have confirmed that CCS can load a copper ion and introduce the disulfide bond in an SOD1 protein even in the presence of a GFP tag (supplemental Fig. 4 and Text 1). As expected, we confirmed that co-expression of CCS increases the fraction of disulfide-bonded G37R and G93R SOD1 (both in the presence and absence of GFP), whereas little change was observed in G85R SOD1 (Figs. 6, A and B). It has been suggested that CCS-dependent formation of a disulfide bond is inefficient in G85R SOD1 (11). In the absence of CCS overexpression, GFP-fused SOD1 with a FALS mutation (G37R, G85R, or G93R) forms inclusion bodies in 3–5% of GFP-positive cells, whereas SOD1(WT) fused with GFP does not (Fig. 6C and supplemental Fig. 5). When CCS is transiently co-expressed with GFP-mutant SOD1, we found a significant alleviation of the G37R and G93R aggregation (less than 1% of total GFP-positive cells) but little reduction in G85R aggregation (Fig. 6C and supplemental Fig. 5). This is consistent with the result that CCS is incompetent for forming disulfide in G85R SOD1 (Figs. 6, A and B). These data show that maturation of SOD1 by CCS reduces the amount of intracellular SOD1.
aggregates, and we thus propose that facilitation of post-translational maturation is a promising idea to reduce intracellular aggregation of SOD1.

**DISCUSSION**

Since the discovery of mutations in SOD1 as a cause of fALS (1, 2), more than 100 types of mutations have been reported. Although several pathogenic mechanisms have been proposed (5), it is still perplexing to identify a common biochemical feature shared by all fALS-mutant SOD1. A copper ion bound in several types of mutant SOD1 functions as an activator of SOD1 expression and as a cofactor that can bind a copper ion still produces an ALS-like phenotype (28). Some fALS mutations lead to protein misfolding by metal ion binding and/or disulfide reduction (35, 42, 43), but it is not known which step(s) of post-translational modifications is not known which step(s) of post-translational modifications malfunctions when SOD1 starts to aggregate. Structural destabilization of apoSOD1 has been considered as a common denominator of fALS-causing mutations but is now controversial; some mutant proteins show comparable melting temperatures to that of WT SOD1 (9).

We propose here that increased intracellular fractions of the apo and disulfide-reduced (E, E\(^{SH}\)) state is a common property shared by fALS-mutant SOD1. Metal deficiency in mutant SOD1 has been implicated as an important factor in protein aggregation (7, 8). In our study we have revealed that disulfide reduction is further required for amyloid-like fibrillar aggregation of SOD1. fALS-causing mutations lead to inefficient post-translational modifications and an increased chance of forming an aggregation-prone E, E\(^{SH}\) state (Fig. 7). Structural destabilization of apoSOD1 by fALS mutations will increase intracellular fractions of E, E\(^{SH}\) but is not absolutely required for protein aggregation because E, E-SOD1(WT)\(^{SH}\) forms fibrillar aggregates under physiological conditions in vitro. As extreme examples, fALS-causing mutations occur at copper/zinc ligands (H46R, H48Q, H80R) and the disulfide-forming cysteine residue (C57R, C146R, and several C-terminal-truncated mutations). In these mutant proteins either metal binding or disulfide formation is intrinsically incompetent. Indeed, transgenic rodent models expressing H46R/H48Q, H48Q, or G127X human SOD1 exhibit prominent SOD1 aggregates, whereas mitochondrial vacuolation is mainly described in transgenic mice expressing G37R and G93A human SOD1 (28, 44–46), which can bind metal ions and form an intramolecular disulfide bond (Figs. 4 and 5). These pathological observations are consistent with our proposal that lack of post-translational modifications promotes SOD1 aggregation.

**SOD1 Fibrillation Occurs before Cysteine Modifications**—In transgenic mice expressing human SOD1\(^{G93A}\), pathological cytoplasmic aggregates containing SOD1 have been shown to possess fibrillar morphology (47). But so far the mechanism of SOD1 fibrillation has been unclear. In acidic conditions, fibrillar aggregates of SOD1 were characterized by incubation of the apo form (48). Crystallographic analysis of mutant apoSOD1 with a disulfide bond (E, E-SOD1\(^{1-S-S}\)) has also revealed higher order assemblies of \(\beta\)-sheets with amyloid-like alignment (49), and a physiological concentration (100 \(\mu\)M) of E, E-SOD1\(^{1-S-S}\) at pH 7.0 forms thioflavin T-positive aggregates after incubation for several days (15). In our study, however, E, E-SOD1\(^{1-S-S}\) does not become insoluble or thioflavin T-positive after overnight agitation, and disulfide reduction is required for facile formation of thioflavin T-positive fibrillar aggregates. The apparent inconsistencies between previous studies and our results are probably due to differences in the experimental conditions. In particular, agitation of protein solutions under our conditions probably expedites protein aggregation. Nonetheless, important roles of E, E-SOD1\(^{1-S}\) in aggregation are supported by a recent study showing that the disulfide-reduced form of mutant SOD1 is enriched in spinal cords of transgenic mice expressing human SOD1\(^{G85R}\) throughout life (21). Although aggregates of E, E-SOD1\(^{1-S-S}\) would have different structural/biochemical features from those of E, E-SOD1\(^{1-S}\), the highest aggregation propensity of E, E-SOD1\(^{1-S}\) implies that oxidation of cysteine residues is not absolutely required for SOD1 fibrillation.

Even though in vitro SOD1 fibrillation does not require cysteine modification, SOD1 multimer cross-linked with disulfide bonds has been detected in the affected spinal cord of fALS model mice (17, 50). Proteasomal inhibition in Neuro2a cells also induces formation of the disulfide-linked SOD1 aggregates (51). Nonetheless, roles of disulfide cross-links in the mechanism of SOD1 aggregation have not been fully described. As shown in Fig. 1A, insoluble fibrillar aggregates prepared from
E,E-SOD1\textsuperscript{SH} in our study become monomers in denaturing conditions, whereas SOD1 aggregates of symptomatic fALS-model mice remain multimeric due to intermolecular disulfide cross-links (17). When disulfide bonds are introduced between protein molecules, the covalent character of disulfide renders aggregates quite resistant to degradation. Accumulation of such degradation-resistant species is considered to compromise proteasomal function, as shown in another neurodegenerative disease model (52). E,E-SOD1\textsuperscript{SH} fibrils themselves may not be toxic but could become harmful to the cell after disulfide-cross-linking caused by oxidative stress. Thus, we hypothesize that disulfide-independent fibrillation of SOD1 occurs first, and subsequent intracellular oxidative stress "locks" SOD1 aggregates by introducing an intermolecular disulfide(s).

**SOD1 Maturation and Its Therapeutic Implications**—Our in vitro studies suggest that mutation of Cys residues as well as zinc binding ligands mimic the SOD1 states with various degrees of post-translational modifications. Because a single amino acid change (i.e. fALS-causing mutation) is sufficient to yield a neurotoxic form of SOD1, there is an important caveat that our non-fALS additional mutations may lead to additional/synergistic effects on neurotoxicity. Based on the mechanism proposed here (Fig. 7), enhancing post-translational modifications will nonetheless suppress SOD1 aggregation and may be a good approach for developing molecular therapies of SOD1-related fALS disease.

CCS is a highly specific metallo-chaperone for maturing SOD1 by introducing a copper ion as well as intramolecular disulfide in disulfide-reduced apoSOD1 (12, 13). Knocking out CCS in the transgenic mouse expressing human SOD1\textsuperscript{G93A}, however, does not affect survival as well as neuropathologies, which is consistent with the observation that significant amounts of SOD1 are still activated even in the absence of the CCS gene (53). It has, thus, been considered that a null mutation in the CCS gene in mammalian cells is not sufficient to increase amounts of immature SOD1.

Surprisingly, overexpression of CCS accelerates the disease course in human SOD1\textsuperscript{G93A} transgenic mice (shortening of mean survival from 242 to 36 days) with severe mitochondrial pathology (54). CCS has been shown to regulate the mitochondrial import of SOD1 (55), leading to the idea that increasing the toxicity of mutant SOD1 with CCS expression may arise from CCS-dependent overloading of mutant SOD1 in mitochondria. Regarding the aggregation pathway, both SOD1\textsuperscript{G93A} and SOD1\textsuperscript{G93A}/CCS mice do not exhibit SOD1-positive protein inclusions at 24 days of age, which is within the end-stage of SOD1\textsuperscript{G93A}/CCS mice (54). Therefore, in rodent models, it remains unanswered whether maturation of SOD1 with CCS protects SOD1 from aggregation. In this study CCS overexpression in *Neuro2a* cells is shown to increase the disulfide form and reduce the number of aggregates of G37R and G93R SOD1 (Fig. 6). It is also possible that the complex formation of SOD1 with CCS contributes to the protection of SOD1 from aggregation in the cell (56). Although modulation of the intracellular concentration of CCS may not be promising for developing therapeutics of fALS disease, we believe that maturation of SOD1 is still one strategy to reduce intracellular aggregation.

To facilitate post-translational modifications in mutant SOD1 without toxicity associated with gross CCS overexpression, CCS-independent activation of SOD1 is an attractive target. Because knock-out of CCS gene still produces the active form of SOD1 in a mouse model (53), compensatory pathways for SOD1 maturation in the absence of CCS have been proposed. In one such pathway, protein disulfide isomerase, which catalyzes the formation and rearrangement of disulfide bonds (57), is a candidate for introducing the disulfide into the SOD1 polypeptide. Although it is an open question whether protein disulfide isomerase forms the correct intramolecular disulfide in SOD1, our proposed mechanism (Fig. 7) accords with previous results that inhibition of protein disulfide isomerase activity increases SOD1 aggregate production (58). In addition to disulfide-forming proteins like protein disulfide isomerase, the protein(s) involved in metal loading in SOD1 will exist in the CCS-independent pathway(s). Further studies are needed to clarify how apoSOD1 acquires metal ions in vivo, particularly in the cytosol, which has extraordinarily high metal-chelating capacity (37, 38). Our results suggest that SOD1 aggregation would also be decreased by physiological processes that stimulate CCS-independent processes of SOD1 maturation. In summary, we have identified E,E-SOD1\textsuperscript{SH} as the most important state for fibrillar aggregation, and this study leads to therapeutic implications that facilitation of post-translational modifications suppresses intracellular SOD1 aggregation.

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