Amyotrophic Lateral Sclerosis Mutations Have the Greatest Destabilizing Effect on the Apo- and Reduced Form of SOD1, Leading to Unfolding and Oxidative Aggregation

Mutant forms of Cu,Zn-superoxide dismutase (SOD1) that cause familial amyotrophic lateral sclerosis (ALS) exhibit toxicity that promotes the death of motor neurons. Proposals for the toxic properties typically involve aberrant catalytic activities or protein aggregation. The striking thermodynamic stability of mature forms of the ALS mutant SOD1 ($T_m > 70 \degree C$) is not typical of protein aggregation models that involve unfolding. Over 44 states of the polypeptide are possible, depending upon metal occupancy, disulfide status, and oligomeric state; however, it is not clear which forms might be responsible for toxicity. Recently the intramolecular disulfide has been shown to be required for SOD1 activity, leading us to examine these states of several disease-causing SOD1 mutants. We find that ALS mutations have the greatest effect on the most immature form of SOD1, destabilizing the metal-free and disulfide-reduced polypeptide to the point that it is unfolded at physiological temperatures ($T_m < 37 \degree C$). We also find that immature states of ALS mutant (but not wild type) proteins readily form oligomers at physiological concentrations. Furthermore, these oligomers are more susceptible to mild oxidative stress, which promotes incorrect disulfide cross-links between conserved cysteines and drives aggregation. Thus it is the earliest disulfide-reduced polypeptides in the SOD1 assembly pathway that are most destabilized with respect to unfolding and oxidative aggregation by ALS-causing mutations.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease caused by selective degeneration of motor neurons in the brain and spinal cord (1). About 10% of total ALS cases are of familial origin, and of these, ~20% are caused by point mutations in the abundant enzyme Cu,Zn-superoxide dismutase (SOD1) (2). SOD1 is a dimer of identical subunits, each of which contains a copper and a zinc ion, and catalyzes conversion of superoxide anion to oxygen and hydrogen peroxide at a dinuclear copper-zinc site (3). Studies of SOD1 knock-out mice reveal no motor neuron disease (4), indicating new toxic functions of ALS mutations. Among the proposed gain of functions is aberrant redox chemistry arising from modification of active copper and zinc sites in ALS mutants. For example, enhanced peroxidase activity (5), protein nitration (6), and superoxide production (7) have been proposed. The requirement of copper ion for toxicity has, however, been put into question, because ALS symptoms are observed in mice expressing the SOD1 mutant in which all of the copper ligands are mutated (8).

Another hypothesis for a toxic function acquired by the mutant SOD1 proteins is an increased propensity for cytoplasmic aggregation, which is one of the pathological features common to familial ALS (9). ALS mutations have been considered to induce protein misfolding and/or destabilization; in fact, ALS mutations provoke a decrease of 1–6 °C in the melting temperature of SOD1 (10), although wild type (WT) SOD1 remains active after treatment at 80 °C (11). Reduced zinc affinity in ALS mutants has been proposed to facilitate destabilization of mature SOD1 (12), and conversely zinc binding has been known to increase the protein stability (11). Many studies have focused on how mutations predispose the active mature protein to adventitious reactions; however, studies of the polypeptide in the earlier stages are now emerging (13–15) as the physiological pathways controlling activation of the enzyme are uncovered. Although it remains unclear how SOD1 acquires zinc ions, the copper chaperone for SOD1 (CCS) typically controls copper acquisition (16–18) and disulfide formation in apo-SOD1 (19). Given that ALS mutants are more susceptible to disulfide reduction compared with the WT protein (20), adventitious reductions have been proposed as an important step in the disease. It is not known, however, if altered regulation of the thiol/disulfide status is associated with ALS mutations. In fact, many of the early physicochemical studies were conducted on mixtures of the SOD1 proteins in various degrees of post-translational modifications. In particular, the status of the essential intra-subunit disulfide and the protein quaternary structure is important but frequently unreported.

In this study, we focus on how the stability of both WT and ALS mutant proteins is influenced by post-translational modifications. Given that four post-translational events precede SOD1 activation, i.e. copper and zinc binding, disulfide formation, and dimerization, it is clear that SOD1 can adopt 44 canonical microstates, where metal binding at incorrect sites are not taken into account. It was not clear which states of the protein are most destabilized by disease-causing mutations. By
examining representative subgroups of these states, we can now establish a hierarchy for the effects of zinc binding and disulfide formation on stability and aggregation of WT SOD1 and three ALS mutants (A4V, G85R, and G93A). The results indicate that the unmodified polypeptide is least stable and show that ALS mutants in this state are unfolded and oligomerized at physiological temperature. Disulfide formation and zinc binding reduce oligomer formation by increasing the thermal stability of SOD1. The disulfide-reduced forms, furthermore, are highly susceptible to disulfide-linked multimerization upon oxidative stress, indicating that regulation of intra- or inter-molecular disulfide formation can determine whether SOD1 aggregates or adopts the mature state. In contrast to many models that focus on altered attributes of the mature SOD1 enzyme, these results now focus attention on aberrant properties of the most immature polypeptide and on the role that post-translational modifications may play in SOD1 aggregation.

**EXPERIMENTAL PROCEDURES**

**Preparation of the Apo and Reduced Human SOD1 Proteins—**Proteins were overexpressed in *Escherichia coli* BL21(DE3) and purified as described previously (19, 21). For preparation of the disulfide-reduced protein, 750 μl of as-isolated human SOD1 (hSOD1) (~3.0 g/liter) was anaerobically treated with 200 μM dithiothreitol (DTT) to reduce disulfide bonds. After anaerobic incubation at 37 °C for an hour, solutions were acidified with 0.4% trifluoroacetic acid to reduce the reactivity of thiol groups and to promote the dissociation of metal ions from SOD1. Following incubation in 15% CH₃CN, 10% CH₃OH at room temperature for an hour, the proteins were purified by reverse-phase high pressure liquid chromatography through a C4 Vydc 214TP54 column as described (19). The isolated apo-SOD1 has less than 0.1 and 1% for copper and zinc ions, respectively, based on the inductively coupled plasma atomic emission spectroscopic analysis. Reduction of the disulfide bond was confirmed by the 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) modification (see below). The resulting apo and reduced SOD1 proteins can be activated by adding copper-bound CCS (data not shown) (19, 22), indicating that the protein tolerates relatively harsh treatment with acid and organic solvents.

The disulfide form of the protein was prepared by oxidizing ~200 μM E,E-hSOD1 with 1 mM K₃[Fe(CN)₆] in the presence of 200 μM ZnSO₄ and 20% glycerol. After 1 h of aerobic incubation the solution was acidified and purified by high pressure liquid chromatography as described above. Protein concentrations were determined with the absorption at 280 nm using 5500 m⁻¹ cm⁻¹ per monomer as the extinction coefficient (23). The Bradford assay with IgG as a standard was also used to routinely determine the protein concentration.

**Thiol Modifications—**To analyze the thiol-disulfide status in hSOD1, modification with either AMS (Molecular Probes, Inc.) or iodoacetamide (IA) was employed (19). Protein was precipitated with 20% trichloroacetic acid, washed with acetone, and dried under vacuum. Protein modification buffer (50 mM HEPES, pH 7.2, 1 mM EDTA, 1 mM bathocuproine disulfonate, 2.5% SDS) containing either 25 mM AMS or 100 μM IA. After anaerobic incubation at 37 °C for an hour, the modified proteins were separated by SDS-PAGE.

**RESULTS**

Human SOD1 (hSOD1) has four cysteine residues; Cys⁵, Cys²⁵, Cys¹¹¹, and Cys¹⁴⁶, among which Cys⁵⁷ and Cys¹⁴⁶ are conserved and form the intra-molecular disulfide bond (24). In this study, X₇-hSOD1₁⁻⁻⁻⁻⁻ signifies protein derivatives in which metal ions have been substituted into the copper-binding site (X) or zinc-binding site (Y). E means empty at the metal-binding site, and the superscripts, SH or S–S, indicate the thiol/disulfide status of the conserved Cys residues. For instance, E,Zn-hSOD1₁⁻⁻⁻⁻⁻ denotes disulfide-reduced protein with a Zn²⁺ ion at the zinc-binding site but no metal ion at the copper-binding site. To reveal the effects of post-translational modifications on protein stability, we first measured the melting temperatures of E,E- and E,Zn-hSOD1₁⁻⁻⁻⁻⁻.

**Post-translational Modifications Can Increase SOD1 Thermal Stability—**Whereas accurate enthalpy values for SOD1 thermal unfolding are not readily obtained from the differential scanning calorimetry (DSC) thermograms (25), comparisons of the relative melting temperature, Tm, reveal relationships among the thermal stabilities of isolated states of SOD1. The unmodified WT protein, E,E-hSOD1₁⁻⁻⁻⁻⁻, melts between 30 and 50 °C and exhibits an endothermic peak at 42.9 °C (Fig. 1A). Given that the holoenzyme (Cu,Zn-hSOD1₁⁻⁻⁻⁻⁻) is one of the most stable proteins known in mesophilic organisms (11), it is interesting that the Tm value of the SOD1 polypeptide in the absence of any post-translational modifications is just above the typical physiological temperature in mammals, ~37 °C. Disulfide formation and zinc binding stabilize the SOD1 polypeptide, increasing the Tm to 49.8 and 58.4 °C, respectively. The Tm value is further increased to 74.6 °C upon formation of the disulfide bond in E,Zn-hSOD1 (Fig. 1, A and B). Fig. 1C graphically shows increase in Tm upon post-translational modifications relative to the unmodified E,E-hSOD1₁⁻⁻⁻⁻⁻ state. Disulfide formation in E,E-hSOD1₁⁻⁻⁻⁻⁻ increases Tm by as much as 6.9 °C, whereas a 15.5 °C increase is observed upon binding of the Zn²⁺ ion. Zinc binding therefore has a larger contribution to SOD1 stability than disulfide formation. These increases of Tm are comparable with those upon disulfide formation or zinc binding in other proteins (26, 27). It is also notable that the ΔTm of 31.7 °C upon both zinc binding and disulfide formation is significantly larger than ΔTm values for the individual modifications (Fig. 1C). Zinc binding and disulfide formation can thus increase the thermal stability of SOD1.

Given that one of the disulfide-bonding cysteine residues, Cys⁵⁷, is positioned in loop IV, reduction of the loop flexibility upon disulfide formation would result in the SOD1 stabiliza-
between 5.1 and 5.7 °C from that of WT ($\Delta T_m^{\text{mut}}$ in Table I). The alterations in $T_m$ observed upon the Cys mutations may be relevant in the ALS mechanism, because disease-associated mutations have been reported for Cys$^6$ and Cys$^{146}$ (www.alsod.org). In order to examine how disease-associated mutations alter the protein stability, we next measured $T_m$ of two common and widely studied ALS mutants, A4V and G93A.

Unmodified ALS Mutant SOD1 Polypeptides Melt at Temperatures Below Physiological Threshold—Previous studies have shown that $T_m$ values of ALS mutants are 3–15 °C lower compared with that of WT protein, although in these cases the thiol/disulfide status was not determined, and mixtures of states are sometimes apparent in the thermograms (10, 29). Most unexpectedly, however, we could not characterize an endothermic transition on the E,E-hSOD1$^{\text{SH}}$ states of ALS mutants were obtained, 35.2 °C (A4V) and 31.2 °C (G93A). These $T_m$ values for the mutant E,E-hSOD1$^{\text{SH}}$ states are significantly lower (by 10.6 °C in A4V and 14.6 °C in G93A) than that of WT protein in the same glycerol-containing buffer ($\Delta T_m^{\text{mut}}$ in Table II). These results show that ALS mutants are completely unfolded in the E,E-hSOD1$^{\text{SH}}$ state at physiological temperature and concentration, even when protein-stabilizing agents are present.

DSC measurements showed that, irrespective of the method, apo-SOD1 would have the disulfide bond. Furthermore, not only Cys$^{57}$ but also all ligands for the Zn$^{2+}$ ion (His$^{53}$, His$^{71}$, His$^{80}$, and Asp$^{183}$) are involved in loop IV. Zinc binding would hence be able to introduce structural restraints around loop IV more significantly than disulfide formation, which is consistent with a larger stabilizing effect due to zinc binding (Fig. 1C). Disulfide formation and zinc binding may stabilize SOD1 through reduction of the loop IV flexibility; in fact, E,E-hSOD1$^{\text{SH}}$ is on the threshold of unfolding at physiological temperature even without any disease-associated mutations (Fig. 1A). This is interesting given that protein aggregates containing WT hSOD1 have been found in a subset of sporadic forms of ALS disease where the molecular defects remain unknown (28).

During DSC measurements, formation of non-native disulfide bonds such as the one between Cys$^6$ and Cys$^{111}$ may also occur (25). We thus further tested for stabilizing effects of the post-translational modifications on hSOD1 by using the following Cys mutants: C6S/C111S and C6S/C57S/C111S/C146S (C$^6$S). The C$^6$S mutant has no Cys residues and can serve as a control for the disulfide-reduced form, whereas the C6S/C111S mutant, which has no free thiol groups when the conserved disulfide bond (Cys$^{57}$–Cys$^{146}$) is intact, can be compared with the disulfide state of WT protein. The apo-form of the C$^6$S mutant and the reduced C6S/C111S mutant exhibit $T_m$ values of 37.2 and 37.8 °C, respectively (Table I). As with WT protein, zinc addition and disulfide formation in the Cys mutants lead to protein stabilization by increasing $T_m$ (Table I). We conclude from these results that the observed stabilization of WT protein upon oxidation arises from formation of the conserved disulfide and not from adventitious disulfides involving Cys$^6$ or Cys$^{111}$. Another important observation in Table I is that mutation of these two Cys residues slightly stabilizes the modified proteins but destabilizes the unmodified polypeptides; the unmodified polypeptides of both Cys mutants represent a destabilization...
probe the SOD1 aggregation states, we characterized each modified state by using the gel filtration chromatography.

**Unmodified Form of ALS Mutants Is Oligomerized—** Gel filtration studies conducted at 4 °C show that E,E-hSOD1SH of WT is a monomer at 30 μM (Fig. 3A), a concentration within the physiological range of 10–100 μM (34, 35). Consistent with our previous findings (36), either disulfide formation, zinc addition, or both leads to dimerization (Fig. 3, A and B). No aggregated forms are observed, which is also consistent with the calorimetry results showing that the WT polypeptide melts at 42.9 °C (Table II).

Unlike the distinct monomeric and dimeric states observed for WT protein, most of E,E-hSOD1SH(A4V) elutes from a gel filtration column with an apparent molecular weight corresponding to 50–60 kDa, and its broad elution profile extends to the region corresponding to the monomeric species (Fig. 3C). Without any modifications, therefore, the A4V mutant is present in oligomerized forms at 4 °C. Even in the presence of 0.2 M NaCl, which would reduce possible electrostatic interactions in the oligomer, the oligomeric fractions are still observed (data not shown). Oligomerization may be related to our inability to observe an endothermic peak in the DSC thermogram of E,E-
hSOD1\textsubscript{SH}(A4V) in the absence of glycerol. In the presence of 20\% glycerol, which allows detection of \(T_m\) (Table II), the oligomeric fractions in E,E-hSOD1\textsubscript{SH}(A4V) were decreased and shifted to the distinct monomeric and dimeric states (supplemental Fig. S1). Even in the absence of glycerol, the extent of the E,E-hSOD1\textsubscript{SH}(A4V) oligomerization is significantly reduced by either zinc binding, disulfide formation, or both (Fig. 3, C and D). Instead, these modifications of the A4V mutant protein favor an equilibrium mixture between the monomeric and dimeric states with correspondingly broad elution profiles. The A4V mutation, therefore, develops the propensity for oligomerization in the E,E-hSOD1\textsubscript{SH} state most likely due to the accessibility of unfolded states at physiological temperatures.

The G93A mutant also exhibits a propensity for oligomerization in its unmodified polypeptide state. E,E-hSOD1\textsubscript{SH} of G93A elutes at 13.8 ml, which corresponds to a significantly higher apparent molecular weight (~55 kDa) than that of the SOD1 dimer (Fig. 3E). Addition of 20\% glycerol can decrease the amount of the oligomeric forms, resulting in distinct monomer-dimer mixtures (supplemental Fig. S1). As with the A4V protein, addition of zinc ion, disulfide formation, or both reduces the G93A oligomeric fractions, and the population of states shifts significantly to the dimer form (Fig. 3, E and F). Because little, if any, oligomer in the E,E-hSOD1\textsubscript{SH} state is observed for WT protein, oligomerization of the most immature form of SOD1 is a conspicuous feature acquired by ALS mutations. Our results also reveal that the extent of the oligomerization is significantly diminished upon post-translational modifications that stabilize the SOD1 protein.

Mild Oxidants Multimerize E,E-hSOD1\textsubscript{SH} via Formation of Inter-molecular Disulfides between Conserved Cysteines—The major intracellular reductant, GSH, is present in the cell with its oxidized species, GSSG, and the sum of their cytosolic concentrations is in the millimolar range for most cells. A mild oxidative stress can therefore be reconstituted by decreasing the GSH/GSSG ratio (37). When \(3 \mu\text{M} E,E\text{-hSOD1}^{\text{SH}}\) is anaerobically incubated in the presence of GSH and GSSG, SOD1 bands in the high molecular region become intense with a decrease in the GSH/GSSG ratio from 100 to 0.5, regardless of WT or ALS mutants (Fig. 4, upper panel). To avoid thiol oxidations within the gel, all samples are treated with the thiol-specific modifier, iodoacetamide (IA), before sample loading on SDS-polyacrylamide gel. When samples are treated with the reductant \(\beta\text{-ME}\) before sample loading on a gel, these high molecular weight bands disappear (Fig. 4, lower panel), suggesting that these SOD1 multimers are stabilized through formation of nonspecific inter-molecular disulfide bonds. The dimer band can still be seen, but its fraction is significantly reduced after addition of \(\beta\text{-ME}\). These disulfide-linked multimers as well as the oligomer seen in E,E-hSOD1\textsubscript{SH} forms of ALS mutants do not bind Congo Red (data not shown), suggesting that the SOD1 oligomers/disulfide-linked multimers are distinct from amyloid-like states (29). The disulfide-linked multimers are insoluble in the absence of detergents such as SDS and Triton X-100 (data not shown), consistent with significant aggregation beyond that shown in Fig. 3. Glutathionylation of SOD1 can also be observed in the mass spectrum (supplemental Fig. S2), and these results strongly suggest that E,E-hSOD1\textsubscript{SH} can readily form multiple disulfide bonds under mild oxidizing conditions.

Oxidative modification of the His and Tyr side chains of SOD1 proteins has been proposed, and such reactions may lead to covalent cross-links between SOD1s (38, 39); however, this was not the case in our current assays. The C\textsuperscript{34}S hSOD1 mutant shows no multimer bands at any of the GSH/GSSG ratios with or without \(\beta\text{-ME}\) treatment (Fig. 5), consistent with the idea that these SOD1 polypeptides are linked via inter-molecular disulfide bonds. To test whether the conserved Cys residues, Cys\textsuperscript{57} and Cys\textsuperscript{146}, are more important in the oxidative multimerization than the other cysteines, we examined two additional Cys mutants, \textit{i.e.} C6S/C111S and C57S/C146S. As seen in Fig. 5, the E,E-form of the reduced C6S/C111S mutant exhibits increasingly high molecular weight bands upon decreasing the

![Fig. 3. E,E-hSOD1\textsubscript{SH} of ALS mutants are oligomeric at physiological concentration. Gel filtration chromatograms of 30 \(\mu\text{M} SOD1\) proteins at 4 °C: A and B, WT; C and D, A4V; and E and F, G93A. The upper panels (A, C, and E) are chromatograms for the disulfide-reduced form, and the disulfide forms are shown in the lower panels (B, D, and F). Metallation state is shown in each panel. Elution for E,E-hSOD1\textsubscript{SH} and E,E-hSOD1\textsubscript{S-S} is conducted in the presence and absence of 1 mM DTT, respectively. E,Zn-hSOD1\textsubscript{SH} and E,Zn-hSOD1\textsubscript{S-S} are eluted with 50 mM K-Pi, 30 \(\mu\text{M} \text{ZnSO}_4\), pH 7.8, in the presence and absence of 1 mM DTT, respectively.](image-url)
GSH/GSSG ratio, indicating that disulfide-linked multimerization readily occurs through Cys57 and Cys146. In contrast, we do not find SOD1 multimers after parallel treatment of the C57S/C146S mutant (Fig. 5). These results suggest that formation of disulfide-linked multimers need not involve the nonconserved Cys residues, Cys6 and Cys111. Instead, the conserved Cys residues, Cys57 and Cys146, play an important role in the E,E-hSOD1SH multimerization upon oxidative stress. Given that zinc ion significantly increases the stability of SOD1 (Fig. 2), we next examined the possibility that zinc binding has a protective role in the SOD1 disulfide-linked multimerization.

**Zinc Binding Decreases the Extent of Disulfide-linked Multimerization in WT but Not in ALS Mutants—** As seen in Fig. 6, formation of intermolecular disulfides is suppressed in E,Zn-hSOD1SH(WT) relative to the zinc-free form (Fig. 4). Even under the most oxidizing conditions (0.5 of GSH/GSSG), a majority of WT protein migrate as a monomer. This result indicates that, under mild oxidative stress, E,Zn-hSOD1SH(WT) preferentially forms intra-molecular as opposed to inter-molecular disulfide bonds. In ALS mutants, however, multimerization of E,Zn-hSOD1SH is readily seen upon mild oxidation (Fig. 6). Zinc binding to the protein is thus an important event that increases stability of both mutant and WT SOD1; yet despite the zinc-induced increase in thermal stability, ALS mutants are significantly more susceptible than WT to forming multimers that are stabilized by incorrect disulfide formation.

**DISCUSSION**

Many of the disease-causing mutations in hSOD1 can give rise to significant amounts of active enzyme; however, other ALS mutations are completely inactive (1). A hallmark of ALS mutants is a dominant "gain of function" that is particularly toxic to motor neurons (9). Whereas cellular targets of the adventitious "function" are not yet clear, two general categories of models for the toxic physicochemical properties of the mature ALS mutants are discussed (1) as follows: (a) deleterious biochemical activities related to the metal cofactors, or (b) the propensity to form toxic aggregates. Here we address two issues of SOD1 stability and folding common to aggregate models. First, we identify the most unstable form of the mutant relative to the WT protein, and we show that it is also the form most prone to oligomerization. Second, we identify an attribute of SOD1 mutants that facilitates oxidative aggregation. The results here suggest that a toxic function acquired by ALS mutations may not be manifested as an attribute of the activated enzyme but of a much earlier stage in the maturation process.

**Oligomer Formation in the Most Immature State of ALS Mutants—** Most previous studies of hSOD1 stability employed mixtures of the holoprotein with protein in intermediate stages of maturation. In particular, the disulfide status has rarely been established; thus, little is known about the effects of ALS mutations on the disulfide-reduced protein. Biochemical methods for isolating and characterizing the distinct disulfide states for *Saccharomyces cerevisiae* SOD1 provide new tools to address these issues (19). As seen in Fig. 2C, ALS mutations in human SOD1 have a pronounced destabilizing effect on the E,E-hSOD1SH state of the disulfide at the "final" stage. The results here suggest that a toxic function acquired by ALS mutations may not be manifested as an attribute of the activated enzyme but of a much earlier stage in the maturation process.
Unfolding and Aggregation of Immature ALS-SOD1 Polypeptides

**Fig. 6.** Addition of zinc protects WT but not ALS mutant SOD1 from oxidative multimerization. Mild oxidation of disulfide-reduced apo-hSOD1 in the presence of ZnSO₄ was examined by Western blot. 3 μM E,E-hSOD1SH in 50 mM HEPES, pH 7.2, was anaerobically incubated with an equimolar amount of ZnSO₄ for an hour at 37 °C, to which GSH and GSSG were then added. After incubation for an hour at 37 °C, the proteins were precipitated with 20% trichloroacetic acid, modified with 25 mM AMS, and then loaded on 12.5% SDS-polyacrylamide gel without β-ME.

Unfolding and Aggregation of Immature ALS-SOD1 Polypeptides

**Disulfide-reduced Forms of SOD1 Are Susceptible to Oxidative Aggregation by Mild Physiological Oxidants.—**We have also identified another attribute of the ALS mutant that becomes apparent upon oxidative stress, namely the propensity to form detergent-soluble multimers cross-linked by *intermolecular* disulfides. SOD1 has an increased susceptibility to oxidative modification during import into the mitochondria (40) or in transport along the meter-long axon in the neuronal cell (41). Exposure of the most immature form of the mutant polypeptides to a mild oxidant, GSSG, leads to multimers containing intermolecular disulfides (Fig. 4). Most interestingly, this is not the only state susceptible to incorrect disulfide formation; ALS mutants in the zinc-loaded and disulfide-reduced state are much more susceptible to mild oxidative aggregation than their WT counterpart (Fig. 6). Thus, more than one of the intermediate states in the maturation pathway of ALS mutants are susceptible to oxidative aggregation. Disulfide bonds have long been recognized as a major factor for stabilizing folded states (42), but recently they have also been found to play a role in the toxicity of some disease-related proteins such as prion (43) and transthyretin (44).

**Immature Reduced Protein: a Branch Point between Oligomerization and Maturation.—**Taken together, the results suggest that the toxic gain of function acquired by the ALS mutant proteins may not be an attribute of the activated enzyme but of much earlier stages in the post-translational pathway. A model for aggregation of the SOD1 polypeptide is shown in Fig. 7, where the unfolded E,E-hSOD1SH form sits at the branch point between productive folding and aggregation pathways. The unmodified WT polypeptide could readily partition between these pathways even under cellular conditions that include extreme fluxes in SOD1 transcription/translation, reducing environment of the cytosol, or metal-cofactor limitation. Under most conditions, in contrast, this state of ALS mutant polypeptides is unfolded and most likely in equilibrium with oligomers in the cell. The ALS mutations clearly lead to a thermodynamic destabilization, but they may also diminish the folding rate, which is known to be affected in β-sheet-containing proteins (45). As proposed in the other human diseases (46, 47), we speculate that the mixture of the various states of mutant SOD1 can be accommodated by the cellular quality control systems, including heat shock, copper chaperone proteins, and degradation processes, but only up to a point. Pre-existing pools of ALS mutants in the holo-state, i.e., a mutant protein that has undergone post-translational modifications via the CCS-dependent or independent pathways (48), may be reduced to give more immature states under certain cellular stress, thus increasing the steady state concentrations of aggregation-prone forms. Once aggregate formation begins, other disulfide-reduced intermediate states in the maturation pathway such as E,Zn-hSOD1SH may also be accumulated via adventitious disulfide formation.

Mutations in approximately two-thirds of the residues of SOD1 can cause ALS. These diverse mutations, however, do not cluster; they are distributed throughout the SOD1 structure. Our model wherein toxicity arises from conformational destabilization and unfolding of the unmodified proteins is compatible with this diverse set of mutations as long as the disease-causing mutations change the folding kinetics or thermodynamics of the immature protein. This type of misfolding/oxidation mechanism is consistent with the previous in vivo studies that deletion of CCS does not modify the onset and progression of the ALS disease in the hSOD1-mouse model (49).

**Impact of Oxidative Aggregation on Potential Targets of the Toxic ALS Mutants—**The involvement of specific intracellular target(s) in the toxicity of the ALS mutant SOD1 proteins has not yet been established. Leading candidates are axonal transport systems (41), the proteasome (50, 51), and the mitochondria (52). It is plausible that SOD1 aggregation retards the slow axonal transport of proteins, promoting motor axon degeneration (53). Unfolding/aggregation of the SOD1 mutants may also facilitate their ubiquitination, through which the proteolysis of the mutants and other proteins will overload proteasomes and inhibit their function (50, 51).

The mitochondrial electron transport chain is a predominant source of reactive intracellular oxidants (54), and mitochondrial abnormalities such as vacuolation have been reported as an early pathological feature in several murine lines expressing ALS mutants (52). SOD1 has been shown to be imported into the intermembrane space of mitochondria (55, 56), and only the most immature form, E,E-hSOD1SH, is taken up into intermembrane space (57). Therefore, in the oxidizing environment of the mitochondrial intermembrane space, unstable and oligomeric forms of the reduced ALS mutants would be subjected to adventitious oxidations that can form the disulfide-linked multimers. During mitochondrial import, ALS mutants in the oligomeric E,E-hSOD1SH state may accumulate at the surface of the mitochondria or inhibit transport of other essential mitochondrial proteins, leading to damage and apoptotic cell death. This mechanism is consistent with recent findings that the ALS mutant is covalently associated with the cytoplasmic surface of the spinal cord mitochondria (40). Given that the spinal cord mitochondria can intrinsically produce higher levels of reactive oxygen species (58), ALS mutants near the spinal cord mitochondria have an increased chance of oxidative insult, which will facilitate the disulfide-linked multimerization and possibly the covalent attachment to crucial mitochondrial components such as an anti-apoptotic Bcl-2 family
protein. These modifications would further lead to mitochondrial injury, release cell death mediators, and contribute to motor neuron cell death. 

**Therapeutic Implications**—Our results show that maturation can provide significant protection against aggregation of SOD1 (Fig. 7). If the in vitro observations showing that zinc binding and disulfide formation deter oligomer formation and oxidative aggregation are borne out by further tests in vivo, there are significant therapeutic implications. Overexpression of proteins like CCS that are involved in SOD1 maturation, protein folding (e.g. HSP70), general disulfide formation (protein-disulfide isomerases), or expression of protein degradation machinery (proteasomes) should diminish the extent of aggregation. This approach of stimulating maturation is supported by other biochemical studies showing that an engineered disulfide bond between subunits that force SOD1 dimerization can reduce aggregation.

In addition, dietary modification that increases copper and zinc delivery to motor neurons, gene therapy, and/or drug-like molecules that lower SOD1 polypeptide levels or that facilitate the post-translational modifications of SOD1 could also be productive approaches to treating ALS. There are, however, two important caveats about the therapeutic applications concerning this (Fig. 7) and other aggregation models. First, if other models for the molecular origin of the disease, such as adventitious catalytic activity of the mature copper-loaded mutant enzyme, are instead borne out, the opposite therapeutic approach will be warranted. Second, it may be possible to rescue some, but not all, of the disease-causing mutations by stimulating the maturation pathway. Among the more than 100 ALS-associated hSOD1 mutants, seven cannot form intrinsically the essential intramolecular disulfide bond but are nonetheless capable of forming the intermolecular cross-links involved in oxidative aggregation. One of the conserved Cys residues, Cys$^{146}$, is missing in the following seven mutants: Val$^{118}$ ins AAAAC (stop at 150), Leu$^{126}$-STOP, Gly$^{127}$ ins TTG (stop at 133), Glu$^{132}$ ins TT (stop at 133), Gly$^{141}$-STOP, and Cys$^{146}$ $\rightarrow$ Arg. It has been reported that minute quantities of SOD1 aggregates can cause the disease in the mice expressing the truncated mutant, Gly$^{127}$ ins TTG (stop at 133) (62). Decreased affinity of Zn$^{2+}$ ion in ALS mutants has also been reported (12). The findings here thus suggest that the combined post-translational modifications of zinc binding and disulfide formation may have a protective role in preventing oligomerization and oxidative aggregation of SOD1 from some but not all mutations. In vivo tests of our current aggregation models are now under way, including characterization of the status of the metallation and thiol/disulfide in the SOD1 protein.

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REFERENCES

1. Bruijn, L. I., Miller, T. M., and Cleveland, D. W. (2004) *Annu. Rev. Neurosci.* **27**, 723–749
2. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O’Regan, J. P., Deng, H. X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Halperin, J. J., Herrfeldt, B., Van den Bergh, R., Hung, W.-Y., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pertic-Vancer, M.-A., Haines, J., Rouleau, G. A., Gusella, J. S., Horvitz, H. R., and Brown, R. H. (1993) *Nature* **362**, 59–62
3. McCord, J. M., and Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055
4. Reaume, A. G., Elliott, J. L., Hoffman, E. K., Rowell, N. W., Ferrante, R. J., Siwek, D. F., Wilcox, H. M., Flood, D. G., Beal, M. F., Brown, R. H., Jr., Scott, R. W., and Snider, W. D. (1996) *Nat. Genet.* **13**, 43–47
5. Wiedau-Pazos, M., Goto, J. J., Rakiszszêl, S., Graula, E. B., Roe, J. A., Lee, M. K., Valentine, J. S., and Brederose, D. E. (1996) *Science* **271**, 515–518
6. Beckman, J. S., Carson, M. Smith, C. D., and Koppenol, W. H. (1993) *Nature* **364**, 584
7. Estevez, A. G., Crow, J. P., Sampson, J. B., Reiter, C., Zhuang, Y. X., Richard-
