Familial Amyotrophic Lateral Sclerosis Mutants of Copper/Zinc Superoxide Dismutase Are Susceptible to Disulfide Reduction*

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We observed that 14 biologically metallated mutants of copper/zinc superoxide dismutase (SOD1) associated with familial amyotrophic lateral sclerosis all exhibited aberrantly accelerated mobility during partially denaturing PAGE and increased sensitivity to proteolytic digestion compared with wild type SOD1. Decreased metal binding site occupancy and exposure to the disulfide-reducing agents dithiothreitol, Tris(2-carboxyethyl)phosphine (TCEP), or reduced glutathione increased the fraction of anomalously migrating mutant SOD1 proteins. Furthermore, the incubation of mutant SOD1s with TCEP increased the accessibility to iodoacetamide of cysteine residues that normally participate in the formation of the intrasubunit disulfide bond (Cys-57 to Cys-146) or are buried within the core of the β-barrel (Cys-6). SOD1 enzymes in spinal cord lysates from G85R and G93A mutant but not wild type SOD1 transgenic mice also exhibited abnormal vulnerability to TCEP, which exposed normally inaccessible cysteine residues to modification by maleimide conjugated to polyethylene glycol. These results implicate SOD1 destabilization under cellular disulfide-reducing conditions at physiological pH and temperature as a shared property that may be relevant to amyotrophic lateral sclerosis mutant neurotoxicity.

Amyotrophic lateral sclerosis (ALS) is an age-dependent degenerative disorder of motor neurons in the spinal cord, brain stem, and brain (1). Approximately 10% of ALS cases are familial, and ~20% of these individuals inherit one of >90 autosomal dominant mutations in the gene encoding copper/zinc superoxide dismutase 1 (SOD1) (2).

SOD1 is a 32-kDa homodimeric enzyme expressed predominantly in the cytosol that decreases the intracellular concentration of superoxide radicals (O2•−) by catalyzing their dismutation to H2O2 and O2. ALS-associated mutations of conserved residues throughout the protein impart a toxic property to the enzyme that appears unrelated to its normal dismutase activity (reviewed in Ref. 3). Whereas transgenic mice that overexpress mutant SOD1s consistently develop lethal motor neuron degeneration (4–8), mice that overexpress the wild type (WT) enzyme exhibit only subtle motor abnormalities (9). In addition, SOD1 knock-out mice are not susceptible to motor neuron loss unless following axonal injury (10).

Mutant SOD1 enzymes have been proposed to facilitate aberrant copper-mediated chemistry, disrupt protein recycling or chaperone function, form toxic aggregates, or induce organelle dysfunction or apoptosis (3, 11, 12), but the precise mechanism of specific motor neuron toxicity has not been elucidated. The observation that some mutant SOD1s exhibit accelerated turnover in vivo or increased proteolytic susceptibility compared with the WT enzyme (13–15) suggests that biologically significant perturbations of mutant SOD1 conformation occur. The induction of chaperone proteins that can protect cultured motor neurons from mutant SOD1 toxicity (16) and appear to associate with SOD1 mutants (17) provides further evidence that destabilization or unfolding of mutant SOD1s in vivo may be related to their toxicity.

We previously purified 14 different biologically metallated ALS mutant SOD1s and observed that one group of “WT-like” mutants (A4V, L38V, G41S, G72S, D76Y, D90A, G93A, and E133A) bound copper in a fully active coordination environment remarkably similar to that of normal SOD1 despite causing a lethal phenotype (18). The other six “metal-binding region” mutants (H46R, H48Q, G85R, D124V, D125H, and S134N) were clearly distinguished from WT SOD1 according to decreased metal ion contents, altered visible absorption spectra, or decreased specific activities. In a further analysis by differential scanning calorimetry, we found that metal-binding region mutants generally exhibited a larger fraction of species that unfolded at low Tm values compared with those of the WT-like mutants and normal SOD1 (19). Although purified SOD1 mutants can lose in vitro metal ion binding specificity following partial denaturation at non-physiological pH (20), the retention of many native properties in the “as-isolated” WT-like mutants suggests that other influences may destabilize these SOD1 mutants in vivo.

Fully metallated bovine SOD1 is active in 4% SDS or 8 M urea (21) and melts in solution at temperatures above 90 °C (22). SOD1 also retains its dimeric quaternary structure upon exposure to 1% SDS in the absence of other denaturing stresses such as heat, urea, reducing agents, or EDTA (23–25). Structural properties of SOD1 that contribute to its extreme thermochanical stability include an eight-stranded β-barrel motif, binding sites for copper and zinc ions, hydrophobic interactions associated with dimerization, and an unusual intrasubunit disulfide bond bridging a loop residue, Cys-57, and Cys-146 of the β-barrel (24, 26). The loop that includes Cys-57 also strongly influences the conformation of Arg-143, which regu-
lates steering of superoxide anion and reactivity of the copper ion via a local hydrogen bond network and the disulfide linkage to Cys-146 (27–29). Furthermore, portions of this loop contribute to the dimer interface and form the zinc ion binding site (26). Conservation of these structural features in all eukaryotic SOD1s suggests that conformational stability of the enzyme, in general, and the disulfide loop, in particular, is critical under physiological conditions.

The ratio of reduced (GSH) to oxidized glutathione ([GSH]/[oxidized glutathione]) of ~30–100:1 in the cytosol (30) normally inhibits the formation of disulfide linkages in cytosolic proteins (31), yet WT SOD1 maintains a strong disulfide bond. We hypothesized that the disulfide linkage in ALS-related SOD1 mutants could be vulnerable to cleavage under cellular reducing conditions, which might further destabilize even WT-like mutants. In this study, we correlated changes in electrophoretic mobility, cysteine accessibility to modifying reagents, and protease susceptibility upon incubation of purified SOD1 mutants with disulfide-reducing agents. We then compared sulfhydryl accessibility of WT versus mutant SOD1 proteins in tissue lysates from transgenic mice expressing WT, G85R, or G93A SOD1 under reducing conditions. Our findings suggest that SOD1 destabilization related to thiol-reducing influences in the spinal cord and brain may contribute to the toxicity of mutant SOD1 enzymes in familial ALS.

EXPERIMENTAL PROCEDURES

Materials—All solutions were prepared using Milli-Q ultrapurified water (Millipore). EDTA was from Invitrogen; sodium and potassium phosphate (monobasic and dibasic), NaCl, and Tris base were from J. T. Baker; ascorbate, reduced glutathione (GSH), iodoacetamide, and human erythrocyte SOD1 were from Sigma; Tris(2-carboxyethyl)phosphine (TCEP) was from Pierce; NaOH was from Mallinckrodt; 1,4-dithiothreitol (DTT), SDS, HCl, methanol, and acetic acid were from EM Science; and Coomassie Brilliant Blue R-250 and bromphenol blue were from Bio-Rad. Formic acid and acetonitrile were from Fisher. Maleimide coupled to polyethylene glycol (Mal-PEG, molecular mass of 5 kDa) was from Shearwater Polymers (Huntsville, AL). Transgenic mouse tissues (spinal cord, brain, brainstem, and peripheral nervous system) were from the Laboratory of Neurogenetics, McLean Hospital, Harvard Medical School. Transgenic mice expressing WT or ALS-related mutant SOD1 proteins (A4V, L34V, L85R, L37V, G34R, G154V, D105N, H48Q, E31K) were obtained from the Jackson Laboratory (Bar Harbor, ME), whereas those overexpressing G85R or G93A SOD1 (7) bred to homozygosity were provided by Dr. Zsuzsanna Xu.

SOD1 Protein Preparation—Human WT or ALS-related mutant SOD1 enzymes (A4V, L34V, L85R, L37V, G34R, G154V, D105N, H48Q, E31K, C93S, and C121A) containing biologically accessible cysteines were isolated from a baculovirus expression system (18). Proteins were purified by size exclusion chromatography and then by metal chelators prior to loading. The method differed from standard SDS-PAGE in that the amount of SDS was lower than usual, the sample was not boiled. Proteins were separated on 15% polyacrylamide Tris-HCl gels (Bio-Rad) run at 12 V/cm (80 V total) for 2 h and stained with Coomassie Blue dye. The sample loading buffer for partially denaturing PAGE contained 62 mM Tris (pH 6.8), 10% glycerol, 0.05% bromphenol blue, and, SDS, reducing agents, or chelators as specified. The standard gel running buffer contained 25 mM Tris (pH 8.0) and 1% SDS. All proteins and samples run under denaturing conditions were boiled for 3 min in denaturing buffer (62 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.01% bromphenol blue) before loading. For the partially denaturing gels (Figs. 1–3), the migration of dimeric WT SOD1 in the absence of disulfide-reducing agent was indicated by an arrow marked M, which corresponded to the migration of a 21.5-kDa denatured marker protein. Gels were photographed with a Kodak DC120 digital camera, and the images were analyzed using Adobe Photoshop and Scion Image 4.0.2 software. Relative protein amounts were estimated by subtracting the background from the total signal for specific protein bands.

Mass Spectrometry—The number of accessible cysteine residues in SOD1 variants was determined by the reaction of SOD1 proteins with iodoacetamide (35, 36) and detection of the number of adducts formed per subunit by electrospray ionization mass spectrometry (ESI-MS). WT or mutant SOD1 was incubated for 1–4 days in the absence or presence of 10 mM TCEP at 0 °C. Proteins were reacted with 5 mM iodoacetamide for 1 h before loading on a liquid chromatography/MS column (C18, 5 μm, 300-Å Vydac column, 1.0 mm, inner diameter × 15 mm) equilibrated with buffer A (1% formic acid, 2% acetonitrile) and were eluted by a gradient to buffer B (1% formic acid, 80% acetonitrile). Protein concentration during incubation was 150 μg/ml, and 3 μl was loaded per injection. ESI-MS was calibrated using porcine insulin (average mass = 5,734.60 Da).

Protease Susceptibility—Digestion buffer contained 5 mM DTT, 150 mM NaCl, and 50 mM sodium phosphate at pH 7.0. WT and mutant SOD1 proteins were divided into aliquots of 3 μg each and incubated at 37 °C in digestion buffer alone for 6 h, in buffer containing 50 μg/ml protease K for 2 h, or in buffer containing 100 μg/ml trypsin for 6 h. The digestion was terminated by the addition of 10 mM phenylmethylsulfonyl fluoride, and the proteins were boiled immediately in denaturing buffer for 3 min before loading for standard SDS-PAGE. Proteins that remained intact were detected by Coomassie Blue staining.

Western Blot Detection of Accessible SOD1 Proteins in Tissue Lysates—Soluble tissue lysates containing 0.375 mg/ml total protein and protease inhibitors (Complete, EDTA-free, catalog number 1873580, Roche Diagnostics) were incubated for 12 h in the presence of 0, 0.3, 1.0, or 3.0 mM TCEP at 25 °C. Mal-PEG (molecular mass of 5 kDa) was then added to a final concentration of 3 mM for covalent modification of accessible cysteines in 1 h at 25 °C (37). The addition of Mal-PEG to accessible cysteines increases the subunit mass of SOD1 by ~5 kDa modification. The reaction was competitively terminated upon addition of loading buffer for denaturing SDS-PAGE that contained 5% β-mercaptoethanol (see above). For Western blot analysis, samples were immediately boiled at 100 °C for 3 min, separated by denaturing SDS-PAGE, and detected using a sheep polyclonal antibody to human SOD1 (catalog number 574597, Calbiochem) as described previously (18).

RESULTS

Both WT-like and Metal Binding Region ALS-related SOD1 Mutants Exhibited Aberrantly Increased Electrophoretic Mobility during Partially Denaturing SDS-PAGE—Human and bovine WT SOD1s retain their homodimeric subunit association, activity, and metal binding in 1% SDS (21, 24, 25, 38). To compare the electrophoretic behavior of biologically metallated human WT and ALS mutant SOD1 variants (18) under partially denaturing conditions, we incubated these enzymes for 30 min with 0.4% SDS and increasing concentrations of the disulfide-reducing agent DTT without boiling prior to PAGE (Fig. 1).

In the absence of DTT (first two lanes of each set), commer-
cially available 32-kDa SOD1 holoenzyme from human erythrocytes (WT-C) migrated as a single band. We showed previously that our WT and WT-like as-isolated recombinant SOD1s typically contained \( \frac{30}{100} \) full copper site occupancy, most probably as a consequence of limited copper availability during overexpression in insect cells (18). Partially metallated recombinant WT SOD1 (\( WT \)) migrated as three distinct species under these conditions. The mobility of all WT species was considerably slowed compared with that of denatured SOD1 near the 21.5-kDa marker during standard SDS-PAGE (18), consistent with low SDS binding under these conditions. Upon exposure to 2–10 mM DTT, a small fraction of the WT SOD1 enzymes exhibited accelerated mobility near the mobility of the denatured WT monomer (Fig. 1, \( \text{arrow marked} \ D \)).

Four WT-like ALS mutants located at one pole of the \( \beta \)-barrel (L38V, G41S, D90A, and G93A) contained amounts of copper and zinc ions comparable with as-isolated WT SOD1 (see Fig. 1 legend) and had normal specific activity (18). In contrast to WT SOD1, this group exhibited reproducible “smearing” when exposed only to 0.1% SDS in the gel-running buffer and in the absence of preincubation with SDS or DTT (Fig. 1, \( \text{first lane in each set} \)). Moreover, these mutants were distinguished from the WT enzyme by an accelerated mobility upon preincubation with DTT. This result suggested that these mutants were more susceptible than WT SOD1 to altered conformation or net charge induced by SDS or DTT.

Mutants that were partially deficient in either zinc (H48Q and G72S) or copper (A4V) compared with WT SOD1 (18) exhibited a component that migrated similarly to denatured WT SOD1 (Fig. 1, \( \text{arrow marked} \ D \)). In addition, two distinct fractions that differed only in metal occupancy were isolated by ion exchange chromatography for both the D76Y mutant and the single-residue deletion mutant E133Δ-1 (18). The D76Y-2 and E133Δ-2 enzymes, which contained \( \frac{50}{100} \)–\( \frac{65}{100} \) less copper and \( \frac{65}{100} \) less zinc than did D76Y-1 and E133Δ-1 (18), were more susceptible to the effects of SDS and DTT on mobility.

Five ALS mutants with substitutions near the active site of SOD1 (H46R, G85R, D124V, D125H, and S134N) were severely copper- and zinc-deficient upon isolation from insect cells (18). These metal-binding region mutants exhibited nearly maximal mobility upon exposure to SDS either in the running buffer only (Fig. 1, \( \text{first lane of each set} \)) or after preincubation in 0.4%...
Disulfide Reduction of ALS Mutant SOD1 Enzymes

According to the following reaction.

\[
(\text{CH}_3\text{CH}_2\text{COOH})_2\text{P} + \text{RS}'\text{SR} + \text{H}_2\text{O} \rightarrow (\text{CH}_3\text{CH}_2\text{COOH})_2\text{P} = \text{O} + \text{RS} + \text{R}''\text{SH}
\]

**REACTION 1**

Fig. 2B shows that 2 mM TCEP was sufficient to fully shift the mobility of A4V SOD1 but had little effect on WT SOD1.

ALS-related SOD1 Mutants Were Susceptible to Incubation with GSH—The cytosolic environment of the nervous system may expose proteins to strong reducing influences. For example, GSH is an abundant intracellular thiol (present at ~1–10 mM in neurons and glia) that can reduce disulfide bonds or be conjugated to accessible cysteine residues (42). Also, among other reducing agents that function as antioxidants by their free radical scavenging activity, ascorbate accumulates in neurons to ~3 mM (43). To determine whether the mobility of ALS mutants was sensitive to these agents, we incubated the purified enzymes with either GSH or ascorbate for 24 h at 37 °C and then analyzed the samples by PAGE. Fig. 3 demonstrates that while WT SOD1 was relatively resistant to 10–20 mM GSH, the mutant enzymes exhibited faster migration suggestive of unfolding or monomerization. In contrast, exposure to ascorbate (up to 100 mM) had no effect on mutant SOD1 mobilities (data not shown). Overall, the findings in Figs. 1–3 demonstrated strikingly distinct behavior during partially denaturing PAGE of purified WT and all of the 14 ALS-related mutants of SOD1 but did not precisely specify the origin of those differences.

**TCEP Preferentially Reduced the Disulfide Bond of SOD1 Mutants and Contributed to Increased Accessibility of a Cysteine Residue within the β-Barrel Core**—We next correlated TCEP-induced changes in SOD1 mobility during native PAGE with altered accessibility of cysteine residues as determined by mass spectrometry. Fig. 4 shows the migration of WT and mutant SOD1 enzymes after native PAGE in the absence or presence of incubation with 10 mM TCEP for 1 day (left panel) or 4 days (right panel). Migration of the WT protein was not significantly altered after 1 day but was accelerated after 4 days, suggestive of increased net negative charge resulting from partial metal ion loss. In contrast, the mutant enzymes A4V, L38V, G85R, D90A, and G93A exhibited species that migrated more slowly on native PAGE after exposure to TCEP for 1–4 days. In the absence of significant conformational change, metal ion loss alone would be expected to accelerate rather than retard mobility of the mutants. For example, the as-isolated G85R mutant contained <5% metal occupancy (18) and migrated more rapidly than WT SOD1 before exposure to TCEP. These native PAGE results suggested that a conformational change related to either disulfide reduction by TCEP or metal ion loss impeded the mobility of the mutant but not WT enzymes.

To determine cysteine accessibility before and after exposure to TCEP, SOD1 proteins were incubated with 5 mM iodoacetamide, which reacts with each accessible sulfhydryl group to form an adduct that adds 57 Da to the subunit mass. Changes in the masses of WT and mutant SOD1 proteins following reaction with iodoacetamide were detected under denaturing conditions by ESI-MS. The deconvoluted spectra in Fig. 5 demonstrate the subunit masses of each resolved species, and the deduced number of modified cysteine residues is shown as a circled number for each peak. In the absence of incubation with TCEP, both the WT and mutant SOD1 proteins exhibited only...
a single prominent modification, which most probably corresponded to adduct formation at the surface-accessible Cys-111 residue.

All 14 mutants were evaluated for cysteine accessibility by mass spectrometry, and all showed increased reactivity to iodoacetamide in the presence of TCEP. Following exposure to TCEP for 1 day, there was no change in cysteine accessibility for WT SOD1, but A4V, L38V, D76Y-1, D76Y-2, G85R, D125H, E133Δ-1, E133Δ-2, and S134N mutants exhibited species containing either three or four modified cysteines (Fig. 5). A total of three modified cysteines would be expected if the disulfide bond (Cys-57 to Cys-146) was cleaved, whereas all four cysteines of the subunit could be modified only if disulfide cleavage was accompanied by at least transient exposure of the side chain of Cys-6, which is normally buried in the interior of the β-barrel. Similar results were also obtained for the mutants G41S, H46R, H48Q, G72S, and D124V after a 1-day incubation with TCEP (data not shown). The results for D76Y and E133Δ (Fig. 5) also clearly indicated that fractions containing lower metal occupancy were more susceptible to reaction with iodoacetamide. For the metal-depleted forms, the increased cysteine accessibility in Fig. 5 correlated with the observed mobility shifts in Fig. 1 and the protease susceptibility in Fig. 6.

After exposure to TCEP for 4 days (Fig. 5), even the WT enzyme exhibited three modified cysteines indicative of disulfide cleavage by TCEP, whereas A4V, L38V, D90A, and G93A not only appeared disulfide-reduced but also exhibited species in which all four cysteines were modified. These results provided strong evidence that the mutant enzymes were more susceptible than WT to disulfide cleavage. Moreover, the reduction of the mutants but not WT SOD1 contributed to exposure of the buried Cys-6 residue and destabilization of the β-barrel itself.

**Mutant SOD1s Were All Susceptible to Digestion by Proteinase K or Trypsin in the Presence of DTT**—WT SOD1 and some ALS mutants expressed in COS-1 cells are resistant to digestion by proteinase K under non-reducing conditions (15). We hypothesized that a disulfide-reducing environment may partially unfold mutant but not WT SOD1 and thereby unmask a sensitivity to protease digestion. Therefore, we compared the fraction of purified WT or ALS mutant SOD1s remaining after exposure to either proteinase K or trypsin in the presence of DTT.

Standard denaturing SDS-PAGE in Fig. 6 revealed that the metal-binding region mutants (H46R, H48Q, G85R, D124V, D125H, and S134N) and the A4V mutant were completely digested by proteinase K or trypsin when incubated with 5 mM DTT at 37 °C. Other mutants at one pole of the β-barrel (L38V, G41S, D90A, and G93A) or in loop regions (G72S, D76Y, and E133Δ) exhibited partial digestion under these conditions, whereas the WT protein remained intact. D90A SOD1, which exhibited relative resistance to the effects of DTT (Fig. 1), TCEP (Figs. 2 and 5), or GSH (Fig. 3), was also the mutant most resistant to protease digestion (Fig. 6). However, as judged by Coomassie Blue staining, less than half of the D90A and G93A SOD1s remained after a 6-h digestion with trypsin.

We hypothesized that increased stability afforded by metal ion binding to SOD1 (19, 21, 22) could contribute to protease resistance despite the presence of a destabilizing mutation. To test this possibility, preparations of D76Y and E133Δ that differed only with respect to metal ion occupancy (18) were exposed to these proteases under reducing conditions (Fig. 6). The preparations with higher metal contents (D76Y-1 and E133Δ-1) were more resistant to proteolysis than were those with lower metal contents (D76Y-2 and E133Δ-2). The results of Fig. 6 also demonstrated that SDS, which was absent from the reaction buffer, was not required to unmask the protease.

**Fig. 3.** SOD1 mutants were susceptible to disulfide reduction by GSH. SDS-PAGE was performed as in Fig. 1 with the exception that samples contained 0.1% SDS and the indicated concentration of GSH. Proteins were incubated for 24 h at 37 °C before loading (3 μg/lane). Metal contents of these SOD1 preparations were as shown in Fig. 2 for WT, A4V, L38V, and G93A, and they were as shown in Fig. 1 for H48Q and D90A proteins.

**Fig. 4.** TCEP slowed the electrophoretic mobility of SOD1 mutants during native PAGE. Native PAGE of SOD1 proteins (5 μg/lane) following incubation for 1 day (left panel) or 4 days (right panel) at 37 °C in the absence or presence of 10 mM TCEP. C, carbonic anhydrase; L, lactalbumin; A, albumin.
FIG. 5. ALS SOD1 mutants exposed to TCEP exhibited greater accessibility of cysteine residues to alkylation by iodoacetamide. WT and mutant SOD1 proteins were exposed to 5 mM iodoacetamide for 1 h before loading on a liquid chromatography/MS column for ESI-MS under denaturing conditions. Shown are deconvoluted spectra with the masses of each protein subunit indicated. Circled numbers for each species indicate the number of cysteine residues alkylated by iodoacetamide as inferred from the measured masses (+57 Da change/modification). Protein concentration was 150 ng/μl, and 3 μl was loaded/injection. Metal contents of these preparations were as described in Fig. 1, and the masses of unmodified subunits were reported previously (18).
sensitivity of the ALS mutant SOD1s upon exposure to DTT.

**TCEP Increased SOD1 Cysteine Accessibility in Neural Tissue Lysates from ALS Mutant but Not WT SOD1 Transgenic Mice**—We next compared SOD1 cysteine reactivity in soluble lysates from transgenic mouse tissues expressing WT, G85R, or G93A SOD1s. Lysates were treated for 12 h with variable amounts of TCEP to simulate a reducing environment, and proteins were then covalently tagged at accessible cysteine residues with Mal-PEG. Reaction with Mal-PEG increased the subunit mass by ~5 kDa/modification, which was resolved by Western blot analysis (37). Fig. 7, left panel, shows that >90% SOD1 in spinal cord lysates from WT SOD1 transgenic mice was modified at a single cysteine residue, in agreement with the results obtained by mass spectrometry for the purified proteins in Fig. 5. Exposure of the WT lysate to TCEP for 12 h did not affect labeling by 3 mM Mal-PEG except at 3 mM TCEP, which inhibited the labeling reaction. In contrast, spinal cord lysates from G85R or G93A SOD1 mutant mice contained SOD1 species that were modified at 1–4 cysteine residues, and labeling at multiple residues was increased by exposure to TCEP (Fig. 7, left panel).

The middle and right panels of Fig. 7 shows that SOD1 in brain (BN), cerebellum (CB), brain stem (BS), skeletal muscle (SK), and heart (HT) tissues from G85R mice also exhibited susceptibility to modification by Mal-PEG. Reaction with Mal-PEG increased the subunit mass by ~5 kDa/modification, which was resolved by Western blot analysis (37). Fig. 7, left panel, shows that >90% SOD1 in spinal cord lysates from WT SOD1 transgenic mice was modified at a single cysteine residue, in agreement with the results obtained by mass spectrometry for the purified proteins in Fig. 5. Exposure of the WT lysate to TCEP for 12 h did not affect labeling by 3 mM Mal-PEG except at 3 mM TCEP, which inhibited the labeling reaction. In contrast, spinal cord lysates from G85R or G93A SOD1 mutant mice contained SOD1 species that were modified at 1–4 cysteine residues, and labeling at multiple residues was increased by exposure to TCEP (Fig. 7, left panel).

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**DISCUSSION**

The conformation and stability of ALS-related SOD1 mutants in affected tissues may be influenced directly by the mutant substitutions or indirectly as a consequence of disulfide bond reduction, decreased metal ion binding, monomerization, or other vulnerabilities under conditions *in vivo*. In this study, we correlated the effects of disulfide-reducing agents upon electrophoretic mobility, cysteine accessibility, and protease sensitivity among purified WT and ALS-related SOD1 variants of known metal ion content (18). We further demonstrated that G85R and G93A mutant SOD1s in transgenic mouse tissue lysates were susceptible to modification at normally inaccessible cysteine residues under reducing conditions.

Our initial observation that all 14 of the ALS mutants exhibited accelerated migration during partially denaturing PAGE (Figs. 1–3) indicated that ALS mutants share properties distinct from WT SOD1. To clarify the nature of those properties, we showed that the mutant proteins were more suscepti-
Disulfide Reduction of ALS Mutant SOD1 Enzymes

selectivity or by creating a novel copper binding site upon exposure of free cysteine residues. On the other hand, disease progression in mutant SOD1 transgenic mice is not affected by genetic ablation of copper chaperone-dependent copper loading into SOD1 (57) or by expression of SOD1 mutants that severely disrupt the copper binding site (58). However, the toxicity of bound copper has not been completely excluded because unshielded copper ions may be toxic at even nanomolar concentrations (59).

Disulfide reduction of the ALS mutants may also contribute to toxicity by mechanisms independent of abnormal copper reactivity. For example, the exposure of hydrophobic residues or reactive cysteines could favor abnormal interactions of SOD1 with itself or with other cellular constituents. Mutant SOD1 can form insoluble protein complexes at early stages in G93A SOD1 transgenic mouse tissues and following proteasome inhibition in cultured cells (60). Similarly, a fraction of total mutant SOD1 in brain and spinal cord tissues from SOD1 mice forms high molecular weight complexes that accumulate with disease progression (61) and mouse tissues can exhibit thioflavin-S-positive inclusions (58). Oxidative stress may also be linked to the accumulation of mutant SOD1 either by decreased proteasome activity or by impaired degradation of oxidatively damaged SOD1 (62). An increased burden of partially unfolded SOD1 proteins or complexes could ultimately impair cellular chaperone capacity (16), perturb mitochondrial function (63), sequester anti-apoptotic factors (12), disrupt protein recycling, or impair an unsustainable metabolic cost to vulnerable tissues (reviewed in Ref. 3).

Our data suggest that cellular disulfide reducing influences at physiologic temperature and pH are sufficient to convert relatively well folded WT-like SOD1 mutants (18) or less stable metal-binding region mutants (19) into more severely destabilized species. These non-native mutant forms might resemble the subset of highly unstable SOD1 C-terminal truncation mutants (15, 64), which also lack the disulfide bond consequent to deletion of Cys-146. Overall, these results implicate susceptibility to conformational destabilization of SOD1 by a cellular reducing environment as a shared property that may be relevant to ALS mutant neurotoxicity.

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