More than 100 different mutations in the gene encoding Cu,Zn-superoxide dismutase (SOD1) cause preferential motor neuron degeneration in familial amyotrophic lateral sclerosis (ALS). Although the cellular target(s) of mutant SOD1 toxicity have not been precisely specified, evidence to date supports the hypothesis that ALS-related mutations may increase the burden of partially unfolded SOD1 species. Influences that may destabilize SOD1 in vivo include impaired metal binding, reduction of the intrasubunit disulfide bond, or oxidative modification. In this study, we observed that metal-deficient as-isolated SOD1 mutants (H46R, G85R, D124V, D125H, and S134N) with disordered electrostatic and zinc-binding loops exhibited aberrant binding to hydrophobic beads in the absence of other destabilizing agents. Other purified ALS-related mutants that can biologically incorporate nearly normal amounts of stabilizing zinc ions (FV, L38V, G41S, D90A, and G93A) exhibited maximal hydrophobic behavior after exposure to both a disulfide reducing agent and a metal chelator, while normal SOD1 was more resistant to these agents. Moreover, we detected hydrophobic SOD1 species in lysates from affected tissues in G85R and G93A mutant but not wildtype SOD1 transgenic mice. These findings suggest that a susceptibility to the cellular disulfide reducing environment and zinc loss may convert otherwise stable SOD1 mutants into metal-deficient forms with locally destabilized electrostatic and zinc-binding loops. These abnormally hydrophobic SOD1 species may promote aberrant interactions of the enzyme with itself or with other cellular constituents to produce toxicity in familial ALS.

Amyotrophic lateral sclerosis (ALS) is a degenerative disease of motor neurons in the spinal cord, brain stem, and brain that causes insidious muscle wasting and death within 3–5 years due to respiratory insufficiency (1). About 10% of ALS cases are familial, and among these, 20% are caused by mutations in the gene encoding Cu/Zn-superoxide dismutase (SOD1) (2, 3) (updated list at www.alsod.org). SOD1 is a thermostable 32-kDa homodimeric enzyme that normally catalyzes the dismutation of superoxide radicals (O2− to O2 and H2O2. The enzyme is abundant in eukaryotic cells and is expressed in human liver at levels 3–5-fold greater than in brain and muscle (4). SOD1 is predominantly located in the cytoplasm, although it is also detected in mitochondria and other organelles (5–12). Transgenic rodents that express high levels of mutant SOD1s develop premature motor neuron degeneration (13–18), while SOD1 knock-out mice do not (19). The time to disease onset and rapidity of motor neuron degeneration in these models is accelerated by increased expression of the mutant SOD1s, suggesting a toxic effect of the mutant proteins.

Mutant SOD1s may ultimately trigger preferential motor neuron death in ALS via complex molecular pathways and cellular interactions. Possible components of toxicity involve aberrant oxidative chemistry, formation of noxious oligomers or aggregates, saturation of proteasome or chaperone functions, impairment of glutamate re-uptake, mitochondrial dysfunction, altered neurofilaments or axonal transport, and triggering of inflammatory or apoptotic cascades (reviewed in Refs. 20–22). Such avenues to motor neuron loss need not be mutually exclusive, and mutant SOD1s may perturb the functions of other cell types with differential severity or temporal course. Because individuals can express mutant SOD1s from birth until middle age before the clinical onset of motor neuron disease, it is apparent that tissues can compensate over years for the effects of mutant SOD1s. A precise understanding of the abnormal properties shared by SOD1 mutants and the defenses against these influences in specific tissues as a function of aging may help us to develop more effective therapies for both familial and sporadic forms of ALS.

Evidence to date suggests that misfolding or partial unfolding of mutant SOD1 proteins in a cellular environment may be related to their toxicity. Subsets of mutant SOD1 enzymes exhibit accelerated turnover in vivo (23, 24), increased proteolytic susceptibility in cellular lysates (25), or decreased zinc binding affinities (26), each of which supports the notion that the mutant proteins may be destabilized in vivo. Stresses that increase the load of mutant SOD1 protein, such as proteasome inhibition (24, 27), acute overexpression in cultured cells (28, 29), or chronic expression in transgenic mice produce insoluble complexes containing SOD1 (27, 30, 31). Moreover, cell lines that are able to tolerate overexpression of mutant SOD1s inducible chaperones such as hsp27, hsp70, and αB-crystallin, while hsp70 introduced by gene transfer can protect cultured motor neurons from mutant SOD1 toxicity (32). These chaperones can associate directly with a small fraction of G93A or G41S mutant but not WT SOD1 in tissue lysates (33), and vulnerable cells such as spinal motor neurons exhibit a weakened endogenous heat shock response that fails to induce hsp70 (34). Accumulating evidence also indicates that purified forms of
ALS-related SOD1 mutants have a more flexible structure than corresponding forms of the wild type (WT) enzyme (35–39). In previous studies, we characterized physicochemical properties of 14 different biologically metallated ALS SOD1 mutants expressed in insect cells (40). A group of “wild type-like” (WTL) mutants (A4V, L38V, G41S, D76Y, D90A, G93A, and E133Δ) are able to bind copper and zinc and retain specific enzymatic activity similar to the normal enzyme. In contrast, a group of “metal-binding region” (MBR) mutants (H46R, G85R, D124V, D125H, and S134N) are deficient in copper and zinc and exhibit dramatic thermal destabilization compared with metallated forms, as measured globally by differential scanning calorimetry (41). Because correct metal coordination is critical to SOD1 stability (42), these results suggested that conditions in vivo which might impair metal binding by WT-like mutants could increase the burden of destabilized SOD1 in tissues. Consistent with this, preparations of several WT-like SOD1 mutants, with metals removed in vitro, unfold more readily upon exposure to chemical denaturants than does apo-WT SOD1 (43).

Furthermore, we observed that both WT-like and metal-binding region SOD1 mutants are more susceptible than the normal enzyme to cleavage of the intrasubunit disulfide bond between Cys-57 and Cys-146 under conditions that mimic the cytosolic reducing environment (44). This important disulfide bond is not fully understood (45), these structural features suggest that it could be important for either SOD1 dimerization or the metal binding process or both (46, 47).

How might decreased global or local stability of mutant SOD1s be related to toxic cellular effects? Zinc-deficient WT or mutant SOD1 is toxic to motor neurons in vitro (48) and is also more susceptible than the WT SOD1 holoenzyme to oxidative damage and aggregation (49). X-ray crystal structures of metal-binding region SOD1 mutants apo-H46R and S134N (37) and the WT SOD1 apoenzyme (50) demonstrate disorder of the zinc-binding loop and electrostatic loop, which are normally stabilized by bound zinc. This loop disorder may cause a reduction of β-sheet edge-strand protection and thereby promote self-association of SOD1 dimers (37, 50) and would also be expected to render the active site more accessible to oxidants. However, it has not been clarified whether SOD1 oxidative modification, dimer self-association, or other interactions of mutant SOD1 related to impaired zinc binding underlie the toxicity of these species in affected tissues.

Recent analyses of mutations that facilitate protein aggregation associated with a variety of diseases have demonstrated that such mutations often increase the hydrophobicity of the protein or decrease its net charge (51). Such changes can promote accumulation of aggregated species if competing equilibria between partially folded conformations and pathways to either native or aggregated states are perturbed. In this study, we observed that ALS-related SOD1 variants share abnormally increased surface hydrophobicity, especially under conditions favoring disulfide reduction and metal ion loss. Furthermore, a fraction of SOD1 in tissue lysates from G85R and G93A mutant but not WT SOD1 transgenic mice displayed strikingly increased binding to hydrophobic media. We propose that exposed hydrophobic residues of SOD1 mutants in vivo may promote aberrant interactions of the enzyme with itself or with other cellular constituents as part of the toxic mechanism in familial ALS.

**EXPERIMENTAL PROCEDURES**

Materials—All solutions were prepared using Milli-Q (Millipore) ultrapure water. EDTA was from In VitroGen; sodium phosphate (monobasic and dibasic), NaCl, and Tris base were from J. T. Baker; Tris-2-carboxyethylphosphine (TCEP) was from Pierce; sodium acetate, sodium deoxycholate, Nonidet® P-40, and urea were from Sigma; 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. Transgenic mice overexpressing either WT SOD1, line B6SJ-TgN(SOD1)2Gur, or G93A SOD1, line B6SJ-TgN(SOD1-G93A)1Gur (13, 49), were purchased from Jackson Laboratory (Bar Harbor, ME), while transgenic expressing G85R SOD1 (15) were generously provided by Dr. Don Cleveland. All mice have been maintained at the University of Massachusetts Medical School animal facility and bred to FVB background for 10 generations.

**SOD1 Protein Preparation**—Human WT or ALS-related mutant SOD1 enzymes (A4V, L38V, G41S, H46R, H48Q, D76Y, G85R, D90A, G93A, E133G, D124V, D125H, G134R, and S134N) containing biologically incorporated metal ions were isolated from a baculoviral expression system (40). Protein concentrations were determined spectrophotometrically (40, 44). Loosely bound copper or zinc ions could conceivably be lost during the SOD1 purification procedure or during storage, especially for metal-binding region mutants such as H46R for which aberrant low affinity metal binding has been suggested (52). We therefore included a small amount of EDTA (0.1 mM) in the isolation buffer to inhibit metal loss before publication and also to strip any loosely bound metals prior to metal content analysis. The metal contents of these proteins were assessed using inductively coupled plasma mass spectrometry (40, 44) and are listed in the figure legends. The fully metallated enzyme is expected to contain two equivalents each of copper and zinc per dimer.

Apo-WT SOD1 was prepared using a demetallation protocol modified from (50). Briefly, WT SOD1 was equilibrated in acetate buffer (pH 3.8) in the presence of 8M urea and 1 mM EDTA for 48 h at 4°C. The protein was then diluted 10-fold into storage buffer containing 10 mM phosphate (pH 7.2) and 1 mM EDTA, followed by exchange and concentration into storage buffer.

**Cell and Tissue Lysates**—N2a mouse neuroblastoma cell lines expressing human WT or mutant SOD1 (54) were rinsed with phosphate-buffered saline and incubated with lysis buffer (10 mM NaCl, 0.1% Triton X-100, 5% glycerol, 16 cells) containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet® P-40, 0.5% deoxycholate, and 1× “Complete, EDTA-free” protease inhibitors (Roche Applied Science) for 5 min with gentle rocking. The lysate was centrifuged at 14,000×g for 10 min at 4°C. 0.2% SDS was added to the supernatant, and samples were stored at −80°C.

Mouse tissues (spinal cord, forebrain, brainstem, cerebellum, skeletal muscle, heart, kidney, or liver) were taken from at least three matched animals that were at the pre-muscle weakness stage (55). For our G93A mice, this stage of disease progression (age <20 weeks) is characterized by abnormal mitochondrial swelling but minimal axonal loss or vacuolar pathology (55). For our G85R mice, muscle weakness became apparent only after 26 weeks of age. Specific ages for mice used in each figure are listed in the legends. Tissues were homogenized in lysis buffer separately using a motorized pestle (~70 mg of tissue per mL of lysis buffer). Lysates for Fig. 4, B and C, were processed as for the N2a lysates above, while lysates for Fig. 5 were centrifuged at 1000×g for 10 min at 4°C, and the supernatant was stored at −80°C. The total protein concentration of lysates was determined by the bicinchoninic acid method (56).

**Polyacrylamide Gel Electrophoresis**—Purified SOD1 proteins or soluble cell or tissue lysates were separated by SDS-PAGE under fully denaturing or partially denaturing conditions as described previously (44). Partially denaturing SDS-PAGE was a modification of native PAGE in which varying amounts of SDS and reducing agent were added to the samples, which were not boiled before electrophoresis. Gels and blots were photographed with a Kodak DC440CF image station and quantitated by Scion Image 4.0.2 software as described (44).
**RESULTS**

**Purified ALS Mutants Bound More Efficiently than WT SOD1 to Nitrocellulose Membranes under Partially Denaturing Conditions**—ALS-related human SOD1 mutants can be produced with high yields in insect cell and yeast expression systems and were initially divided into two broad groups. WTL mutants bound copper and zinc and exhibited normal specific activity, while MBR mutants exhibited poor metal binding and low activity (40, 58). Biologically metallated "as-isolated" WTL mutants and WT SOD1 could target copper and zinc to the native metal-binding sites, while several purified WTL mutants to which metal ions were subsequently added (40, 44) exhibited mislocalization of metal ion binding (58). For this reason, we have focused our studies on the as-isolated SOD1 preparations that more closely resemble the proteins in vivo, even though these preparations may exhibit variable extents of metal loading. We showed previously that ALS-related SOD1 mutants were more susceptible than WT SOD1 to partial unfolding, especially under disulfide reducing conditions, where they exhibited accelerated gel mobility, increased vulnerability to protease digestion, and greater accessibility of cysteine residues to alklylation by iodoacetamide (44).

To examine whether as-isolated SOD1 mutants exhibit abnormal surface properties, we performed partially denaturing SDS-PAGE under nonreducing or reducing conditions, followed by nondenaturing electrotransfer to nitrocellulose membranes (Fig. 1). The purified mutant SOD1 enzymes (3 μg per lane, incubated with or without 2 mM DTT) adhered to blots to a much greater extent than did WT SOD1, as detected by a general protein stain. The continuous range of migration for mutant SOD1 in gels is indicated by an arrow marked D. The migration of fully denatured and reduced monomeric WT SOD1 in gels is indicated by an arrow marked M, which also corresponds to the migration of a 21.5-kDa denatured marker protein. The metal contents of these proteins were determined previously by inductively coupled plasma mass spectrometry (40, 44) and were (in equivalents per dimer) WT, 0.67 copper, 1.62 zinc; A4V, 0.33 copper, 1.81 zinc; G41S, 0.52 copper, 1.35 zinc; G85R, 0.00 copper, 0.01 zinc; D90A, 0.34 copper, 1.77 zinc; and G93A, 0.45 copper, 1.46 zinc. The fully metallated enzyme is expected to contain 2 equivalents each of copper and zinc per dimer.

**Aberrant Hydrophobicity of ALS Mutant SOD1s**

Following electrophoresis, proteins were electroblotted to a nitrocellulose membrane using ice-cold nondenaturing transfer buffer (25 mM Tris and 192 mM glycine (pH 8.3)). Immunodetection was performed as described previously (44) using either a sheep polyclonal anti-SOD1 antibody (Calbiochem catalog number 574597) or a rabbit polyclonal antibody raised against a peptide containing amino acids 125–137 of human SOD1 (23). The efficiency of protein transfer was visualized for blots using SYPRO Ruby fluorescent stain from Molecular Probes (57).
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Because protein denaturation may affect either the membrane transfer efficiency or antibody recognition (58), we immunostained parallel blots with a polyclonal antibody against human SOD1 (Fig. 1B, 100 ng of protein per lane). A detection pattern similar to that of Fig. 1A confirmed the inefficient binding of WT compared with mutant SOD1 and indicated that the bound SOD1 variants retained epitopes necessary for antibody recognition. Under partially denaturing conditions, the migration of some mutants (e.g. A4V and G41S) was accelerated by exposure to DTT without substantially altering the efficiency of epitope recognition.

Purified Metal-deficient SOD1 Mutants Bound Most Effectively to Hydrophobic Beads in the Presence of SDS—To address whether the retention of mutant SOD1s by nitrocellulose in Fig. 1 was related to hydrophobic interactions, we next examined binding of mutant SOD1s to hydrophobic beads. Phenyl-Sepharose binds exposed hydrophobic residues of proteins and is typically employed in hydrophobic interaction chromatography. During hydrophobic interaction chromatography, proteins bound to the matrix under high salt conditions are eluted in order of increasing hydrophobicity as the salt concentration is decreased, since more local water molecules become disordered and available to solvate the hydrophobic regions (60).

As-isolated SOD1 proteins of varying metal contents were incubated overnight at 25 °C with phenyl-Sepharose beads in a binding buffer containing 0.1% SDS with or without 2 mM DTT. After washing the beads twice, bound SOD1 was eluted under denaturing conditions and visualized by SDS-PAGE (Fig. 2).

Metal-deficient SOD1 mutants (H46R, G85R, D124V, D125H, and S134N) showed the greatest retention by the hydrophobic beads. These mutants were bound under either nonreducing or reducing conditions, suggesting that disulfide reduction was not required for aberrant hydrophobic exposure in the context of prior metal ion loss. In contrast, WT SOD1 was not bound by the hydrophobic beads under the same conditions, despite exposure to SDS and DTT.

Other SOD1 mutants known to be partially metallated (A4V, H48Q, D76Y, and E133Δ) bound to the hydrophobic surface to varying degrees, depending on the metal contents and reducing conditions. In general, binding was enhanced for these mutants after exposure to 2 mM DTT. Both the D76Y mutant and the single-residue deletion mutant E133Δ were isolated by ion exchange chromatography as two distinct fractions that differed only in metal occupancy (40). The D76Y-1 and E133Δ-1 enzymes, which contained more copper and zinc than did D76Y-2 and E133Δ-2, respectively, bound less efficiently to the hydrophobic beads. This suggested that stabilization afforded by metal ion binding decreased the aberrant hydrophobic exposure and that this stabilization could be partially overcome by disulfide reduction.

Other mutants (L38V, G41S, D90A, and G93A) that contained as-isolated copper and zinc amounts similar to WT SOD1 and showed similar biochemical and biophysical properties (40) did not bind efficiently to hydrophobic beads under these conditions. A small amount of binding was observed for these mutants upon exposure to 2 mM DTT, but the reducing agent alone was insufficient to appreciably increase the hydrophobicity of these mutants. It is noteworthy that these preparations of DTT-resistant mutants and the WT enzyme contained higher contents of zinc (68–96% of full occupancy) than copper (17–34% occupancy), while the DTT-sensitive mutants (A4V, H48Q, D76Y, and E133Δ) contained relatively low zinc (26–64%) but similarly low copper (11–32%) occupancy.

WT-like SOD1 Mutants Incubated with a Metal Ion Chelator under Reducing Conditions Showed Increased Hydrophobic Binding Compared with WT SOD1—Although the WT-like mutants retained the ability to bind stabilizing metal ions, we hypothesized that these variants may become destabilized under conditions in vivo which promote metal loss and may then behave similarly to the metal-binding region mutants. Partially denaturing SDS-PAGE in Fig. 3A shows that the electrophoretic mobility of the WT-like mutants (A4V, L38V, G41S, D90A, and G93A) was aberrantly accelerated in the presence of a metal chelator (1 mM EDTA) to a greater extent than was WT SOD1. Concurrent exposure of mutants or WT SOD1 to a disulfide reducing agent (10 mM TCEP) could facilitate the formation of these abnormal rapidly migrating species, which was most complete for the A4V variant. Under these conditions, WT SOD1 maintained the greatest preservation of dimeric species, although the D90A and G93A mutants were also relatively resistant to the destabilizing effects of EDTA and 10 mM TCEP. Three distinct bands were observed for differentially metallated dimeric WT SOD1 species, as seen previously (44). Exposure to 50 mM TCEP produced nearly complete conversion of even the most resistant WT-like mutant SOD1s (D90A and G93A) and WT SOD1 to faster migrating species. In contrast, the metal-free apoenzyme form of WT SOD1 migrated as a monomer even without exposure to TCEP. These results suggested that loss of bound metal ions, facilitated by exposure to TCEP, could destabilize a fraction of each SOD1 variant to a monomeric form.

Given that the WT-like mutants with high zinc content in Fig. 2 did not exhibit hydrophobic binding, we next asked whether destabilization by EDTA with or without TCEP could increase the hydrophobic character of these mutants and whether this required SDS. In Fig. 3B (top panel), SOD1 proteins were treated with 1 mM EDTA with or without 10 mM TCEP for 48 h followed...
by hydrophobic binding to phenyl-Sepharose beads in the absence of SDS. After exposure to EDTA without TCEP, the A4V, L38V, and G41S SOD1 mutants showed increased binding to the hydrophobic beads compared with that of WT SOD1. Concurrent treatment with TCEP greatly increased the fraction of hydrophobic binding for the A4V mutant and also facilitated binding for the other WT-like mutants. The extent of SOD1 binding to hydrophobic beads, except in the case of apo-WT SOD1, generally correlated with the fraction of aberrant monomeric species observed in Fig. 3A.

In Fig. 3B (bottom panel), 0.1% SDS was added to the bead binding assay to mimic the conditions during partially denaturing SDS-PAGE (Figs. 1 and 3A). In the presence of SDS, even apo-WT or WT SOD1 exposed to the combination of TCEP and EDTA became strongly hydrophobic (Fig. 3B, bottom). In contrast, metallated WT SOD1 that was not exposed to EDTA or TCEP failed to exhibit significant hydrophobic binding (Fig. 3B, lane 1 in each panel). Overall, these results indicate that reduction of the intrasubunit disulfide bond and metal ion loss increase the hydrophobic character of purified WT-like mutants to resemble that of the metal-binding region mutants.

**ALS Mutant but Not WT SOD1s in N2a Cell Lysates Exhibited Aberrant Binding to Nitrocellulose Upon Incubation with DTT**—The metallability or stability of SOD1 mutants overexpressed and purified from insect cells may differ from that of WT SOD1 in a mammalian cellular environment. Accordingly, we next examined the behavior of SOD1 in lysates from undifferentiated mouse N2a neuroblastoma cells stably transfected with either WT or human mutant SOD1 cDNAs (54). The soluble fraction of lysates was either boiled in denaturing/reducing buffer (Fig. 4A, lane 1 of each set) or incubated without boiling in buffer containing 0.4% SDS and either 0 or 5 mM DTT (lanes 2 and 3, respectively) prior to electrophoresis. Proteins were then transferred to nitrocellulose, and SOD1 species were detected using an antibody that recognizes both mouse (m) and human (h) SOD1.

In lane 1 of each set (Fig. 4A), both mouse and human denatured SOD1s transferred efficiently to the blot, as expected after sample reduction and boiling. In lanes from the unboiled lysates not preincubated with DTT (lane 2 of each set); however, neither WT nor mutant SOD1 proteins adhered to the blots. After exposure of lysates to DTT, however, the unboiled G37R and G41D mutants exhibited a striking increase in binding compared with WT human or mouse SOD1s (lane 3 of each set). The G85R mutant in lysates behaved similarly, although this mutant migrates faster (to the same extent as mouse SOD1) in standard Tris-glycine gels (23). The failure to detect appreciable mouse SOD1 in lane 3 for WT, G37R, and G41D lysates strongly suggested that if unfolded mouse SOD1 were present in lane 3 for the G85R lysate, it should compose only a minor fraction of the detected protein. Overall, these results indicated that mutant SOD1s in N2a cell lysates, similar to the purified mutant proteins, are more susceptible than WT SOD1 to increased hydrophobicity upon exposure to exogenous disulfide-reducing agents.

**G85R and G93A Mutant Mouse Tissue Lysates Contained Hydrophobic SOD1 Species in the Absence of Exogenous DTT Exposure**—We next examined SOD1 in spinal cord lysates obtained from transgenic mice overexpressing human WT, G85R, or G93A SOD1 and from nontransgenic littermate controls under similar partially denaturing Western blot conditions. Immunodetection of SOD1 revealed that both the mouse and human WT SOD1 proteins were retained only poorly by blots unless denatured by boiling (Fig. 4B). In contrast to the results obtained using N2a cell lines, both G85R and G93A SOD1 mutants in spinal cord lysates transferred to the blot with high efficiency even in the absence of preincubation with DTT (lane 2 for each set). Exposure of the soluble fraction of these lysates...
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Fig. 4. Mutant but not WT SOD1s in cell and tissue lysates exhibited aberrant hydrophobic properties on partially denaturing Western blots. The soluble fraction of cell or tissue lysates (10 μg per lane) was boiled in denaturing buffer for 3 min (lane 1 of each set) or was incubated in loading buffer containing 62 mM Tris (pH 6.8), 10% glycerol, 0.05% bromphenol blue, 0.4% SDS, and either no DTT (lane 2) or 5 mM DTT (lane 3) for 30 min at 25 °C prior to SDS-PAGE. Following electrophoresis and transfer to nitrocellulose blots, SOD1 proteins were detected using a rabbit polyclonal antibody raised against a peptide containing amino acids 125–137 of human SOD1 (23). The human SOD1 proteins (h, upper band) migrate more slowly than the endogenous mouse SOD1 (m, lower band), except for G85R SOD1. Arrows marked D and M are the same as described in the legend to Fig. 1. A, lysates from undifferentiated mouse N2a neuroblastoma cells stably transfected with either WT or mutant human SOD1 cDNAs. B, spinal cord lysates from nontransgenic (Non-TG, age 15 week) or transgenic mice expressing WT (15 week), G85R (5.7 week), or G93A (15 week) human SOD1. C, tissue lysates (SC = spinal cord, BN = forebrain, BS = brainstem, CB = cerebellum, SK = skeletal muscle, HT = heart muscle, K = kidney, and L = liver) from transgenic mice expressing WT (age 15 week), G93A (15 week), or G85R (27 week) human SOD1.

To determine whether mutant SOD1 from transgenic mice exhibited aberrant hydrophobic behavior in other tissues besides spinal cord (SC), we prepared soluble lysates from forebrain (BN), brainstem (BS), cerebellum (CB), skeletal (SK), and cardiac (HT) muscle, kidney (K), and liver (L) tissues. Immunodetection using the antibody to SOD1 (Fig. 4C) was compared in lysates after either boiling in denaturing buffer to indicate total SOD1 (lane 1 of each set) or incubating in non-denaturing buffer without boiling to indicate hydrophobic species (lane 2 of each set). Similar to the results of Fig. 4B, a large fraction of SOD1 from spinal cord overexpressing G93A but not WT SOD1 migrated heterogeneously and was retained on the blot. A large fraction of total G85R SOD1 in lysates from spinal cord, brain regions, and muscle tissues exhibited abnormal hydrophobic membrane retention. Interestingly, only a small amount of aberrantly hydrophobic G85R SOD1 was detected in kidney or liver lysates, despite abundant expression of the mutant SOD1 in these tissues. Overall, these results suggested that partially unfolded SOD1 mutants as a fraction of total SOD1 were most abundant in lysates from affected parts of the nervous system and least abundant in lysates from liver or kidney tissues.

Aberrantly Hydrophobic Mutant G85R and G93A SOD1s Were Prominently but Not Exclusively Detected in Spinal Cord and Brain Tissues—Because electrophoresis or transfer conditions could have affected SOD1 properties in the experiments of Fig. 4, we next measured the fraction of SOD1 retained by phenyl-Sepharose beads in tissue lysates from transgenic mice overexpressing human SOD1s (WT-Tg, G85R-Tg, or G93A-Tg) or from nontransgenic controls (Fig. 5). The upper blot in Fig. 5A shows the amount of SOD1 from G93A-Tg or WT-Tg mouse tissues retained by hydrophobic beads following incubation with 100 μg of input lysate protein. The lower blot in Fig. 5A shows the SOD1 expression level in each tissue lysate (i.e., 2 μg of lysate protein per lane or 2% of the input for the binding assay). We observed that 0.15–0.3% of the total input SOD1 from G93A-Tg mouse spinal cord, brain regions, skeletal muscle, and liver tissues was retained by the beads after three washes, while SOD1 retained from WT-Tg mouse lysates was barely detectable under the same conditions. Although only a small fraction of the total SOD1 from G93A-Tg mouse spinal...
cord and brainstem lysates was retained by the beads after repeated washing, those hydrophobic species were >15-fold more abundant in G93A-Tg compared with WT-Tg lysates, despite comparable levels of total SOD1 expression.

In Fig. 5B, bead-bound SOD1 (upper blot) and total SOD1 in 10% of the input lysates (lower blot) from G85R-Tg or nontransgenic mouse tissues were compared. Similar to that observed for lysates from G93A-Tg mice, the lysates from G85R-Tg mice contained aberrantly hydrophobic SOD1 species. Hydrophobic SOD1 was most abundant in G85R-Tg spinal cord (~4% of input SOD1) but was also present in other CNS regions and muscle tissues. In contrast, hydrophobic SOD1 was not appreciably detected in kidney or liver lysates from G85R-Tg mice or in any of the nontransgenic mouse tissue lysates. Hydrophobic SOD1 species were not restricted to spinal cord tissue and brain regions but were also observed in cardiac and skeletal muscle (and liver for G93A-Tg). In nontransgenic mice, total SOD1 was most abundant in liver (0.2% of total lysate protein) and 3.2-fold less abundant in spinal cord. Compared with expression in spinal cord lysates from nontransgenic mice, total SOD1 was increased by 3.3-fold in G85R-Tg (0.2% of soluble protein), 36-fold in G93A-Tg (2.2% of soluble protein), and 32-fold in WT-Tg (1.9% of soluble protein) spinal cord lysates. The results from Fig. 5 suggest that hydrophobic and potentially toxic conformations of the abundant mutant SOD1 proteins may accumulate, at a minimum, to levels near ~0.01% of total soluble protein in the central nervous system. Given possible inefficiencies of the bead binding assay, the local concentration of these species may be much greater, especially if hydrophobic mutant SOD1 is distributed focally within tissues.

**DISCUSSION**

Understanding the physicochemical properties shared by a large number of SOD1 variants associated with ALS may provide insights regarding the toxicity of these proteins *in vivo* to cause preferential motor neuron death. Given that a variety of disease states may be caused by protein aggregation related to increased hydrophobicity or decreased net charge (51), we compared the hydrophobicity of ALS-related SOD1 mutants by measuring retention of the proteins to uncharged nitrocellulose membranes and to hydrophobic phenyl-Sepharose beads. We observed that SOD1 mutants in purified preparations and in tissue lysates from transgenic mice exhibited abnormally increased hydrophobic binding under near-physiological buffer conditions. Among purified SOD1 variants, the degree of association with hydrophobic beads correlated with the extent of metal ion deficiency and the presence of species that migrated as monomers on partially denaturing SDS-PAGE. Hydrophobic binding could be enhanced by metal chelation with concurrent disulfide bond reduction and by partial denaturation of the mutants with SDS. WT SOD1 was much more resistant than the mutants to the destabilizing influences that produced these hydrophobic species.

Metal-binding region mutants known to be deficient in copper and zinc (e.g. H46R, G65R, D124V, D125H, and S134N) showed the greatest degree of hydrophobic binding and did not require disulfide reduction, exposure to strongly acid pH, or reaction with strong oxidants to elicit this behavior (Fig. 2). It is of interest that crystal structures have been obtained for some of these metal-deficient mutants (H46R and S134N) that indicate a well preserved β-barrel structure but disorder of the zinc-binding and electrostatic loops (37). Furthermore, this loop disorder exposes part of the β-barrel surface that is normally buried, and this may facilitate the observed polymerization of dimers within crystals via non-native loop interactions (37). Whether SOD1 dimer filamentous packing occurs *in vivo* or not, the pronounced degree of hydrophobic binding we observed for these ALS mutants supports the notion that impaired metal binding may greatly influence aggregation or other aberrant interactions.

How might WT-like mutants with preserved metal incorporation, catalytic activity, and loop conformations also be vulnerable to potentially toxic hydrophobic exposure? In our experiments, stresses that facilitated metal loss (TCEP + EDTA or SDS) increased the extent of hydrophobic binding for WT and WT-like mutants that were not initially metal deficient. We showed previously that ALS-related SOD1 mutants are more susceptible than the WT enzyme to cleavage of the disulfide linkage between Cys-57 of the disulfide loop and Cys-146 of the β-barrel (44). Because the disulfide loop (residues 48–62) forms part of the dimeric interface and is also contiguous with the zinc-binding loop (residues 63–83), reduction of the disul-
Increased hydrophobicity, a shared property of ALS-related SOD1 mutants, may contribute to aberrant interactions that promote cellular toxicity in ALS. Zinc-deficient SOD1 species that exhibit disorder of the zinc-binding and electrostatic loops may arise from direct structural perturbation of the zinc-binding site by ALS mutations (MBR mutants) or from indirect effects as a consequence of greater susceptibility in vivo to disulfide reduction or oxidative damage. In this study, zinc-deficient MBR SOD1 mutants were hydrophobic even without disulfide reduction, while zinc-loaded WTL SOD1 mutants converted more readily than WT SOD1 into hydrophobic species upon exposure to a disulfide reducing agent and a metal chelator. Weakened subunit-subunit contacts or loss of β-sheet edge strand protection by loop disorder may expose hydrophobic patches as indicated. These partially unfolded SOD1 species may be detoxified by adaptive mechanisms of refolding, degradation, or sequestration until age-related declines in these capabilities occur. An increased burden of hydrophobic SOD1 may contribute to toxicity by aberrant interactions with cellular constituents in motor neurons or supporting cells.

Although most ALS-related SOD1 mutations are missense substitutions, at least 10 mutations produce C-terminal truncation in exon 5 of the SOD1 protein. These interesting variants can delete a major portion of the electrostatic loop (residues 120–143), the disulfide anchor at Cys-146, and the terminal strand at the dimeric interface (residues 148–153). Truncation before Cys-146 would preclude formation of the disulfide bond and would also be expected to decrease dimer stability by removing interactions between Gly-150-Ile-151 and Phe-50-Gly-51 of the partner subunit. Truncation of the electrostatic loop in these mutants would also be expected to expose hydrophobic residues of the β-barrel and thereby increase biological turnover and the propensity for aggregation. Indeed, the SOD1 truncation variant L126GQRWKX (mutation: G127delTT) (61) has a greatly decreased half-life in N2a cells (25), and other truncation mutants also do not accumulate to high levels (62, 63). Another mutant, G127GGQRWKX (mutation: G127insTGGG) is present at only low levels in spinal cord and brain but accumulates in detergent-soluble proteoaggregates, detergent-resistant aggregates, and cellular inclusions (64).

We observed that lysates from kidney (for G85R-Tg and G93A-Tg mice) and liver (for G85R-Tg mice) contained a relative inclusions (64). SOD1 truncation variants decreased half-life in N2a cells (25), and other truncation mutants also do not accumulate to high levels (62, 63).Another mutant, L126GQRWKX (mutation: G127delTT) (61) has a greatly decreased half-life in N2a cells (25), and other truncation mutants also do not accumulate to high levels (62, 63). Another mutant, G127GGQRWKX (mutation: G127insTGGG) is present at only low levels in spinal cord and brain but accumulates in detergent-soluble proteoaggregates, detergent-resistant aggregates, and cellular inclusions (64).

We observed that lysates from kidney (for G85R-Tg and G93A-Tg mice) and liver (for G85R-Tg mice) contained a relatively smaller fraction of hydrophobic mutant SOD1 species compared with other tissue lysates, despite high expression levels of total SOD1 (Figs. 4C and 5). The amount of hydrophobic SOD1 detected in these lysates should reflect a balance between accumulation and clearance of abnormal SOD1 species. Mutant mice show age-dependent accumulation of mutant SOD1, with brain and spinal cord showing higher accumulation than other tissue types (31, 64). Our data and studies by others suggest that tissues such as kidney and liver may have more effective mechanisms for hydrophobic SOD1 clearance, which might minimize the accumulation of hydrophobic or aggregated forms. A better understanding of how some tissues, such as kidney, succeed in minimizing the burden of hydrophobic SOD1 species may suggest new protective approaches.

How might increased SOD1 hydrophobicity contribute to neurodegeneration in familial ALS? Our results suggest that hydrophobic SOD1 species may arise in vivo from direct failure to bind zinc (MBR mutants) or from disulfide reduction and zinc loss of natively folded SOD1 (WTL mutants), as summarized in Fig. 6. Even WT SOD1 would be expected to exhibit increased hydrophobicity if oxidative damage facilitates loss of zinc binding. In addition, nascent SOD1 molecules fold into apoenzyme forms that are susceptible to aggregation pathways that compete with the native folding pathways (65, 66), especially when zinc binding is impaired.

Our observation of a fairly widespread tissue distribution of soluble hydrophobic mutant SOD1 species is distinct from reports of high molecular weight complexes or insoluble aggregates that preferentially accumulate in spinal cord and brain (27, 30, 31, 64). We propose that the soluble hydrophobic species, which are detected at early presymptomatic stages (Fig. 4B), may be precursors to higher molecular weight complexes or aggregates found in clinically affected tissues. It remains unproven whether such aggregates are directly toxic. It is also

![Fig. 6. Increased hydrophobicity, a shared property of ALS-related SOD1 mutants, may contribute to aberrant interactions that promote cellular toxicity in ALS. Zinc-deficient SOD1 species that exhibit disorder of the zinc-binding and electrostatic loops may arise from direct structural perturbation of the zinc-binding site by ALS mutations (MBR mutants) or from indirect effects as a consequence of greater susceptibility in vivo to disulfide reduction or oxidative damage. In this study, zinc-deficient MBR SOD1 mutants were hydrophobic even without disulfide reduction, while zinc-loaded WTL SOD1 mutants converted more readily than WT SOD1 into hydrophobic species upon exposure to a disulfide reducing agent and a metal chelator. Weakened subunit-subunit contacts or loss of β-sheet edge strand protection by loop disorder may expose hydrophobic patches as indicated. These partially unfolded SOD1 species may be detoxified by adaptive mechanisms of refolding, degradation, or sequestration until age-related declines in these capabilities occur. An increased burden of hydrophobic SOD1 may contribute to toxicity by aberrant interactions with cellular constituents in motor neurons or supporting cells.](image-url)
plausible that hydrophobic SOD1 in certain subcellular locations, such as mitochondria or axons, might be less readily removed by adaptive responses and could accumulate or interact to produce toxicity. Reactive oxygen species are known to be increased in ALS tissues, and zinc-deficient, disulfide-reduced SOD1 mutants may also be vulnerable to oxidative cross-linking (65, 66). Potentially toxic interactions of aberrantly hydrophobic SOD1 include filamentous self-association (37) or other accumulation into aggregates, overload of chaperone defenses (65, 66). Potentially toxic interactions of aberrantly hydrophobic SOD1 in certain subcellular locations, such as mitochondria or axons, might be less readily removed by adaptive responses and could accumulate or interact to produce toxicity. Reactive oxygen species are known to be increased in ALS tissues, and zinc-deficient, disulfide-reduced SOD1 mutants may also be vulnerable to oxidative cross-linking (65, 66). Potentially toxic interactions of aberrantly hydrophobic SOD1 include filamentous self-association (37) or other accumulation into aggregates, overload of chaperone defenses (65, 66).

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