Decreased Metallation and Activity in Subsets of Mutant Superoxide Dismutases Associated with Familial Amyotrophic Lateral Sclerosis*§

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‡ The abbreviations used are: ALS, amyotrophic lateral sclerosis; ESI-MS, electrospray ionization mass spectrometry; ICP-MS, inductively coupled plasma mass spectrometry; O$_2^-$, superoxide anion; SOD, superoxide dismutase; SOD1, copper/zinc superoxide dismutase; WT, wild type.

Over 90 different mutations in the gene encoding copper/zinc superoxide dismutase (SOD1) cause ~2% of amyotrophic lateral sclerosis (ALS) cases by an unknown mechanism. We engineered 14 different human ALS-related SOD1 mutants and obtained high yields of biologically metallated proteins from an SF21 insect cell expression system. Both the wild type and mutant “as isolated” SOD1 variants were deficient in copper and were heterogeneous by native gel electrophoresis. By contrast, although three mutant SOD1s with substitutions near the metal binding sites (H46R, G85R, and D124V) were severely deficient in both copper and zinc ions, zinc deficiency was not a consistent feature shared by the as isolated mutants. Eight mutants (A4V, L38V, G41S, G72S, D76Y, D90A, G93A, and E133*) exhibited normal SOD activity over pH 5.5–10.5, per equivalent of copper, consistent with the presumption that bound copper was in the proper metal-binding site and was fully active. The H48Q variant contained a high copper content yet was 100-fold less active than the wild type enzyme and exhibited a blue shift in the visible absorbance peak of bound Cu(II), indicating rearrangement of the Cu(II) coordination geometry. Further characterization of these as-isolated SOD1 proteins may provide new insights regarding mutant SOD1 enzyme toxicity in ALS.

Amiotrophic lateral sclerosis (ALS,1 Lou Gehrig’s disease) is an age-dependent, degenerative disorder of motor neurons in the spinal cord and brain. Progressive dysfunction of both upper and lower motor neurons causes death from respiratory paralysis, usually within 5 years. Investigation of the causes of familial ALS, which comprises ~10% of cases, may contribute insights relevant to the pathophysiology of sporadic ALS and of other motor neuron diseases (1, 2).

A subset of autosomal dominant ALS is caused by over 90 mutations in the gene encoding copper/zinc superoxide dismutase (SOD1) (3, 4) (see an updated list of all mutations on the World Wide Web at www.alsod.org). SOD1 is a 32-kDa homodimeric enzyme that functions as an antioxidant, converting two molecules of superoxide anion (O$_2^-$) to O$_2$ and H$_2$O$_2$. This redox cycle involves alternate reduction (reaction 1) and reoxidation (reaction 2) of the catalytic copper ion by O$_2^-$.

$$
\text{SOD1-Cu(II)} + O_2^- \leftrightarrow \text{SOD1-Cu(I)} + O_2 \quad (1) 
$$

$$
\text{SOD1-Cu(I)} + O_2^- + 2H^+ \leftrightarrow \text{SOD1-Cu(II)} + H_2O_2 \quad (2)
$$

** REACTIONS 1 and 2 **

SOD1 contains an eight-stranded β-barrel motif, an intrasubunit disulfide bond, and a zinc binding site that contribute to its extreme thermochemical stability (Fig. 1). The mutant residues are scattered throughout the protein, including some residues important for copper or zinc coordination, others located near the dimer interface or at either pole of the β-barrel, and several in the charged loop near the C terminus that may guide O$_2^-$ to the active site. Although most are missense substitutions, some are predicted to truncate the C terminus of the protein, including the charged loop. No null mutations have been described.

Mutant SOD1 most likely causes motor neuron death by gain of an unknown toxic property rather than by deficiency of dismutase activity (reviewed in Ref. 5). Numerous studies in yeast and mammalian cultures implicate a proapoptotic function of mutant SOD1, while SOD1 knockout mice do not acquire ALS pathology (6). Moreover, transgenic mice that overexpress ALS mutant SOD1s consistently develop severe motor neuron pathology despite increased total SOD1 activity (7–11), whereas mice that overexpress the WT enzyme develop much milder and less specific pathology (12). An increase or a decrease of wild type (WT) SOD1 expression does not affect the course of the disorder in G85R mutant SOD1 mice (13), suggesting that cellular O$_2^-$ levels do not directly influence mutant SOD1 toxicity.

To clarify biophysical and biochemical properties that differ between mutant and normal SOD1, we engineered 14 different...
ALS mutant SOD1s and expressed each protein in an SF21 insect cell/baculovirus system. The insect cell system was chosen because, like yeast but unlike bacterial systems, it produces very high expression of soluble SOD1 variants with correctly acetylated amino-terminal groups (14). In addition, while previous reports of mutant SOD1s expressed in yeast have described abnormal metal ion coordination (15) and normally low copper/zinc ratios (16), it remains unclear whether altered metal incorporation is a function of the expression system, the \textit{in vitro} remetallation procedure, or the mutations themselves.

A standard procedure for obtaining a homogeneous population of fully metallated WT SOD1 after purification involves removal of metal ions by chelation at pH 3.8 followed by remetallation with copper and zinc (17). However, previous studies have shown that, in contrast to WT SOD1, remetallated ALS mutant enzymes exhibit an altered zinc binding site and abnormal copper coordination geometry (15, 18–20). Moreover, it has recently been demonstrated that the removal of metals under acidic conditions \textit{in vitro} from some ALS SOD1 mutants grown in yeast, followed by remetallation, may result in metal ion mislocalization to the wrong binding site (19). It is important to distinguish whether these abnormalities arise in biologically metallated mutant SOD1s or are related to irreversible denaturing influences during the remetallation procedure.

We have therefore analyzed \textit{as-isolated} SOD1 enzymes, without attempting to deplete or substitute bound metal ions. Human SOD1 mutants were expressed and purified from insect cells and then compared with the WT enzyme obtained under similar conditions. In this report, we identified differences in electrophoretic mobility, copper and zinc ion content, copper coordination geometry, and specific SOD activity of these SOD1 variants containing biologically incorporated metals.

**EXPERIMENTAL PROCEDURES**

**Materials**—All solutions were filtered through a 0.2-μm filter and were prepared using water that had been purified with a Millipore deionizing system. KCl, NaCl, (NH₄)₂SO₄, β-mercaptoethanol, Tris base, and mono- and dibasic potassium phosphate were from J. T. Baker Inc.; EDTA, glycerol, and SDS were from EM Science; HNO₃ and copper and zinc standards were from Fisher. Ultrapure water for preparation of the washes was from CASCO NERL Diagnostic.

**Site-directed Mutagenesis**—cDNA fragments encoding 14 specific human mutant SOD1s associated with familial ALS (A4V, L38V, G41S, G85C, G93A, E122G, R152H, G178R, L285R, A311T, A316V, V354I, S376C, and S377A) were used as templates for mutagenesis. The EcoRI/BglII restriction enzymes from were from J. T. Baker Inc.; EDTA (10 μg/ml) and penicillin G (100 units/ml) were from Pharmingen and used as a template for mutagenesis. For each mutant, the region of each clone was sequenced to verify the desired substitution. For moderate scale protein expression, suspension cultures of SF21 insect cells were propagated in 0.5–1-liter spinner flasks (Belco) at 27 °C in serum-free medium (SFM II-900; Life Technologies, Inc.) supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. The impeller was set at 95 rpm and cultures in log phase cultures in log phase growth were inoculated with 1.5–10⁶ cells/ml were inoculated with baculovirus stock at a multiplicity of 5–10. During inoculation, either 50, 150, or 300 μl of CuCl₂ and ZnCl₂ was added to the medium from separate 150 ml stocks (pH 7.4). Metal ion concentrations greater than 500 μM in the serum-free medium were excessively toxic to SF21 cells. During the expression period, cell viability was monitored by trypan blue exclusion. At 60 h following infection, the cell pellet was harvested by centrifugation at 1000 × g for 10 min and then stored at −80 °C.

**Purification of Human WT and Mutant SOD1**—We employed hydrophobic interaction chromatography to isolate highly pure SOD1 rapidly from SF21 cells. The cell pellet from 2 liters of culture was resuspended on ice in 100 ml of a lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (“Complete, EDTA-free”; catalog no. 1873580; Roche Diagnostics). The cells were thoroughly disrupted after six cycles of homogenization using a Beadbeater (BioSpec, Bartlesville, OK) and 1-mm glass beads for 30 s followed by cooling on ice for 30 s. The lysate was centrifuged at 30,000 × g for 30 min at 4 °C. (NH₄)₂SO₄ crystals were added over 1000 min to the supernatant to 20% saturation (31.4 g/100 ml). After an additional 30 min of gentle stirring on ice, and the centrifugation was repeated. 0.25 volumes of a saturated (NH₄)₂SO₄ solution prepared at 25 °C was added to adjust the supernatant to −64% saturation at 4 °C, and the centrifugation was repeated. The supernatant was diluted with 0.215 volume of water to a final concentration of 2.0 mM (NH₄)₂SO₄ and loaded onto a column containing 30 ml of phenyl-Sepharose 6 Fast Flow high sub (Amersham Biosciences). The column was washed extensively with a high salt buffer containing 2 mM (NH₄)₂SO₄, 50 mM sodium phosphate (pH 7.0), 150 mM NaCl, 0.1 mM EDTA, and 0.25 mM dithiothreitol, adjusted to a final pH of 7.0 with NaOH. Proteins were eluted using a 300-ml linearly decreasing salt gradient toward a low salt buffer that was identical to the high salt buffer except for omission of (NH₄)₂SO₄. WT or mutant SOD1 was released with high specificity from the column between 1.6 and 1.1 M ammonium sulfate (240–170-milliemons conductivity).

Fractions containing SOD1 were identified by SDS-PAGE, pooled, and exchanged to a low salt buffer containing 10 mM Tris (pH 8.0) using a Centricron-80 spin concentrator (Millipore Corp.). The proteins were then loaded onto a HiTrap Q-Sepharose anion exchange column (Amersham Biosciences) and eluted using a 200-ml linearly increasing salt gradient consisting of 30 ml NaCl and 10 mM Tris (pH 8.0). SOD1 proteins typically eluted between 50 and 100 mM NaCl (6–13-milliemons conductivity) as one major peak that contained >90% of the total SOD1 protein. A minor peak (~10% of total SOD1) eluted at higher salt concentrations. Fractions containing SOD1 were pooled, concentrated, and stored at 4 °C for short term use or at −80 °C.

**SDS-PAGE and Western Blotting**—SDS-PAGE was performed using 15% polyacrylamide gels and a running buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 1.0% SDS. The sample loading buffer contained 62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.007% bromphenol blue. After electrophoresis, gels were either stained with a solution containing 0.1% Coomassie Blue R-250 in 50% methanol, 10% glacial acetic acid or electrophoblotted to nitrocellulose membranes (Amersham Biosciences) for Western blot analysis. Blots were incubated for 1 h each at 25 °C with a 1:2,000 dilution of a primary sheep anti-human SOD1 polyclonal antibody (Calbiochem catalog no. 574597) and a 1:10,000 dilution of secondary rabbit anti-sheep IgG (Calbiochem catalog no. 402100; peroxidase conjugate) according to the ECL (Amersham Biosciences) protocol. For native PAGE using 7.5% polyacrylamide gels, the loading buffer did not contain SDS or β-mercaptoethanol, the samples were not boiled, and the running buffer did not contain SDS.

**Characterization by Electrospray Ionization Mass Spectrometry (ESI-MS)**—The purity and molecular weight of the isolated proteins were determined using a Finnigan LCQ electrospray ionization, quadrupole ion trap mass spectrometer. Data were collected and analyzed at the Mass Spectrometry Facility (E. K. Shriver Center, Waltham, MA). Data were acquired over a mass range (10 μg/ml) and penicillin G (100 units/ml) were from Pharmingen and used as a template for mutagenesis. For each mutant, the region of each clone was sequenced to verify the desired substitution and to rule out spurious mutations.

**Expression of Human SOD1 Proteins in SF21 Insect Cells**—To produce baculovirus stocks, cells from \textit{Spodoptera frugiperda} (SF21) were grown as adherent cultures at 27 °C in Grace’s medium containing 10% fetal bovine serum and 1% antibiotic–antimycotic (10 μg/ml gentamicin. Baculovirus DNA (0.1 ng of BaculGold; Pharmingen) was co-transfected with pVL1392 (containing WT or mutant SOD1 sequence) into SF21 cells by calcium phosphate precipitation and incubated for 5 days. Recombinant viruses in the supernatant were amplified by two or three rounds of infection to obtain a high titer viral stock of 5–10 × 10⁷ pfu/ml, as estimated by the end point dilution method.
SOD1 variant after purification was quantitated using inductively coupled plasma mass spectrometry (ICP-MS). SOD1 proteins were diluted to 1.5–4 μM in 0.1% nitric acid. The mass spectrometer was programmed to detect Cu-65 and Zn-68, and standard curves were constructed from solutions of known copper and zinc content. Protein concentrations were based on absorbance at 280 nm of the purified enzymes, as described under “Spectroscopy.” Measurements were made in duplicate or triplicate with an experimental error of <5% using the ICP-MS instrument at the Caltech Environmental Analysis Center and a Perkin-Elmer Elan 5000A instrument.

Spectroscopy—UV-visible spectra from 240–900 nm were obtained using a Hewlett-Packard model 8453 diode array spectrophotometer. Purified proteins in 100 mM phosphate buffer (pH 7.2) were centrifuged in 1 min at 12,000 × g before obtaining spectra. Dimeric SOD1 concentrations were determined using an extinction coefficient of ε280 nm = 1.08 × 10^4 M^-1 cm^-1 (16), except for the D76Y mutant, for which ε280 nm = 1.38 × 10^4 M^-1 cm^-1 was used to account for the additional tyrosine absorbance (23). For clarity in Fig. 6, artifacts produced at 486, 628, and 656 ± 2 nm by the spectrophotometer lamp were omitted.

Superoxide Dismutase Activity by Pulse Radiolysis—SOD activity was measured directly by pulse radiolysis as described previously (16). All activity measurements were conducted in buffer containing Millipore-deionized water, 10 mM formate, 10 mM phosphate, and 10–25 μM EDTA. The pH was adjusted by the addition of NaOH or H2SO4 (both of highest purity commercially available). By monitoring the change in absorbance at 260 nm with respect to time, a first-order rate for the catalytic disproportionation of O2 was measured directly by pulse radiolysis as described previously (16). All activity measurements were conducted in buffer containing Millipore-deionized water, 10 mM formate, 10 mM phosphate, and 10–25 μM EDTA. The pH was adjusted by the addition of NaOH or H2SO4 (both of highest purity commercially available). By monitoring the change in absorbance at 260 nm with respect to time, a first-order rate for the catalytic disproportionation of O2 was measured directly by pulse radiolysis as described previously (16).

RESULTS

Selection of ALS-related SOD1 Mutants—We produced a diverse set of 14 human SOD1 variants for biophysical and biochemical analysis (Fig. 1). A brief summary of the structural aspects of these highly conserved residues is presented here to facilitate the description of our experimental findings. Five mutants within the β-barrel (A4V, L38V, G41S, D90A, and G93A) were constructed (Fig. 1A). A4V SOD1, which causes an aggressive motor neuron degeneration with median survival of 11 months (24), was selected because it could potentially disrupt SOD1 dimerization or stability of the β-barrel (25). L38V, G41S, D90A, and G93A were chosen because they are located within important turns near one pole of the β-barrel. L38V may disrupt packing of the β-barrel “plug,” while loss of left-handed glycines in G41S and G93A may reduce the flexibility of their respective loops. Substitution of Gly-93 with any side chain will perturb the carbonyl oxygen of Leu-38 and may disrupt the local backbone structure (26). In addition, the Asp-90 side chain may accept a hydrogen bond from the amide nitrogen of residue 92 to stabilize the tight β-hairpin turn formed by residues 90–93 (26).

Six mutants located near the active site (H46R, H48Q, G72S, D76Y, G56R, and D124V) were selected because they may alter metal binding or reactivity (Fig. 1B). His-46 may normally position the copper ion in a strained coordination geometry that favors rapid redox cycling between the Cu(1) and Cu(II) forms in Reactions 1 and 2 (27–29). In addition, His-46 participates with Asp-124 and the zinc ligand His-71 to form an extended bridge between the copper and zinc ions (Fig. 1B). Non-ALS mutant substitutions of Asp-124 are known to bind zinc poorly (30). The left-handed Gly-85 residue interacts through a conserved water molecule with both Asp-124 and Gly-72, which may influence both the copper and zinc binding sites. The G85R mutation was selected because it may severely destabilize this interaction, or may disrupt the local backbone structure that positions Asp-83 for zinc coordination (25). G72S and D76Y, located within the zinc-binding loop, were constructed to examine the degree to which they may specifically impair zinc binding.

D125H, E133Δ, and S134N were selected because they may alter the charged loop that forms one face of the channel leading to the active site. E133A denotes a deletion of the Glu-133 residue (31) rather than a truncation of the protein. These three residues interact with each other and with Thr-137 through the local backbone and a conserved hydrogen bond network (Fig. 1B). Perturbation of the channel geometry or its flexibility could alter the steering of O2 or influence reactivity of the catalytic copper ion.

Expression and Purification of Human SOD1 Variants Associated with ALS—Human WT or mutant SOD1 proteins were...
We observed that SOD1 mutants D76Y and E133A human SOD1 were separated by SDSPAGE and stained with Coomassie Blue. SOD1 was enriched among proteins that remained soluble in (NH₄)₂SO₄ at 64% saturation (lane 3 in each panel) and subsequent ion exchange (lane 5 in each panel; 2 µg/lane) and showed further SOD1 concentration. Western blot analysis of duplicate gels, loaded with 5% of the amount of protein as in A and detected with an anti-SOD1 polyclonal antibody.

Expressed in Sf21 insect cells using a baculoviral system and isolated using an improved SOD1 purification procedure. In Fig. 2A, the soluble cellular lysate from uninfected Sf21 cells (lane 1) or cells expressing either WT, G85R, or G93A human SOD1 (lane 2 in each group) were separated by SDSPAGE and stained with Coomassie Blue. SOD1 was enriched among proteins that remained soluble in (NH₄)₂SO₄ at 64% saturation (lane 3 in each panel, 10 µg/lane). Pooled SOD1-containing fractions after hydrophobic interaction chromatography (lane 4 in each panel, 2 µg/lane) and subsequent ion exchange (lane 5 in each panel, 2 µg/lane) showed further SOD1 concentration. B, Western blot analysis of duplicate gels, loaded with 5% of the amount of protein as in A and detected with an anti-SOD1 polyclonal antibody.

As-isolated Recombinant Human SOD1 Proteins Exhibited Heterogeneous Migration During Native PAGE—Fig. 3A demonstrates the migration of purified WT and mutant SOD1s by denaturing SDSPAGE using a standard Tris-glycine-SDS running buffer. Whereas most mutants demonstrated mobility comparable with that of WT SOD1, we observed a small but consistently accelerated migration for the mutants G85R and D90A, in agreement with previous reports (32, 33). When the samples were run on Tris-Tricine-SDS gels (34), this differential mobility disappeared as a result of the WT and other mutant SOD1s migrating faster relative to marker proteins (not shown). A possible explanation for the relative acceleration of G85R and D90A on standard gels is that these mutants may have bound slightly more SDS than the WT protein as a result of more complete unfolding.

In contrast to the relatively homogeneous appearance of the SOD1 proteins on denaturing PAGE, both WT and mutant as-isolated proteins migrated as a heterogeneous mixture of species during electrophoresis under native conditions (Fig. 3B). Commercially available human erythrocyte SOD1 (WT-C), which is almost fully metallated with copper and zinc ions, migrated as a single band on native PAGE. However, the as-isolated human WT protein from insect cells (WT; both panels of Fig. 3B) consisted of at least three distinct species, two bands that migrated more rapidly and a third faint band of comparable mobility to the WT-C holoenzyme. One subset of mutants, including H46R, G85R, D124V, D125H, and S134N, migrated much more rapidly, while D90A migrated more slowly than WT SOD1 on native PAGE (Fig. 3B). Several factors may affect the mobility of SOD1 in native gels, including 1) conformational changes such as unfolding or monomerization, 2) altered net charge caused by mutant amino acid variation, and 3) differences in molecular weight.

**Fig. 2.** Purification of human WT and ALS mutant SOD1 enzymes from insect cells. A, soluble lysates (10 µg/lane) obtained from uninfected Sf21 cells (lane 1) or cells expressing either WT, G85R, or G93A human SOD1 (lane 2 in each group) were separated by SDSPAGE and stained with Coomassie Blue. SOD1 was enriched among proteins that remained soluble in (NH₄)₂SO₄ at 64% saturation (lane 3 in each panel, 10 µg/lane). Pooled SOD1-containing fractions after hydrophobic interaction chromatography (lane 4 in each panel, 2 µg/lane) and subsequent ion exchange (lane 5 in each panel, 2 µg/lane) showed further SOD1 concentration. B, Western blot analysis of duplicate gels, loaded with 5% of the amount of protein as in A and detected with an anti-SOD1 polyclonal antibody.

**Fig. 3.** As-isolated SOD1 variants after denaturing or native PAGE. A, SDSPAGE of purified WT and mutant SOD1 enzymes (2 µg loaded/lane) as detected with Coomassie Blue. The growth medium during protein expression was supplemented with copper and zinc ions at either 50 µM (for D90A and E133A), 150 µM (for A4V and S134N), or 300 µM (for all other SOD1s). B, native PAGE showed that each recombinant SOD1 enzyme preparation consisted of a mixture of species with differing mobilities. WT-C was commercially available human SOD1 holoenzyme (Sigma). Each lane contained 5 µg of protein. Standards (STD) in the first lane included lactalbumin (L; 14.2 kDa), carbonic anhydrase (C; 29 kDa), and albumin (A; 45 kDa), which also exhibited migration rates dependent on charge and conformation under native conditions.

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substitutions or differences in metal occupancy, or 3) covalent modifications of the enzyme. To identify the cause(s) of the observed heterogeneity, we used mass spectrometry to examine the molecular weight and the metal ion contents of each as-isolated SOD1 enzyme.

Each As-isolated SOD1 Mutant Protein Appeared Homogeneous by ESI-MS—The purity and molecular mass of each SOD1 enzyme was assessed by electrospray ionization mass spectrometry (ESI-MS) in the presence of 50% acetonitrile at acidic pH. Under these strongly denaturing conditions, both WT and mutant SOD1s lost their ability to retain metal ions and dissociated into monomers. Fig. 4 displays representative raw spectra (left panels) and deconvoluted spectra (right panels) for WT, A4V, L38V, and G85R SOD1 enzymes. Mutant SOD1s acquired more charges than did WT SOD1, as indicated by a mild but reproducible shift in the envelope of charged species to lower m/z values in sequential scans under identical electrospray ionization conditions. More extensive ionization suggested, but did not prove, that mutant SOD1 proteins were more susceptible to unfolding under these conditions compared with the WT enzyme.

The raw m/z spectra were deconvoluted to obtain the mass of each protein species (Fig. 4, right panels), which corresponded for all mutants to the expected mass of amino-terminal acetylated SOD1 monomers devoid of metal ions (Table I). In addition, the two charge variants resolved by ion exchange chromatography for D76Y and E133/K904 SOD1s each had the same mass, indicating that the protein species of each pair did not differ. This suggested that differential metallation was the most likely explanation for the charge heterogeneity of D76Y and E133/K904 SOD1 proteins.

A Subset of ALS Mutant Proteins Exhibited Copper and Zinc Contents Comparable with WT SOD1, whereas Others Were More Severely Metal-deficient—To compare metal loading of WT and mutant SOD1 enzymes in SF21 cells under similar growth conditions, we analyzed copper and zinc contents of each as-isolated protein by inductively coupled plasma mass spectrometry (ICP-MS). Fig. 5 shows the metal occupancy per
Metallation and Activity of ALS Mutant SOD1 Enzymes

### Table I

| SOD1 protein        | Location          | Expected mass | Measured mass<sup>a</sup> | Copper<sup>b</sup> (mutant/WT) | Zinc<sup>b</sup> (mutant/WT) | Copper coordination geometry<sup>c</sup> | Specific activity<sup>d</sup> |
|---------------------|-------------------|---------------|---------------------------|-------------------------------|-------------------------------|---------------------------------|-----------------------------|
| WT-like mutants     |                   |               |                           |                               |                               |                                 |                             |
| WT                  |                   | 15,845        | 15,874                    | 100                           | 100                           | Normal                          | Normal                      |
| A4V                 | Dimer interface   | 15,873        | 15,875                    | 48                            | >90                           | Normal                          | Normal                      |
| L38V                | β-Barrel          | 15,831        | 15,833                    | 75                            | 85                            | Normal                          | Normal                      |
| G41S                | β-Barrel          | 15,875        | 15,872                    | 84                            | 80                            | Normal                          | Normal                      |
| G72S                | Zinc loop         | 15,875        | 15,873                    | >90                           | 46                            | Normal                          | Normal                      |
| D76Y-1              | Zinc loop         | 15,893        | 15,891                    | >90                           | 76                            | Normal                          | Normal                      |
| D76Y-2              | Zinc loop         | 15,893        | 15,891                    | 58                            | 49                            | Normal                          | Normal                      |
| D90A                | β-Barrel          | 15,801        | 15,803                    | >90                           | >90                           | Normal                          | Normal                      |
| G93A                | β-Barrel          | 15,859        | 15,858                    | 87                            | >90                           | Normal                          | Normal                      |
| E133A-1             | Charged loop      | 15,715        | 15,719                    | >90                           | 84                            | Normal                          | Normal                      |
| E133A-2             | Charged loop      | 15,715        | 15,721                    | 52                            | 56                            | Normal                          | Normal                      |
| Metal-binding region mutants |       |               |                           |                               |                               |                                 |                             |
| H46R                | Active site       | 15,715        | 15,719                    | 52                            | 56                            | Normal                          | Normal                      |
| H48Q                | Active site       | 15,836        | 15,834                    | 75                            | 85                            | Normal                          | Normal                      |
| G85R                | Active site       | 15,944        | 15,946                    | 64                            | 64                            | Planar<sup>e</sup>              | ~1%                         |
| D124V               | Active site       | 15,829        | 15,826                    | 80                            | 80                            | Planar<sup>e</sup>              | ~1%                         |
| D125H               | Charged loop      | 15,867        | 15,865                    | 15                            | 21                            | Planar<sup>e</sup>              | ~17%                        |
| S134N               | Charged loop      | 15,872        | 15,870                    | 40                            | 24                            | Planar<sup>e</sup>              | ~35%                        |

<sup>a</sup> Monomeric protein masses measured by ESI-MS under strongly denaturing conditions, in which the metal ions were removed. In all cases, the measurement error was 0.025% or less. Masses were consistent with amine-terminal acetylation of all SOD1 proteins.

<sup>b</sup> Metal ion content of as-isolated SOD1 preparations, as determined by ICP-MS (Fig. 5), relative to the metal ion content of WT SOD1 expressed with the same metal ion supplementation (50, 150, or 300 μM) to the insect cell growth media.

<sup>c</sup> Normal = Cu(II) absorbance peak centered at 680 nm. Planar<sup>e</sup> = absorbance peak at 610 nm.

<sup>d</sup> Activity is expressed per equivalent of bound copper.

<sup>e</sup> E133A denotes a deletion of the Glu-133 residue alone (31) rather than a truncation of the protein.

<sup>f</sup> ND, not determined.

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**FIG. 5. WT and mutant SOD1 as-isolated variants exhibited differences in both copper and zinc content.** The content of copper (gray bars) and zinc (black bars) measured by ICP-MS from each purified SOD1. Fully metallated SOD1 holoenzyme contains 2.0 equivalents of copper and zinc per dimer. The concentration of copper and zinc ions (50, 150, or 300 μM) added to the culture medium during expression increased total copper ion incorporation in a dose-dependent manner. As shown in Fig. 5, increasing exogenous Cu<sup>2+</sup> and Zn<sup>2+</sup> from 50 to 300 μM approximately doubled the copper content of WT, A4V, and L38V SOD1s without greatly affecting zinc content. In contrast, for mutants that bound metal ions poorly such as H46R or G85R, supplementation of the medium did not improve the metal deficiency of the enzymes (Fig. 5).

Cu(II) Exhibited a WT-like Coordination Geometry in All Mutant SOD1 Enzymes Except for H48Q—We measured the UV-visible spectrum of the as-isolated WT and mutant SOD1 proteins to confirm differences in Cu(II) content and to evaluate possible changes in the coordination geometry of Cu(II) in these enzymes (Fig. 6). We observed a broad absorbance peak centered at 680 nm, a characteristic property of Cu(II) binding to the native copper site of SOD1 in a distorted tetragonal geometry (15). For the WT and nine SOD1 mutants that bound appreciable amounts of Cu(II) ions (A4V, D90A, and G93A) each contained 80–90% of full zinc occupancy under comparable conditions. The addition of metal ions to the media during protein expression increased total copper incorporation in a dose-dependent manner. As shown in Fig. 5, increasing exogenous Cu<sup>2+</sup> and Zn<sup>2+</sup> from 50 to 300 μM approximately doubled the copper content of WT, A4V, and L38V without greatly affecting zinc content. In contrast, for mutants that bound metal ions poorly such as H46R or G85R, supplementation of the medium did not improve the metal deficiency of the enzymes (Fig. 5).

The H48Q mutant appeared distinct compared with the WT and other mutant SOD1 enzymes. We observed a blue shift of the visible electronic absorption peak for Cu(II) from 680 to 610 nm (Fig. 6), suggesting rearrangement of the distorted tetragonal copper coordination geometry toward a more regular geometry. A similar blue-shifted absorbance has been observed for the non-ALS mutant H63A of yeast SOD1, which lacks the histidine bridge between the copper and zinc ions (35).

A Subset of ALS Mutant SOD1s Exhibited Decreased Specific Activity Normalized to Copper Content—Rates for dismutation...
of superoxide catalyzed by SOD1 derivatives at pH 5.5–10.5 were determined by pulse radiolysis (36). Because only copper-containing enzymes are active, specific activities were based on the concentration of copper-bound enzyme (16) as determined by ICP-MS (Fig. 5). Correctly metallated WT SOD1 exhibits nearly constant activity in the pH range 5–9, with a steep decline at pH > 9 (36).

All but three of the as-isolated mutants containing copper exhibited WT-like SOD activity between pH 5.5 and 10.5, consistent with binding of copper to the proper metal-binding site (Fig. 7). WT and eight of the ALS mutant SOD1s (A4V, L38V, G41S, G72S, D76Y, D90A, G93A, and E133Δ) exhibited specific SOD activities between 1 and 2 × 10^6 M^−1 s^−1 at physiological pH. These results were consistent with recent studies of the as-isolated human WT, A4V, L38V, and G93A SOD1s purified from a yeast expression system (16, 19). The activity measurements demonstrated that over half of the ALS mutants were capable of binding copper in a redox-active conformation.

In contrast to the above mutants, H48Q SOD1 was less active by ~100-fold at neutral pH despite binding a similar amount of copper as the WT enzyme. H48Q SOD1 also exhibited a greater pH sensitivity, with activity declining 10-fold as the pH was increased from 5.8 to 6.8 (Fig. 7). The copper- and zinc-deficient mutants S134N and D125H exhibited specific SOD activities that were 3–5-fold lower than WT SOD1 at physiological pH, even after accounting for their decreased copper content. The mutants H46R, G85R, and D124V exhibited severe metal deficiency, which precluded accurate calculation of copper-based specific activities.

**DISCUSSION**

Models of mutant SOD1 toxicity in familial ALS have been divided into catalytic and noncatalytic mechanisms (5, 37–39), implicating aberrant reactions by copper (40–43) or abnormal protein interactions (13, 44–46). A loosened structure of SOD1 is presumed for both mechanisms, but the activity of copper and the thermochemical stabilities among a large group of SOD1 mutants have not been compared.

In this study, we isolated a set of 14 biologically metallated human SOD1 mutants associated with ALS for detailed physicochemical analyses. One group of “WT-like” mutants (A4V, L38V, G41S, G72S, D76Y, D90A, G93A, and E133Δ) exhibited fully active bound copper in a coordination environment remarkably similar to that of WT SOD1 (Table I). The other six “metal binding region” mutants (H46R, H48Q, G85R, D125H, and S134N) differed from WT SOD1 according to metal ion contents, visible absorption spectra, or specific activities.

All of our as-isolated SOD1 proteins, including WT, were copper-deficient (Fig. 5), in agreement with studies of human WT, A4V, L38V, and G93A SOD1s in yeast (16, 19). The observed reductions in metal content may depend on biological metal ion availability and do not necessarily correspond to measures of intrinsic metal binding affinity. Indeed, in our view, the most likely explanation for the copper deficiency of our as-isolated SOD1 enzymes is that copper availability was limited in the insect cells during SOD1 overexpression. Although metal ions could have been removed from the enzymes by chelators during purification, this appears unlikely. The affinity of WT SOD1 for copper ions is ~7,000-fold greater than that for zinc ions (47), yet even our WT enzyme was much more copper-deficient than zinc-deficient. Further, if metals were removed during purification, one would not expect an increase of metal incorporation dependent upon the degree of prior copper supplementation to the medium. A similar dose-dependent increase of WT SOD activity has been observed in Sf21 cells exposed to exogenous CuCl_2 (14).
SOD1 mutants at the pole of the β-barrel (L38V, G41S, D90A, G93A) or involving some loop residues (D76Y or E133A) had metal contents most comparable with WT SOD1 (Table I). Other mutants exhibited either partial copper deficiency (A4V) or partial zinc deficiency (G72S) compared with the WT enzyme expressed and isolated under similar conditions. The properties that all eight of these mutants shared were spectral properties indicative of a WT-like Cu(II) coordination geometry (Fig. 6) and full SOD activity of bound copper (Fig. 7).

Previous studies have documented the activities of some as-isolated ALS mutant SOD1 enzymes, but most have not correlated these results to the metal content of the proteins. The A4V mutant expressed in COS-1 cells (32) was ∼50% as active as WT SOD1, whereas the G85R mutant did not exhibit detectable activity in native gels, in agreement with our results when copper content is considered. The H46R mutant expressed in Escherichia coli contained <5% of the copper expected for the holoenzyme as determined by electron spin resonance (ESR) and exhibited only 1–2% of the catalytic activity of WT SOD1 (48). SOD1 purified from ALS patients homozygous for the D90A mutation exhibited nearly normal metal content and catalytic activity (33), whereas H46R and H48Q mutants expressed in COS-1 cells had activity below the limits of detection (49). These diverse results, obtained from different expression systems, agree with our present findings from the human enzymes isolated from insect cells.

Most mutant SOD1s with substitutions near the metal binding sites, with the notable exception of H48Q, contained only low amounts of both copper and zinc ions (Table I). These mutants (H46R, G85R, D124V, D125H, and S134N) also exhibited the most rapid mobility on native PAGE (Fig. 3F), presumably because metal deficiency rendered them more negatively charged or altered their conformation. Mutation of three residues (His-46, Gly-85, or Asp-124) that participate in an important hydrogen bond network closest to the active site (Fig. 1F) each resulted in the most severe deficiency of both copper and zinc (<10% of WT levels). The charged loop mutants D125H and S134N exhibited abnormally low catalytic activities despite correcting for their low copper contents (Fig. 7), suggesting that bound copper was not fully active. Because we saw no evidence of altered copper coordination for these mutants, the most likely explanation for the decreased activity was that O$_2^-$ steering or accessibility to the active site was perturbed.

ALS mutant SOD1 proteins that are remetallated in vitro can undergo structural rearrangements of the zinc binding site (15). In addition, a competitive chelation study suggests that four human ALS mutants (A4V, A4T, I113T, and L38V) expressed in E. coli exhibit an 18–30-fold decrease in zinc affinity compared with WT SOD1 (47). However, we observed that several of the biologically metallated ALS mutants, including A4V and L83V studied by Crow et al. (47), contained amounts of zinc comparable with that bound by WT enzyme (Fig. 5 and Table I). This implies that in the insect cell milieu, these mutant enzymes effectively competed for binding of available zinc ions and retained them during purification.

Both the highly conserved SOD1 protein structure and zinc binding are likely to impose constraints on copper accessibility or coordination geometry that influence its redox reactivity. Exposure to either WT or mutant SOD1 species loaded with copper but lacking zinc is toxic to motor neurons in vitro (42). However, it has not been established that these SOD1 species occur in vivo or are relevant to ALS (50). Two of our mutants, H48Q and G72S, fit this pattern in that they were moderately zinc-deficient yet contained as much copper as WT SOD1. However, the five mutants that were most severely zinc-deficient (H46R, G85R, D124V, D125H, and S134N) generally contained correspondingly low amounts of copper (Table I). Although zinc loss may be one mechanism that could perturb the reactivity of bound copper, neither selective zinc deficiency nor altered SOD activity was a common characteristic among our mutant SOD1 enzymes.

In this study, the H48Q mutant contained as much copper as WT SOD1 (Fig. 5), yet, as previously reported by Liochev et al. (51), exhibited only ∼1% of normal SOD activity (Fig. 7). In the crystal structure of the yeast H48C SOD1 mutant (29), the three remaining histidine ligands and a chloride ion bind copper equatorially in a square plane. This may greatly reduce SOD activity by impeding the conformational change associated with copper ion reduction during Reaction 1. Similarly, the yeast H48Q SOD1 mutant structure reveals copper ion binding directly to the remaining three histidines and indirectly to Gln-48 via a tightly associated water molecule to adopt a square planar orientation. 2 Our spectroscopic evidence of a shift in the visible absorption maximum to 610 rather than 680 nm (Fig. 6) suggests that this planar geometry of the Cu(II) center is also present in human H48Q SOD1. H48Q SOD1 thus stands out as unique among the familial ALS SOD1 mutants that we and others have characterized in its very low SOD activity despite substantial levels of copper and zinc, its blue-shifted visible absorption spectrum, and its ability to catalyze superoxide-driven peroxidative chemistry (51).

In summary, we did not observe a consistent abnormality in copper or zinc ion contents, visible absorption spectra, or specific activity of bound copper within this diverse set of biologically metallated SOD1 mutants. Recent studies imply that reduced copper loading into mutant SOD1 does not ameliorate its toxicity in transgenic mouse models (52). While these observations do not exclude potential catalytic mechanisms of toxicity, an important hypothesis that merits further scrutiny is that mutant SOD1 toxicity may be related to a primary disruption of SOD1 structural stability. These considerations have prompted us to undertake a direct investigation of the thermodynamic stability of ALS mutant SOD1 enzymes in an accompanying report (53).

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