Oxidation-induced Misfolding and Aggregation of Superoxide Dismutase and Its Implications for Amyotrophic Lateral Sclerosis*

The presence of intracellular aggregates that contain Cu/Zn superoxide dismutase (SOD1) in spinal cord motor neurons is a pathological hallmark of amyotrophic lateral sclerosis (ALS). Although SOD1 is abundant in all cells, its half-life in motor neurons far exceeds that in any other cell type. On the basis of the premise that the long half-life of the protein increases the potential for oxidative damage, we investigated the effects of oxidation on misfolding/aggregation of SOD1 and ALS-associated SOD1 mutants. Zinc-deficient wild-type SOD1 and SOD1 mutants were extremely prone to form visible aggregates upon oxidation as compared with wild-type holo-protein. Oxidation of select histidine residues that bind metals in the active site mediates SOD1 aggregation. Our results provide a plausible model to explain the accumulation of SOD1 aggregates in motor neurons affected in ALS.

ALS is a fatal neuromuscular disease that presents as weakness, spasticity, and muscle atrophy. The disease is caused by selective degeneration of motor neurons in the brain, brainstem, and spinal cord. Although ALS presents mostly as a sporadic disease, a familial form of ALS is seen in ~10% of cases. Twenty percent of familial ALS (FALS) cases are caused by point mutations in the SOD1 gene. More than 90 distinct amino acid mutations spread throughout the sequence of this 153-residue protein have been identified (1). The finding that many FALS-associated SOD1 mutants possess full specific enzyme activity (2) suggests that the disease is not caused by loss of normal dismutase activity. Further support for this idea has come from transgenic mouse studies. Transgenic mice that harbor FALS-associated SOD1 mutations develop ALS-like symptoms despite having greater than normal levels of SOD1 activity, including the normal complement of endogenous mouse SOD1 enzyme (3). Furthermore, SOD1 knockout mice do not develop ALS-like symptoms. Thus, it has been proposed that mutations in SOD1 cause FALS by a gain, rather than a loss, of function (reviewed in Ref. 1).

One proposed gain of function involves free radical generation by SOD1. Because the dismutase action of SOD1 runs in a reversible catalytic cycle with a number of different possible substrates (4–6), under some conditions, SOD1 may catalyze the reverse reaction and generate radical species. It has been proposed that certain FALS-associated SOD1 mutants have lower $K_m$ values for hydrogen peroxide in the reverse reaction and therefore possess greater free radical generating activity than do wild-type enzymes. This makeup ultimately allows a greater number of cytotoxic peroxydation reactions to occur in these mutants (4, 5).

The exact species responsible for oxidative damage, however, has recently come under question. Fridovich and co-worker (7) showed that the production of hydroxyl radicals would be negligible because of competition with bicarbonate ions for hydroxyl radicals bound to copper in SOD1.

Another possible gain of function implicates the formation of zinc-deficient enzyme as the common toxic entity derived from all mutants. One property shared by many FALS-associated SOD1 mutants is a decreased affinity for Zn$^{2+}$ (8, 9). It has been proposed that reduced Zn$^{2+}$ binding destabilizes the structure of SOD1, increasing the rate of abnormal reduction of bound Cu$^{2+}$ to Cu$^+$ by intracellular reducing agents. This reduced structure of SOD1 could then catalyze the reverse enzymatic reaction and become a net producer of superoxide anion. In the absence of a well defined protein fold, the electrostatic gradient that is normally present in SOD1 (10) does not exist to prevent diffusion of the resultant radical anion. Therefore, in the presence of nitric oxide, which reacts five times faster with superoxide than does SOD1 itself, zinc-deficient SOD1 becomes a net producer of peroxynitrite (11). Thus, the zinc-deficient SOD1 hypothesis maintains that peroxynitrite is the final mediator of oxidative neuronal injury and works by either nitrating and/or oxidizing critical cellular targets.

Active site copper plays a critical role in both of the proposed mechanisms for a gain of function of FALS-associated SOD1 mutants described above. A recent study that used transgenic mice that expressed FALS-associated SOD1 mutants but lacked the gene for the copper chaperone protein (CCS) investigated whether alterations in copper loading would affect disease pathology (12). CCS facilitates the incorporation of Cu$^{2+}$ into SOD1 in vivo (13, 14), and copper is essential for normal dismutase activity as well as for any gained functions that are oxidant-mediated. The transgenic study found that knocking out the CCS gene reduced copper incorporation into FALS-associated SOD1.
In the present study, we sought to elucidate physiologically relevant environmental factors that may trigger aggregation of SOD1 in motor neurons. SOD1 aggregates seen in ALS patients and transgenic mouse models are limited to neural tissue (motor neurons and, occasionally, neighboring astrocytes) and are not seen in other cell types. Given that SOD1 is present in high concentrations in all cells, an environmental factor must exist within motor neurons that induces aggregation specifically in this cell type. Two differences between SOD1 molecules in motor neurons and other cells are its long half-life and higher concentration. Concentration of SOD1 is greater in motor neurons than in other neurons and glial cells, and it is found not only in the cell body of motor neurons but also within axons and nerve termini (19). To reach the nerve termini, SOD1 is transported through the axon by using the slow component b of the anterograde axonal transport system (20), which has a rate of 2–8 mm/day. Thus, the transport time for motor neurons with a meter-long axon could approach 500 days, and the life span of the protein must exceed the transport time. The long life span of this protein increases the chances of oxidative modification by reactive oxygen species; one possible byproduct of oxidative modification is induction of protein aggregation. The greater life span of SOD1 in motor neurons means that it would have more opportunity to accumulate oxidative modifications and to be altered in ways that could increase its own propensity to aggregate. In support of this hypothesis, markers of oxidative damage were shown to be significantly elevated in neural tissue of ALS patients as compared with controls (21, 22). To explore the possibility that oxidation triggers SOD1 aggregation, we examined the effects of oxidation on fully metallated wild-type SOD1 (holo-SOD1), on zinc-deficient SOD1, and on four SOD1 mutants.

**EXPERIMENTAL PROCEDURES**

In Vitro Aggregation of SOD1—Wild-type Cu-Zn SOD1 from human erythrocytes was obtained from Sigma. Mutant and zinc-deficient SODs consisted of 10 mM SOD1, 4 mM ascorbic acid, and 0.2 mM CuCl2 in 10 mM Tris, 10 mM acetate buffer, whereas control reactions were 10 mM SOD1 in buffer. Reactions were incubated at 37°C for 48 h. The pH was 7.0 unless stated otherwise.

**Inhibition of in Vitro Aggregation**—To readily recognize inhibition of SOD1 aggregation, the most aggregation-prone SOD1 species (zinc-deficient SOD1) was used. SOD1 aggregation mixtures (10 mM SOD1, 4 mM ascorbic acid, 0.2 mM CuCl2, 10 mM Tris acetate, pH 7) were incubated with 2 mM EDTA, 10 mM mannitol, or 10 mM DMPO as probes for the reactive oxygen species. Anaerobic conditions were achieved by degas-

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**TABLE I**

Comparison of relative amino acid composition of oxidized to control SOD1

| Amino acid | Wild-type SOD1 | Zinc-deficient SOD1 |
|------------|----------------|---------------------|
| Aspartic acid | 1.00 | 1.01 |
| Threonine | 1.02 | 1.03 |
| Serine | 1.01 | 1.00 |
| Glutamic acid | 1.06 | 1.00 |
| Glycine | 1.00 | 1.03 |
| Valine | 1.00 | 1.03 |
| Isoleucine | 0.95 | 1.02 |
| Leucine | 0.99 | 0.95 |
| Phenylalanine | 0.94 | 0.94 |
| Histidine | 0.63 | 0.62 |
| Lysine | 0.95 | 0.84 |
| Arginine | 0.97 | 0.87 |
| Proline | 0.91 | 0.93 |
| Alanine | 1.07 | 1.00 |

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2 M. J. Strong, W. L. Strong, B. P. He, M. M. Sopper, and J. P. Crow, personal communication.
Congo Red Spectral Shift Assay—SOD1 aggregates were diluted to a final concentration of 3 μM (~100 μg/ml) and incubated with 6 μM Congo red for 30 min before measuring near-UV and visible absorbance.

Circular Dichroism (CD)—Zinc-deficient SOD1 aggregates were centrifuged for 5 min at 13,000 × g, and the supernatant was removed and replaced with 20 mM sodium phosphate buffer, pH 7.0. Aggregates were then resuspended by vortex and sonication before CD spectra were recorded on an Aviv CD spectrometer model 62 DS at 25 °C.

RESULTS AND DISCUSSION

Metal-catalyzed Oxidation of SOD1—We used metal-catalyzed oxidation with CuCl₂ and ascorbic acid to generate reactive oxygen species because of the physiological relevance of this system. Metal-catalyzed oxidation is the principal source of hydroxyl radicals under normal physiological conditions (23), and it is especially important under conditions of oxidative stress (24). The concentrations of ascorbic acid used in this study (2–4 mM) are well within the normal concentration range (0.5–10 mM) found in neurons and glial cells (25). We examined the effects of oxidation on three different ALS-associated mutants of SOD1: A4V, D90A, and G93A, as well as a site-directed mutant (D124N) that has decreased zinc-binding affinity (26) and serves as a model of zinc-deficient SOD1. A4V is the most common mutation that causes FALS, D90A causes a rare autosomal recessive form of FALS (1), and G93A is the mutant most widely used for the transgenic mouse model of ALS. We examined the effect of oxidation on the zinc-deficient form of wild-type SOD1, because this species has been implicated in neurotoxicity associated with ALS (11) and because it can use ascorbate to produce superoxide and hydrogen peroxide directly.

We found that at a neutral pH, oxidation of each of the three SOD1 mutants and zinc-deficient wild-type SOD1 induces the formation of large aggregates that scatter light (Fig. 1A). The zinc-deficient protein displayed the most robust aggregation reaction and, interestingly, D90A, the mutation that causes an autosomal recessive form of FALS, displayed the least amount of aggregate formation. Oxidation of wild-type SOD1 under identical conditions did not induce the formation of aggregates detectable by right-angle light scattering (i.e. visible aggregates >350 nm in diameter). With the exception of zinc-deficient SOD1, aggregates did not form in control samples that lacked oxidants. The small amount of aggregate observed in control samples of zinc-deficient SOD1 suggests that this form of the protein has an intrinsic aggregation tendency. The aggregation reaction displays distinct pH dependence, with reduced aggregation at pH < 5.5 (Fig. 1B). Similar pH dependence has been observed in the oxidation-induced aggregation of human relaxin, in which oxidation of a single His residue apparently accounts for the pH dependence (27). Performance of the oxidation reaction under anaerobic conditions or in the presence of EDTA inhibited aggregation and revealed that copper and oxygen are an absolute requirement for oxidation-induced aggregation (Fig. 1C). On the other hand, the addition of the free radical scavengers mannitol and DMPO did not inhibit aggregation (Fig. 1C). Similar results have been obtained with copper-catalyzed, oxidation-induced aggregation of both human relaxin (28) and hamster prion protein (29). The insensitivity to free radical scavengers and the pH dependence of the oxidation-induced aggregation are consistent with the

### Table II. Summary of mass spectroscopic analysis of tryptic fragments of oxidized SOD1

| Tryptic peptide sequence | Position | Theoretical mass [M + H]⁺ | Experimental mass | Modified amino acid |
|--------------------------|----------|---------------------------|-------------------|--------------------|
| HVGDGLGNVTADK            | 80–91    | 1225.6                    | 1225.6            | His80              |
| TLVVHEK                  | 116–122  | 825.5                     | 825.5             | His120 + His16     |
|                          |          | 841.5                     |                   |                    |

Fig. 2. Oxidative modification sites of SOD1 revealed by tryptic digestion and mass spectrometry. SOD (30 μM) was incubated with 2 mM ascorbate, 25 μM copper, 10 mM sodium acetate, pH 5.0, at 37 °C for 24 h. The protein was reduced and alkylated with dithiothreitol and iodoacetamide in 6 M guanidine hydrochloride and then digested with trypsin (25:1 substrate to enzyme ratio) at 38 °C for 50 h and analyzed by capillary LC-MS/MS. The ribbon diagram was created from the PDB coordinates 1SPD with use of the program PYMOL (Delano Scientific). Side chains of modified His residues (90 and 120) are orange, the copper ion is blue, and the zinc ion is gray.
site-specific metal-catalyzed oxidation mechanism. This mechanism requires a metal ion binding site that is in close spatial proximity to the modification sites (23). In this type of oxidation reaction, very few residues are modified.

**Characterization of Oxidative Modification Sites**—Amino acid analysis was performed on oxidized wild-type protein and on oxidized and aggregated zinc-deficient SOD1 (Table I). The most striking feature of the amino acid analysis of both types of oxidized protein was the loss of histidine residues. Amino acid analysis suggested that three of the eight histidine residues of the SOD1 subunit were modified. It is known that metal-catalyzed oxidation of proteins leads to conversion of histidine residues to 2-oxohistidine, 4-hydroxy-glutamate, aspartate, or asparagine (23). Because the glutamate and aspartate contents do not appear to be altered by oxidation, it is likely that histidines have been largely converted to 2-oxohistidines. Further support for the conversion to 2-oxohistidine was obtained by sequencing tryptic peptides of oxidized wild-type SOD1 by LC-MS/MS (Table II). The masses of two tryptic peptides were increased by 16 mass units, which is consistent with the formation of 2-oxohistidines. Peptide sequencing revealed that both His 80 and His 120 contain an additional 16 mass units; these residues are located at the zinc and copper binding sites, respectively, of SOD1 (Fig. 2).

**Morphology and Structure of SOD1 Aggregates**—The results presented here demonstrated that oxidation of select His residues induces misfolding and aggregation of SOD1. However, the question remains, do these in vitro aggregates represent aggregates seen in ALS? Examination of ALS inclusion bodies by light, electron, and immunoelectron microscopy have shown them to be a unique feature of ALS and distinct from the amyloid plaques and neurofibrillary tangles seen in Alzheimer’s disease and the intracellular deposits seen in Parkinson’s disease (30–32). In particular, ALS inclusion bodies are not stained by the amyloid dye Congo red (30). Instead, the inclusion bodies seen in COS7 cells that express ALS mutants of SOD1 (15), transgenic mouse models of ALS (3, 33), and ALS patients (34–37) are all composed of a mixture of granular aggregates and some thick fibers as compared with the thin fibrils seen in amyloid diseases (38).

Our atomic force microscopy examination of aggregates formed by oxidation of zinc-deficient SOD1 revealed large amorphous aggregates (<10 μm diameter) that were composed of smaller globular particles (0.2–0.5 μm diameter) (Fig. 3A). These heterogeneous aggregates were composed of amorphous aggregates along with fibrous aggregates that were 40 nm in diameter and several micrometers long (Fig. 3B). These fibrous aggregates are thicker than the amyloid fibrils formed by the Alzheimer amyloid peptide, which are 60–90 Å in diameter (38).

Dye binding experiments with thioflavin T and Congo red, as well as CD, were also used to determine whether the SOD1 aggregates possessed amyloid characteristics. A 2-fold enhancement of thioflavin T fluorescence was observed with the aggregates produced from zinc-deficient SOD1 (Fig. 4A); however, the fluorescence enhancement seen with amyloid fibrils is usually 3 orders of magnitude higher (39). On binding to Congo red, very little, if any, increase was seen in absorbance or spectral shift (Fig. 4B), which would have been expected had the aggregates in fact been amyloid (40). This lack of increase is in keeping with the failure of Congo red to bind SOD inclusion bodies in vivo (30).

The CD spectrum of SOD1 undergoes a large change on oxidation-induced aggregation (Fig. 4C). However, the CD spectrum of SOD1 aggregates indicates random coil rather than the characteristic β-sheet spectrum of amyloid. Thus, although it appears that oxidative damage of SOD1 results in misfolding and aggregation, the resultant aggregates do not appear to be amyloid. Indeed, the morphology of the aggregates observed in vitro in this study compare favorably with that of granular SOD1 inclusions observed in ALS models and patients.

**Structural Changes to SOD1 before Aggregation**—To determine whether susceptibility to oxidation-induced aggregation of zinc-deficient SOD1 and SOD1 mutants results from an altered conformation, ANS dye binding experiments were performed on untreated unoxidized protein samples. ANS binding is a probe of exposed hydrophobic surfaces in proteins. Zinc-deficient SOD1 bound the most ANS, wild-type SOD1 did not show any ANS binding, and the SOD1 mutants displayed vary-

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**Figure 3.** A, atomic force microscope height image of an aggregate formed by zinc-deficient SOD1. Aggregates are large and amorphous. Horizontal scale bar = 10 μm, vertical scale = 2 μm. Inset: close-up of protein aggregate shows that the aggregate is made up of smaller particles. Horizontal scale bar = 2 μm, vertical scale = 1 μm. B, transmission electron micrograph of SOD1 incubated in the presence of 25 μM copper and 2 mM ascorbate in 10 mM sodium acetate buffer, pH 5.0, for 48 h at 37 °C. Scale bar = 400 nm.
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intermediate ANS binding may result from heterogeneity in the metallation status of the mutants, in which mutant preparations that show the greatest ANS binding contain significant quantities of incompletely metallated protein, much of which could be zinc-deficient.

Concluding Remarks—We have shown that zinc-deficient SOD1, a site-directed mutant with low zinc binding affinity, low zinc content (D124N), and three FALS-associated SOD1 mutants, is much more susceptible to oxidation-induced aggregation than the fully metallated wild-type protein. These findings, coupled with the long half-life of SOD1 in motor neurons and the high levels of oxidative damage that are known to occur in neural tissues of ALS patients (21), provide a possible explanation for the SOD1 aggregates observed in ALS. Although it still remains to be established whether the SOD1 aggregates are intrinsically toxic, evidence is mounting that protein aggregates exhibit a general toxicity that is independent of the function of the protein in its native state (43). Our data are also consistent with the recent model put forward by Okado- Matlak et al. (15) to which are potential factors such as heat shock proteins are sequestered by abundant misfolded/aggregated proteins, such as SOD1 or other misfolded proteins induced by oxidation/nitration, leading to apoptosis.

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