Variation in the biochemical/biophysical properties of mutant superoxide dismutase 1 enzymes and the rate of disease progression in familial amyotrophic lateral sclerosis kindreds

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Mutations in superoxide dismutase 1 (SOD1) polypeptides cause a form of familial amyotrophic lateral sclerosis (FALS). In different kindreds, harboring different mutations, the duration of illness tends to be similar for a given mutation. For example, patients inheriting a substitution of valine for alanine at position four (A4V) average a 1.5 year life expectancy after the onset of symptoms, whereas patients harboring a substitution of arginine for histidine at position 46 (H46R) average an 18 year life expectancy after disease onset. Here, we examine a number of biochemical and biophysical properties of nine different FALS variants of SOD1 polypeptides, including enzymatic activity (which relates indirectly to the affinity of the enzyme for copper), polypeptide half-life, resistance to proteolytic degradation and solubility, in an effort to determine whether a specific property of these enzymes correlates with clinical progression. We find that although all the mutants tested appear to be soluble, the different mutants show a remarkable degree of variation with respect to activity, polypeptide half-life and resistance to proteolysis. However, these variables do not stratify in a manner that correlates with clinical progression. We conclude that the basis for the different life expectancies of patients in different kindreds of sod1-linked FALS may result from an as yet unidentified property of these mutant enzymes.

INTRODUCTION

Sporadic and familial amyotrophic lateral sclerosis (FALS) are characterized by paralysis, muscular atrophy, spasticity and a variety of other motor signs, all of which are caused by the dysfunction and death of large α-motor neurons of the brainstem and spinal cord (for a review, see refs 1,2). Although the etiology of the more common ‘sporadic’ form of ALS is not well defined, mutations in sod1 have been associated with a subset of inherited cases (3,4).

Superoxide dismutase 1 (SOD1) polypeptides form a homodimeric enzyme that catalyzes the detoxification of intracellular superoxide (5). In yeast, SOD1 enzyme also plays a role in maintaining cytosolic Cu2+ homeostasis (6). Although some disease-linked mutations may diminish these functions, data from several studies argue that most FALS mutations in sod1 cause disease by imparting toxic properties to the enzyme. For example, many FALS-associated mutations do not substantially reduce enzymatic activity in in vitro assays (7). Moreover, many mutant SOD1 enzymes can rescue the growth defects associated with deletion of endogenous yeast sod1 (sod1A yeast) (8). Although some FALS-linked mutations severely diminish polypeptide half-life in non-neuronal cells, other mutations have much less pronounced effects on protein stability and, moreover, these diminutions are superimposed on a very long-lived protein (9). Heterodimers of mutant and wild-type enzyme retain activity (7,9) and, even when expressed in excess, mutant subunits do not appear to alter the activity or stability of the wild-type subunits (9–12). Thus, although some FALS mutations may diminish the specific activity or longevity of SOD1 enzymes, the mutations tested thus far do not eliminate enzyme activity (8,13).

Studies in mice provide additional data to support the idea that loss of SOD1 enzymatic activity is not the cause of disease. For example, the targeted deletion of sod1 in mice does not lead to developmental defects or cause motor neuron disease (14). In contrast, mice expressing moderate to high levels of FALS mutant SOD1 polypeptides (G37R, G93A and G85R) show motor neuron loss, muscle atrophy, paralysis and death (10–12,15). In all cases, the levels of superoxide scavenging activity are not diminished (in G37R and G93A mice levels are greatly increased). High level expression of wild-type human (Hu) SOD1 polypeptides does not cause disease (10,11). Thus, a large body of evidence supports the view that FALS mutant

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SOD1 enzymes cause disease by acquiring properties that are particularly toxic to motor neurons.

In FALS kindreds with mutations in sod1, the age of onset of weakness varies greatly but the duration of illness appears to be characteristic for a particular mutation (16–18). For example, in patients inheriting the A4V substitution, the average life expectancy is 1.5 years after the onset of symptoms, whereas patients harboring the H46R substitution have an average life expectancy of 18 years after disease onset. In view of the evidence supporting the idea that FALS variants of SOD1 enzymes acquire toxic properties, the variations in the duration of illness in different kindreds might arise because each mutation imparts different degrees of toxicity to the mutant protein. In this setting, a long duration of illness would be associated with a lesser degree of toxicity. In the present study, we have examined a number of biophysical and biochemical properties of FALS mutant SOD1 enzymes to determine whether we could identify a feature of mutant SOD1 enzymes that correlates with the relative toxicity of the enzyme (as indicated by the duration of illness in sod1-linked FALS kindreds). Mutant enzymes, including mutations that eliminate crucial histidine residues that coordinate Cu²⁺ binding, were assayed for: (i) the ability to rescue the growth defects of yeast lacking endogenous SOD1 enzymes; (ii) polypeptide half-life in mammalian cells lines; (iii) resistance to proteolysis; and (iv) solubility. Our data indicate that the biochemical/biophysical properties of FALS mutant SOD1 enzymes vary significantly, but do not stratify in a manner that correlates with relative toxicity.

RESULTS

FALS-linked mutations at His46 and His48 dramatically reduce enzymatic activity

The histidine residues at positions 46 and 48 are two of the four histidines that participate in the coordinate binding of copper (19). Mutations at each of these histidines have been linked to FALS (20,21). Yeast lacking SOD1 enzymes (sod1Δ yeast) display a number of growth defects, including an inability to grow on medium lacking methionine and lysine in the presence of atmospheric levels of oxygen (22,23). To determine the impact of the FALS-linked mutations at His46 and His48 on enzyme activity, we transformed sod1Δ yeast with expression plasmids encoding wild-type, Gly37→Arg (G37R) mutant, His46→Arg (H46R) mutant and His48→Glu (H48Q) mutant Hu SOD1 enzyme. Transformed colonies were first grown in anaerobic conditions then plated onto medium lacking lysine and grown at 30°C for 3 days in the presence of atmospheric levels of oxygen (Fig. 1A). In contrast to yeast expressing wild-type and G37R Hu SOD1 enzymes, yeast expressing the two histidine mutants grew poorly. Importantly, immunoblot
analyses of yeast expressing the H46R and H48Q mutants revealed that these polypeptides accumulate to levels similar to wild-type Hu SOD1 enzyme (Fig. 1B). Yeast require as little as 2% of normal levels of SOD1 polypeptides to grow in air in the absence of methionine and lysine (13). Thus, these data suggest that the histidine mutants retain very little activity. Similarly, Liochev et al. (24) demonstrated that reconstituted H48Q SOD1, purified from recombinant Escherichia coli, retained little superoxide scavenging activity.

To assay for whether the H46R and H48Q mutants show activity in mammalian cells, we transiently transfected COS-1 cells with expression plasmids encoding these two mutants. In addition, we included in the analysis another interesting mutant that is C-terminally truncated as a result of a 2 bp insertion at codon 126 (25), resulting in a shift in the reading frame and termination three amino acids downstream from codon 126 (FS126). The levels of activity in cell extracts were assayed by gel assay (7). In contrast to wild-type enzyme and the G37R variant, none of the three mutants showed activity (Fig. 2A, lanes 5–7). Immunoblot analysis of cell extracts showed that mutant enzymes accumulate to levels comparable with wild-type Hu SOD1 enzymes (Fig. 2B, lanes 2–5); the FS126 mutant co-migrates with endogenous COS-1 polypeptide (Fig. 2B, lane 7). Thus, despite expression at relatively high levels, the H46R, H48Q and the FS126 mutants fail to show activity in assay gels.

The FALS mutation at Gly85 may reduce polypeptide affinity for copper

In examining the activity of FALS mutants expressed in yeast, we discovered that the G85R variant, which completely rescues the growth of sod1 null yeast (8,13) and shows activity in solution assays of yeast extracts (Fig. 3A), shows no activity in non-denaturing assay gels (Fig. 3B, lane 5). In comparison, the A4V and G37R FALS variants extracted from yeast (Fig. 3, lanes 3 and 4) both show activity. Because the G37R and G85R mutations create similar charge changes, we would expect these variants to show similar mobilities in these assay gels, but extracts from yeast transformed with G85R expression plasmids never showed activity in gel assays (Fig. 3B, lane 5). Although there are several possible explanations for these findings, one simple explanation is that during electrophoresis, the bound copper cofactor for the G85R variant is extracted. The positively charged copper ion would tend to migrate towards the negative pole, whereas the polypeptide is migrating into the gel towards the positive pole. Thus, it is possible that during migration, the G85R variant is inactivated by the extraction of the bound Cu²⁺ cofactor.

The H46R and H48Q FALS variants are long-lived, whereas the FS126 variant is very short-lived

To examine the relative polypeptide stabilities of these three mutants, mouse neuroblastoma N2a cells were transiently transfected with expression plasmids, metabolically radiolabeled and then chased for varied intervals before lysis and immunoprecipitation of Hu SOD1 polypeptides. The use of the mouse N2a cells allowed us specifically to immunoprecipitate...
the Hu wild-type and mutant SOD1 polypeptides with a Hu-specific SOD1 (H-SOD1) antisera that was raised against a synthetic peptide that is unique to Hu SOD1 (12). The half-lives of the H46R and H48Q variants were very similar to that of wild-type Hu SOD1 polypeptides (~24 h) (Fig. 4A), whereas the half-life of the FS126 mutant was estimated to be only 5 h (Fig. 4B).

**FALS mutant SOD1 polypeptides retain a high degree of protease resistance**

To assess the relative resistance of a variety of FALS mutants to proteolytic degradation, we transiently transfected COS-1 cells with expression plasmids, then lysed the cells in 0.1% NP-40 with freeze–thaw and digested the extracts with 10 µg/ml proteinase K for 30 min at 37°C as described in Materials and Methods. Wild-type and several of the FALS mutant SOD1 polypeptides (G37R, G93C and I113T) were very resistant to digestion by proteinase K (Fig. 5, upper panel). However, the A4V, G41D and G85R mutants were far less resistant to digestion. Notably, in a previous study, these mutants showed relatively short polypeptide half-lives (7). Coomassie blue staining of gels, containing one-half of the digested extracts, demonstrated that the majority of cellular proteins [as well as the bovine serum albumin (BSA) that was added as a marker] were degraded by the 1 mg/ml digest with proteinase K (Fig. 5, lower panel). Overall, our data suggest that the A4V, G41D and G85R mutations may cause significant changes in protein conformation that result in increased sensitivity to protease digestion, but the protease resistance of many FALS mutants is not severely altered by mutation.

**FALS variants of SOD1 polypeptides do not aggregate spontaneously into sedimentable structures**

Focal increases in SOD1 immunoreactivity have been observed in the motor neurons of both FALS patients and transgenic mice expressing the FALS SOD1 variants (12,26–29). In mice expressing the G85R variant, the cytoplasm of both motor neurons and astroglia of the spinal cord show SOD1-immunoreactive inclusions (12). Similar inclusions, although more rare, have been seen in the cell bodies and proximal axons of spinal motor neurons in mice expressing the G37R variant (11,26). Spinal cord sections of patients harboring sod1 alleles with the A4V mutation (28) and the FS126 mutation (27,29) show motor neurons filled with SOD1 immunoreactivity.

In view of these findings, we thought it possible that some of the ALS variants might be more prone to aggregate spontaneously. To test this possibility, we transiently transfected COS-1 cells with pEF-BOS expression plasmids, encoding both wild-type and FALS mutant Hu SOD1 polypeptides. Cells were lysed by freeze–thaw in 0.1% NP-40 and centrifuged at 10,000 g to remove nuclear material. This initial low-speed pellet fraction contained very little SOD1 polypeptide (data not shown). To determine whether the SOD1 protein in the low-speed supernatant is aggregated into sedimentable structures, the supernatants were centrifuged at 200,000 g for 30 min. Although a very small percentage of some of the mutants appeared to sediment, in all cases the vast majority of mutant protein was soluble (Fig. 6A). A similar analysis of SOD1 polypeptides in the spinal cords of mice expressing the G37R variant (lane 42), and showing clinical signs of illness, demonstrated that the majority of this mutant does not aggregate into sedimentable structures (Fig. 6B). In other disorders, such as the prion diseases and Alzheimer’s disease, prion proteins (30,31) and β-amyloid peptides (32–34) form sedimentable aggregates. Thus, it is not clear whether the clustering of SOD1 immunoreactivity that has been described in affected tissues of transgenic mice (11,12,26,27) and FALS patients represents a true aggregation.

**DISCUSSION**

Our data show that the biophysical/biochemical properties of FALS variants of SOD1 enzymes vary greatly. In examining the data (Table I), we find that no particular property (or properties) of the mutants correlates well with relative toxicity (as defined by the duration of disease in various kindreds). Disease
with duration of 4 years or less (A4V, H48Q, I113T and FS126) is associated with mutants that: (i) retain the ability to scavenge superoxide (A4V and I113T) or are inactive (H48Q); (ii) display short (A4V and FS126) or long (H48Q and I113T) polypeptide half-lives; and (iii) show low (A4V) or high (I113T) resistance to proteolytic digestion. All mutants tested showed no great propensity to aggregate spontaneously into sedimentable structures. Moreover, several mutants (G37R, G93C and I113T) displayed biochemical and biophysical properties that were remarkably similar to those of the wild-type enzyme. Among this cohort of mutants, the G37R variant is associated with a long duration of illness, whereas the I113T mutant is associated with a short duration (16). Thus, none of the biochemical/biophysical parameters of FALS mutant SOD1 enzymes that we have examined here appears to correlate with duration of illness.

Is the duration of illness in different sod1-linked kindreds an indicator of mutant enzyme toxicity?

Because the duration of illness within an sod1-linked FALS kindred appears to be remarkably consistent, it has been suggested that the duration of illness is an indicator of the relative toxicity of the mutant SOD1 enzymes (16). For example, the H46R and G37R variants are thought to be less toxic because patients inheriting these mutations show a slower rate of decline over a very protracted clinical course (average 16–18 years) (Table 1). In contrast, the A4V mutation is thought to be highly toxic because individuals inheriting this mutation uniformly show a very rapid decline over a 2 year period (Table 1). Notably, the average age of onset in the A4V kindred is 50 years, whereas individuals in G37R kindreds develop disease at an average age of 45 years (16). The only clinical feature that appears to vary significantly among kindreds is the duration of illness (16).

Studies in transgenic models of FALS provide further support for the notion that different mutants possess slightly different toxic properties. For example, the dying motor neurons of mice expressing high levels of the G37R and G93A mutants show predominantly vacuolar pathology that appears to result from the swelling of mitochondrial and endoplasmic reticulum membranes (11,35,36). In contrast, in mice expressing the G85R mutation,
motor neurons and astroglia of affected spinal cords contain inclusions that are immunoreactive for SOD1 polypeptides and ubiquitin; vacuolar pathology is absent in these animals (12,27). These comparisons are complicated by the fact that the level of transgene expression plays an important role in the manifestation of pathology: vacuolar pathology is much less prevalent in mice expressing low levels of G93A (37). Nevertheless, the absence of uniformity in the neuropathological findings from these mice is consistent with the notion that individual mutants possess slightly different properties.

One reservation, however, is that many FALS kindreds contain only a few affected individuals, raising the possibility that the duration of illness in different kindreds reflects the activity of modifying factors rather than the inherent toxicity of the enzyme. Arguing against this notion are the observations that the duration of illness in A4V individuals is uniformly short, that >53 affected individuals have been described and that this mutation has occurred independently in divergent kindreds (18). However, there is emerging evidence that other genes can modify the phenotype of sod1-linked FALS. In Swedish kindreds with the D90A mutation in sod1, the disease is inherited in an autosomal recessive manner and exhibits partial penetrance (38). In these recessive kindreds, no individual heterozygous for the D90A mutation develops disease. However, in unrelated families elsewhere in the world, the D90A mutation can be associated with a dominant inheritance pattern (39). Recent studies now show that the recessive kindreds with the D90A mutation all derive from a common founder (40). Additional mapping studies indicate that some of the dominant kindreds may be related to the recessive kindreds, with a crossover event ~2.5 cM from the sod1 locus distinguishing the recessive and dominant kindreds (40). One interpretation of these data is that in the recessive Swedish kindreds the activity or inactivity of a gene very near the sod1 locus modifies the phenotype of the D90A mutation. If this modifying locus is polymorphic, then some of the heterogeneity in the duration of illness among different sod1-linked FALS kindreds may be due to the action of a modifier locus near the sod1 locus. Whether all the variations among kindreds can be explained in full by the action of a single tightly linked modifier locus remains to be determined.

How do the mutant enzymes cause disease?

Two competing, but not mutually exclusive, hypotheses have emerged to explain the toxicity of FALS mutant SOD1 enzymes. One hypothesis suggests that the mutations may enhance the ability of the enzyme to react with \( \mathrm{H}_2\mathrm{O}_2 \) (41,42), leading to increased levels of hydroxyl radical and oxidative damage. Another suggests that the mutations may enhance the ability of the enzyme to catalyze nitration, leading to the modification/inactivation of protein targets (43–46). In both of these scenarios, the chemistry involved in generating toxic species depends upon the Cu\(^{2+}\) cofactor of the SOD1 enzyme. Thus, if these reactions are a major factor in mutant SOD1 enzyme toxicity, then one would anticipate that one important variable governing toxicity would be the inherent affinity of the enzyme for copper and the structure of the polypeptide backbone around the copper-binding site [a structure that better excludes \( \mathrm{H}_2\mathrm{O}_2 \) and/or peroxynitrite (\( \cdot\mathrm{ONOO}^{-}\)) would be less toxic].

Histidine residues 46 and 48 are two of four histidines that coordinate the Cu\(^{2+}\) cofactor (19), and in two kindreds of FALS these residues are mutated to arginine and glutamine, respectively (20,21). A previous study of the ability of the His46 mutant (H46R) to rescue E.coli lacking endogenous SOD1 enzymatic activity suggested that this mutant retains some level of normal activity (47). However, it has become clear recently that proteins, termed copper chaperones, are required to load SOD1 enzyme with its Cu\(^{2+}\) cofactor (48), and, thus, the data from E.coli may not be relevant, as bacterial and mammalian SOD1 enzymes utilize different metal cofactors (49,50). An examination of purified H46R enzyme suggested that this mutant can bind copper in vitro (51). However, our data here and elsewhere (13) demonstrate that in yeast, the H46R and H48Q variants retain little superoxide scavenging activity. The inability of the H46R and H48Q mutants to rescue yeast is not due to an incompatibility of the yeast copper chaperone for SOD1 enzyme (CCS; termed Lys7 in yeast) and the mutant Hu SOD1 enzyme, because the co-expression of human CCS with the H46R and H48Q mutants failed to rescue sod1 null yeast (V.C. Culotta, unpublished observation). Thus, in yeast, the H46R and H48Q mutants are either unable to acquire Cu, less able to hold onto the ion once loaded by the CCS or inactive despite the presence of bound Cu.

In a previous study, Corson et al. (13) examined whether the H46R and H48Q mutants can acquire Cu under conditions that might increase the availability of Cu in the cytosol. Many strains of yeast, including the ones used in the present study, contain multiple copies of Cu-metallothionein. When the H46R and H48Q mutants were expressed in a metallothionein null background, both mutants showed low levels of superoxide scavenging activity (13). One interpretation of these data is that, in the presence of proteins that compete for Cu\(^{2+}\), the H46R and H48Q mutants may be less able to hold onto the bound Cu once loaded by CCS. Similarly, our data, showing that the G85R variant loses activity during gel electrophoresis, are consistent with the idea that this mutant is also less able to hold on to the bound copper cofactor. Thus, a subset of FALS variants of SOD1 enzymes may possess a diminished affinity for Cu. Notably, the H46R variant is associated with a slowly progressing phenotype (Table I), whereas the H48Q variant is associated with a rapidly progressing phenotype (Table II). Collectively, these observations suggest that the relative toxicity of the enzyme may not stratify with the relative affinity of the mutant enzymes for Cu (indirectly assayed by enzymatic activity).

If Cu chemistry is not involved in the disease, then what are the alternatives? The prevalence of SOD1-immunoreactive inclusions in transgenic mice expressing FALS variants (11,12,26,27) and a subset of human sod1-linked FALS cases (12,27,28) has led to the suggestion that mutant enzymes may aggregate (12,27,52). Recent immunocytochemical studies of cells transfected with FALS variants of SOD1 suggest that mutant enzyme may form cytoplasmic aggregates when over-expressed in embryonic motor neurons (53) and that co-expression of heat shock protein 70 ameliorates both SOD1-mediated toxicity and SOD1 aggregation (54). However, we find no evidence that the mutants we tested rapidly aggregate into sedimentable structures in our cultured cell models. Similarly, we could not demonstrate sedimentable SOD1 polypeptides in extracts from G37R mice although it is possible that...
our extraction conditions did not fully solubilize aggregates that might have been present. In addition, in the G37R mice, we cannot exclude the possibility that most of the SOD1 extracted from spinal cord does not derive from motor neurons (26). In summary, it remains uncertain whether the SOD1 inclusion pathology that is prominent in some of the transgenic mice and some FALS patients (27) is a direct result of SOD1 aggregation or the consequence of a dysfunction in some other cell function.

Conclusions

In summary, we present data on the biochemical and biophysical properties of FALS mutant SOD1 enzymes. Our measures of enzymatic activity, polypeptide half-life, polypeptide resistance to proteolysis and enzyme affinity for Cu2+ all failed to identify a property of the mutants that correlates with the relative toxicity of the enzyme as defined by the duration of illness in sod1-linked FALS kindreds. Moreover, some mutants (G37R and I113T, for example) are nearly indistinguishable from wild-type enzyme in all of the properties we assessed. It is possible that the in vitro assays do not reflect accurately on the biology of the enzyme in vivo. Nevertheless, on the surface, these findings would suggest that the toxicity of mutant SOD1 enzymes may not arise from the corruption of one of the known facets of SOD1 enzyme biology but instead may be a consequence of the alteration of a heretofore unrecognized property or function of the protein.

MATERIALS AND METHODS

SOD1 expression plasmids and SOD1-specific antibodies

The expression plasmids for transfection of mammalian cells were generated with the pEF.BOS vector (55). The pEF.BOS.SOD1 plasmids encoding wild-type, A4V, G37R, G41D, G85R, G93C and I113T Hu SOD1 cDNA have been described previously (7). To generate the pEF.BOS plasmids encoding H46R and H48Q Hu SOD1 cDNA, we amplified, via PCR, segments of Hu SOD1 cDNA with primers that span residues 46 and 48, encoding single nucleotide mutations that result in a shift in coding from histidine to arginine at codon 46, or from histidine to glutamic acid at codon 48. The entire sequence of Hu SOD1 cDNA fragments generated by these methods was confirmed by nucleotide sequencing prior to the generation of expression plasmids. Mammalian expression plasmids were transfected into COS-1 and mouse neuroblastoma N2a cells as described previously (7,9).

The expression plasmids for the transfection of yeast were generated with a vector [pSM703 kindly provided by S. Michaelis (Department of Cell Biology, Johns Hopkins University, Baltimore, MD)] that utilizes a 2 µm plasmid and promoter sequences from the phosphoglycerate kinase (PGK1) gene of yeast (13). cDNAs encoding wild-type, A4V, G37R, G41D, G85R, H46R and H48Q Hu SOD1 were subcloned from pEF.BOS or pBluescript plasmids into the pSM703 vector (13). Yeast were transformed by lithium acetate and electroporation (56).

The SOD1 antibodies raised against synthetic peptides have been described previously and have been demonstrated to be specific for SOD1 polypeptides. The M/H SOD1 antibody was raised against a synthetic peptide that is identical in sequence in mouse and human SOD1 polypeptide (7,57). The H-SOD1 antibody, specific for human SOD1 polypeptides, was raised against a synthetic peptide that shows considerable differences in sequence between mouse and human SOD1 proteins and, therefore, distinguishes between human and mouse SOD1 polypeptides (12). In addition to these antibodies, we used an SOD1 antiserum that was raised against whole Hu SOD1 polypeptide (purified Hu SOD1 enzyme was obtained from Sigma, St Louis, MO).

Gel assay of SOD1 enzymatic activity

The activities of wild-type and mutant Hu SOD1 enzymes were examined by gel assay. Confluent 60 mm dishes of COS-1 cells were transfected with 5 µg of expression plasmid, following previously described procedures (58). After 48 h, the cells were lysed in TN (50 mM Tris–HCl pH 7.5, 150 mM NaCl) with 0.1% NP-40 (TP–NP-40) by three cycles of freeze (−20°C)–thaw. After centrifugation at 14 000 g for 10 min, 50–75% of the supernatant volume was mixed with an equal volume of loading buffer (0.125 M Tris–HCl pH 6.8, 20% glycerol, 0.025% bromophenol blue and 0.1% NP-40), loaded onto 10% polyacrylamide assay gels (7) and electrophoresed until the bromophenol blue reached the bottom of the gel. The activity of the enzymes was then assayed in these gels as described previously (59).

To determine the relative amounts of SOD1 polypeptides in extracts from transfected cells, a portion of the extract (1/10) prepared for gel assay was analyzed by SDS–PAGE on 15% polyacrylamide gels then immunoblotted with H-SOD1 antiserum (1:2500 dilution). Bound SOD1 antibodies were visualized by incubation with protein A coupled to horseradish peroxidase (HRP; Sigma) and chemiluminescence reagents (Pierce, Rockford, IL).

Metabolic radiolabeling and immunoprecipitation

Confluent 60 mm dishes of mouse neuroblastoma N2a cells were transfected with 5 µg of expression plasmid as described above. After 24 h, each dish was trypsinized and split into three 30 mm wells. After 24 h, the cells were incubated in cysteine-free medium (with 2% dialyzed fetal calf serum) for 1 h, then labeled with cysteine-free medium containing 300 µCi/ml of [35S]cysteine. After 30 min, the cells were washed with phosphate-buffered saline (PBS) then incubated (chased) in standard cell culture medium for varied intervals before extraction in TNE (50 mM Tris–HCl pH 7.5; 150 mM NaCl, 5 mM EDTA) with 0.5% NP-40 and 0.5% deoxycholate. The cell extracts were centrifuged at 14 000 g for 10 min. The supernatants were collected and mixed with SDS (final 0.2%) then boiled. After 10 min, the extracts were centrifuged for 10 min at 14 000 g. The supernatants were collected and mixed with SDS (final 0.2%) then boiled. After 10 min, the pellets were collected and mixed with SDS (final 0.2%) then boiled. After 10 min, the pellets were centrifuged at 14 000 g for 5 min. The supernatants were centrifuged for 5 min. The pellets were transferred to a clean microcentrifuge tube, washed twice more in IP buffer, then dispersed in 1× Laemmlli buffer, boiled for 10 min and separated by
SDS–PAGE on 15% gels. After fixation in methanol/acetic acid (30/10%), the gels were soaked in Amplify (Amersham, Arlington Heights, IL), dried and exposed to film or PhosphorImager plates (Molecular Dynamics, Sunnyvale, CA).

**Sedimentation assays**

Confluent 60 mm dishes of COS1 cells were transfected with 5 µg of expression plasmid as described above. After 48 h, the cells were lifted from the culture dish by incubation in PBS with 5 mM EDTA. The suspended cells were then pelleted at 3000 g for 5 min, then resuspended in 100 µl of TN–NP-40 and lysed by two cycles of freeze–(–20°C)–thaw. A 50 µl aliquot of the lysate was then centrifuged at 10 000 g. Both the pellet and the supernatant were collected. The supernatant was then centrifuged in a Beckman Airfuge for 30 min at 200 000 g, and both the pellet and the supernatant were collected. The pellets were resuspended individually in 50 µl of TN–NP-40. The collected supernatants and the resuspended pellets were all mixed with an equal volume of 2 x sample buffer and boiled for 10 min before SDS–PAGE (20 µl of total volume per lane) and immunoblot analysis with M/H SOD1 antisemur and 125I-labeled protein A (or anti-mouse IgG coupled to HRP and chemiluminescence).

The solubility of SOD1 polypeptides in tissues harvested from transgenic mice was assessed via a similar protocol. Freshly harvested tissues were dispersed in TN–NP-40 buffer by homogenization in a small glass dounce. After two freeze–thaw cycles, the homogenates were fractionated by centrifugation as described above.

**Proteasease K-sensitivity assays**

Confluent 60 mm dishes of COS-1 cells were transiently transfected with 5 µg of pEF.BOS-SOD1 vectors as described above. After 48 h, each dish of cells was lysed in 100 µl of TN–NP-40 and subjected to three freeze–thaw cycles as described above. The lysates were then centrifuged at 14 000 g for 10 min before collecting the supernatant. Portions of 10 µl from each extract were then supplemented with 5 µg of BSA, to provide a uniform protein marker to determine the extent of proteolysis, and digested with proteinase K (10 µg/ml and 1 mg/ml; Boehringer Mannheim, Indianapolis, IN) in a total volume of 20 µl of TN–NP-40. After 30 min at 37°C, the digests were terminated by the addition of phenylmethylsulfonyl fluoride (Sigma) to a final concentration of 5 mM. Following the addition of an equal volume of 2x Laemmlil sample buffer, each digest was boiled for 10 min, divided into two aliquots, and each was separated by SDS–PAGE. One gel was immunoblotted with the M/H SOD1 peptide antisemur and 125I-labeled protein A and the other gel was stained with Coomassic blue. The amount of SOD1 polypeptides remaining after proteinase K digestion was quantified by PhosphorImaging.

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