Mechanisms of Biosynthesis of Mammalian Copper/Zinc Superoxide Dismutase

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Abstract

Copper/zinc superoxide dismutase (SOD1) is an abundant intracellular enzyme with an essential role in antioxidant defense. The activity of SOD1 is dependent upon the presence of a bound copper ion incorporated by the copper chaperone for superoxide dismutase, CCS. To elucidate the cell biological mechanisms of this process, SOD1 synthesis and turnover were examined following $^{64}$Cu metabolic labeling of fibroblasts derived from $CCS^{+/+}$ and $CCS^{-/-}$ embryos. The data indicate that copper is rapidly incorporated into both newly synthesized SOD1 and preformed SOD1 apoprotein, that each process is dependent upon CCS and that once incorporated, copper is unavailable for cellular exchange. The abundance of apoSOD1 is inversely proportional to the intracellular copper content and immunoblot and gel filtration analysis indicate that this apoprotein exists as a homodimer that is distinguishable from SOD1. Despite these distinct differences, the abundance and half-life of SOD1 is equivalent in $CCS^{+/+}$ and $CCS^{-/-}$ fibroblasts, indicating that neither CCS nor copper incorporation has any essential role in the stability or turnover of SOD1 in vivo. Taken together, these data provide a cell biological model of SOD1 biosynthesis that is consistent with the concept of limited intracellular copper availability and indicate that the metallochaperone CCS is a critical determinant of SOD1 activity in mammalian cells. These kinetic and biochemical findings also provide an important framework for understanding the role of mutant SOD1 in the pathogenesis of familial amyotrophic lateral sclerosis.
**Introduction**

Copper/zinc superoxide dismutase\(^1\) (SOD1) is a ubiquitously expressed, homodimeric intracellular enzyme essential for antioxidant defense (1,2). SOD1 catalyzes the disproportionation of superoxide into oxygen and hydrogen peroxide and this reaction is dependent upon a single bound copper ion present in each monomeric subunit. Previous studies in yeast have revealed that under physiological circumstances intracellular copper availability is extraordinarily restricted (3). As a result, the delivery of copper to specific pathways within the cell is mediated by a family of proteins termed metallochaperones that function to provide copper directly to target pathways while protecting this metal from intracellular scavenging (4-6). Consistent with this concept, recent studies have revealed that the incorporation of copper into SOD1 in yeast and mammals is mediated by a metallochaperone termed the copper chaperone for superoxide dismutase (CCS) (7,8).

Biochemical and structural studies indicate that the process of copper incorporation into SOD1 is accomplished via direct protein-protein interaction involving a CCS-SOD1 heterodimeric intermediate (9-17). Despite these findings, the precise cell biological mechanisms of CCS function in intracellular copper homeostasis remain unknown. Although the strong affinity of the SOD1 dimer would suggest that CCS inserts the metal co-translationally or immediately following de novo SOD1 synthesis, studies in yeast demonstrate that CCS can insert copper post-translationally into SOD1 *in vivo*, suggesting that pre-existing apo dimers of SOD1 exist within the cell and may serve as a target for CCS (12). Furthermore, there is currently no understanding of how CCS obtains copper within the cell, if there is any intermediate copper pool and once incorporated into SOD1 if this copper is available for
exchange. In this current study a cellular model of CCS deficiency has been utilized to address these questions and to elucidate the cell biological mechanisms of CCS function.
Experimental Procedures

Materials. General chemicals and reagents were purchased from Sigma. Protein A beads were purchased from Repligen. DNA restriction and modifying enzymes were purchased from Promega and used according to the manufacturer's specifications. Hybridization membranes and ECL reagents were purchased from Amersham Biosciences. Polyclonal rabbit antisera to human and rat SOD1 were purchased from Stressgen. Additional antibodies to human SOD1 and CCS were prepared and used as described previously (9). To generate additional antisera to human CCS, an amino acid peptide corresponding to the carboxyl terminal forty amino acids of human CCS was synthesized and used to produce polyclonal antisera in rabbits (Alpha Diagnostics International, San Antonio, TX). Experiments were performed with antisera that was affinity purified against the CCS peptide coupled to cyanogen bromide activated Sepharose 4B column. \(^{[35\text{S}]}\)Methionine and \(^{[35\text{S}]}\)Cysteine Translabel and \(^{[35\text{S}]}\)Cysteine were purchased from ICN Radiochemicals. \(^{64}\text{Cu}\) (750 Ci/mmol) was obtained by fast neutron bombardment of a natural zinc target and used as described previously (8).

Cell Culture and Metabolic Labeling. HeLa and HepG2 cells were obtained from the American Type Culture Collection and cultured in basal growth media composed of DMEM with 10% bovine serum as described (9). CCS\(^{-/-}\) and SOD\(^{-/-}\) mice were obtained utilizing transgenic methodology as previously described (8,18). For some cell culture experiments, CCS\(^{+/+}\) and CCS\(^{-/-}\) mouse embryonic fibroblasts (MEFs) from E12.5 embryos were isolated and cultured in basal media. After the first passage these MEFs were genotyped by PCR analysis as described (8). Freshly isolated MEFs from SOD\(^{+/+}\) and SOD\(^{-/-}\) E12.5 embryos were isolated and allowed to adhere to tissue culture plates for four hours, then used immediately for metabolic experiments. Cells were pulse-labeled for 3 hours with 20 µCi/ml \(^{35\text{S}}\)cysteine and chased with serum-free
medium for the indicated time points, followed by collection of media and lysate for immunoprecipitation as described previously (9). Protein concentration was then determined by bicinchoninic acid assay (Sigma). Samples were subjected to 15% SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by PhosphoImager (Molecular Dynamics) or exposure to Eastman Kodak Co. MR film. Signal intensity was quantified with Un-scan-it imaging software (Silk Scientific Corporation). For copper labeling, cells were incubated in Optimem media containing 100 µCi/mL $^{64}$Cu for 3 hours, and chased with serum-free medium for the indicated time points, followed by collection of media and lysate as described previously (19,20). Samples were prepared in Laemmli sample buffer with or without reducing agents as indicated, subjected to electrophoresis in 4-20% Tris-HCl polyacrylamide gels, and gels exposed directly to a PhosphoImager. $^{64}$Cu signal intensity was quantified as noted above. In some experiments, to maximize copper incorporation into SOD1, cells were preincubated for 48 hours with 50 µM of the copper chelator tetraethylenepentamine (TEPA), followed by extensive washing in phosphate-buffered saline (PBS) prior to the addition of $^{64}$Cu as described previously (20). Counts per minute (cpm) of $^{64}$Cu per µg cell lysate were measured using a Cobra-II Auto-Gamma counter (Packard). Experiments utilizing $^{64}$Cu incorporation in mice were carried out as previously described (8). In studies examining $^{64}$Cu-SOD1 turnover, samples were stored and counted at the same time to allow for the half-life of the isotope.

**Immunoblot Analysis and SOD Activity Gels.** Cell lysates were prepared in 50 mM HEPES, pH 7.4/0.1% Nonidet P-40 (NP-40)/150 mM NaCl supplemented with protease inhibitor cocktail (Calbiochem Corp.) on ice for 15 min, followed by centrifugation for 10 min at 6,000 X g at 4°C. Protein concentration for all samples was determined by Bradford’s method (BioRad). For specified experiments, lysates were heated at 100°C for 10 min in the presence of SDS sample
buffer containing β-mercaptoethanol (β−ME) and centrifuged for 5 min at 16,000 X g at 4°C before loading the supernatant onto SDS-PAGE (9). For specific experiments, immunoblot analysis was performed following non-denaturing electrophoresis of lysates in Criterion 4-20% Tris-HCl polyacrylamide gels, transfer to Hybond ECL nitrocellulose membranes (Amersham Pharmacia), followed by UV crosslinking, boiling membranes in 2% SDS/50 mM Tris, pH 7.6 for 10 minutes and extensive washing in PBS. In all cases immunoblot analysis was carried using either the SuperSignal West Pico or West Femto Chemiluminescence kits (Pierce) with goat anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibody (Pierce). SOD activity gels were performed by electrophoresing lysates on SDS-PAGE gels followed by washing in deionized water for 1 hour and then processing for SOD activity as previously described (8).

**Gel filtration chromatography.** Gel filtration chromatography was performed at 4 °C with an ÄKTA FPLC system and a Superose 12 column (Amersham Pharmacia) equilibrated with PBS. Protein standards were used to calibrate molecular mass to elution volume. 5 mg of liver lysates from CCS+/+, CCS−/−, SOD+/+ and SOD−/− mice were prepared in 250 µL of PBS, applied to the column and then eluted with PBS in 0.5 mL fractions at a flow rate of 0.5 mL/min. Individual fractions were monitored for the presence of specific proteins by immunoblot analysis as indicated above.

**Guanidine denaturation.** 10 µg of purified human SOD1 (Sigma) was resuspended in PBS with 6 M guanidine at 4 °C for increasing incubation times from 0 to 9 hours (20). Protein samples were then precipitated with 2 volumes of ice-cold 100% EtOH by incubation at −80 °C for 10 min and centrifugation at 10,000 g for 5 min at 4 °C. Pellets were then washed with 2 volumes of
ice-cold 80% EtOH, dried and resuspended in PBS followed by SDS-PAGE under non-denaturing conditions and detection of protein with Coomassie blue dye.
Results

To analyze copper incorporation into SOD1, $^{64}$Cu was injected into SOD1$^{+/+}$ and SOD1$^{-/-}$ mice followed by non-denaturing, non-reducing PAGE of tissue lysates. A single $^{64}$Cu-labeled band was observed in lysates from SOD1$^{+/+}$ mice that co-migrated with SOD1 dismutase activity and immunoreactivity (Fig. 1A lanes 1,3,5) and was absent in identical samples from SOD1$^{-/-}$ mice (Fig. 1A lanes 2,4,6). These data confirmed that the labeled species observed under these conditions is murine SOD1, thus permitting an analysis of the kinetics of copper incorporation into this protein. To determine these kinetics, immortalized mouse embryonic fibroblasts derived from wild-type CCS$^{+/+}$ mice were pulse-labeled with $^{64}$Cu for five minutes, chased in media with 100-fold excess non-radioactive copper for the times indicated and cell lysates examined as above. As can be seen in Fig. 1B, $^{64}$Cu-SOD1 was observed at the earliest chase time and the abundance of this protein did not exchange over the chase period. Similar experiments in human HepG2 cells also identified a $^{64}$Cu-labeled band co-migrating with SOD1 dismutase activity and immunoreactivity (Fig. 1C lanes 1,2) that was eliminated by pre-incubation of lysates with SOD1-specific antisera (data not shown) and pulse-chase analysis of copper incorporation into SOD1 in these cells revealed identical kinetics to that observed in murine fibroblasts (Fig. 1C, lanes 3-6). In some experiments, cells were preincubated for 48 hours with 50 µM of the copper chelator tetraethylenepentamine (TEPA) prior to incubation with $^{64}$Cu. Although pretreatment with TEPA increased the amount of copper incorporated into SOD1, in all cases the results of pulse and pulse-chase experiments were identical regardless of prior chelation (data not shown).

These kinetic data reveal that copper is almost immediately incorporated into SOD1 and that no exchange of this metal occurs in this initial chase period. To determine if incorporated copper is available for exchange into other proteins over time, the half-life of SOD1 was determined
following metabolic labeling of CCS\textsuperscript{+/+} fibroblasts with \([^{35}\text{S}]\text{cysteine or }^{64}\text{Cu}.\) This analysis revealed similar half-lives for SOD1 labeled either at the protein backbone or copper site (half-lives of 38.7 \(\pm\) 0.6 and 34.2 \(\pm\) 4.5 hours respectively), indicating that once incorporated into SOD1, copper is unavailable for exchange, remaining with SOD1 until this protein is degraded (Fig. 1D). Identical results were obtained when these experiments were repeated in other mammalian cell lines (data not shown).

The kinetic data indicate that copper is rapidly incorporated into SOD1 without prolonged equilibration in a cytosolic pool and that no exchange of this metal from SOD1 occurs following this process. To determine the mechanisms of copper incorporation, cell lines were treated with cycloheximide prior to pulse labeling with \(^{64}\text{Cu}.\) Under conditions that reduced total protein synthesis to greater than 95\%, \(^{64}\text{Cu}\) incorporation into SOD1 was reduced (Fig. 2A). As anticipated from the half-life of SOD1, this treatment was without effect on steady-state SOD1 activity or total SOD1 protein (Fig. 2A). \(^{64}\text{Cu}\) incorporation into newly synthesized SOD1 was undetectable in immortalized mouse embryonic fibroblasts derived from CCS\textsuperscript{-/-} mice regardless of cycloheximide treatment, consistent with the essential role of this chaperone in copper incorporation into SOD1 (Fig. 2A). Human and murine SOD1 migrate at different rates under these conditions, accounting for the apparent size difference (Fig. 2A). Quantitative analysis revealed that copper incorporation into SOD1 was consistently reduced to about 50\% of normal following abrogation of new protein synthesis with cycloheximide (Fig. 2B). In all cases this effect of cycloheximide was reversible and without effect on either the initial uptake of copper or the time required for detectable \(^{64}\text{Cu}\) incorporation into SOD1 (data not shown).

The cycloheximide data reveal that new protein synthesis is required for copper incorporation into a significant portion of cellular SOD1 and that this process is dependent upon CCS. As the
half-life of this copper chaperone is greater than 19 h (Bartnikas and Gitlin, unpublished observations) and this protein is the only critical factor for copper incorporation under these conditions, the cycloheximide-sensitive process must represent copper incorporation into newly synthesized SOD1, with the cycloheximide-resistant pool representing preformed apoSOD1. To directly examine this possibility, these experiments were repeated in cells preincubated with TEPA to render the SOD1 pool entirely inactive apoSOD1, as indicated by the absence of SOD1 activity (Fig. 3A). Cycloheximide had no detectable effect on $^{64}$Cu incorporation into SOD1 revealing that under these conditions copper is preferentially incorporated into the preformed apoSOD1 pool (Fig. 3A,B). To further test this concept, CCS$^{+/+}$ fibroblasts were preincubated with excess copper, followed by pulse labeling with $^{64}$Cu in the absence or presence of cycloheximide. Quantitative analysis revealed that $^{64}$Cu incorporation into SOD1 is linear with respect to the length of the pulse time in untreated cells but not in cycloheximide-treated cells, consistent with the concept that copper incorporation in occurs into both newly synthesized SOD1 and an intracellular pool of apoSOD1 (Fig. 3C).

The above data suggest that at steady state a portion of cellular SOD1 consists of preformed apoprotein. To further address this issue, CCS$^{+/+}$ and CCS$^{-/-}$ mice were injected with $^{64}$Cu and tissue lysates analyzed for $^{64}$Cu-SOD1 (Fig. 4A lanes 1,2), SOD1 oxidase activity (Fig. 4A lanes 3,4) and SOD1 protein (Fig. 4A lanes 5,6) following nonreducing, nondenaturing PAGE. When SOD1 was denatured in situ following transfer to nitrocellulose, two distinct SOD1 immunoreactive bands were identified in liver lysates from CCS$^{+/+}$ mice, while only the more rapidly migrating band was observed in identical lysates from CCS$^{-/-}$ animals (Fig. 4A, lanes 5,6). The upper band migrates in the identical position as enzymatically active, $^{64}$Cu-SOD1 while the lower band is only observed under conditions where SOD1 is copper-free and enzymatically
inactive, suggesting that these two SOD1 species correspond to holo and apo SOD1 respectively. Consistent with this concept, equivalent amounts of SOD1 were detected in liver lysates from CCS\(^+/+\) and CCS\(^{-/-}\) mice when analyzed by reducing SDS-PAGE (Fig. 4A, lanes 5,6, lower panel marked SOD1). Furthermore, identical results were observed in all tissues from these mice and both bands were eliminated by prior incubation with multiple different antisera specific to SOD1 (data not shown). Similar findings were observed in HepG2 cells following pretreatment with TEPA, where copper chelation resulted in abrogation of SOD1 activity and resultant conversion of the immunodetectable SOD1 under nondenaturating conditions to the more rapidly migrating band corresponding to apoSOD1 (Fig. 4B). As was observed in the murine experiments above, the amount of SOD1 in these cells was equivalent when examined by SDS-PAGE (Fig. 4B), supporting the concept that the differences in SOD1 mobility and abundance observed under nondenaturing conditions reflect conformational changes related to copper incorporation into this protein.

To determine the reason for this observed difference in electrophoretic mobility of holo and apoSOD1, purified human SOD1 was treated with 6M guanidine followed by analysis for SOD1 dismutase activity, total SOD1 protein and holo and apoSOD1 as above. Initially, purified SOD1 existed in equilibrium between these two species (Fig. 4C, lane 1), with holo and apoSOD1 migrating in the identical position as observed in HepG2 cells following copper chelation (Fig. 4C, lanes 5,6). Over time, guanidine denaturation progressively decreased the abundance of holoSOD1 with a concomitant increase in apoSOD1 associated with the progressive loss of oxidase activity (Fig. 4C, lanes 2-4). Although these methods allow for the detection of apo and holoSOD1, it is important to note that the signal observed following denaturation SOD1 in situ is not quantitative. This is apparent when examining the differences in the amounts of apo and
holoSOD1 detected with this technique versus total SOD1 determined following reducing, SDS-PAGE (Fig. 4B). Such differences likely reflect incomplete denaturation with the in situ technique as the SOD1 antisera does not detect native SOD1. Nevertheless, the methodology clearly demonstrates the presence of both apo and holoSOD1 in the tissue and cell lysates and reveals that the abundance of each species is directly related to the copper content. Importantly, in these experiments, as with the $^{64}$Cu studies, the terms apo and holoSOD1 refer only to the observations on copper incorporation, as zinc metallation is not able to be examined under these circumstances. Nevertheless, the requirement of zinc for SOD1 activity suggests that the holoSOD1 observed here represents SOD1 with both copper and zinc.

These findings suggest that under steady state conditions SOD1 exists in the cell as both apo and holoprotein and that the equilibrium of these two species is directly dependent upon the availability of intracellular copper provided by the metallochaperone CCS. Although numerous in vitro studies have revealed that SOD1 exists as a stable homodimer, it is uncertain if this is the case in vivo for both the holo and apo forms and it is unclear which species is the target for interaction with CCS. To directly examine these questions, the molecular mass of SOD1 was determined by gel filtration chromatography of liver lysates from CCS$^{+/+}$ and CCS$^{-/-}$ mice. As can be seen in Fig. 5A, this analysis revealed that SOD1 exists as a homodimer in both CCS$^{+/+}$ and CCS$^{-/-}$ liver lysates with an elution profile similar to that for purified erythrocyte human SOD1. Although immunoblot analysis following SDS-PAGE revealed that SOD1 eluted in equivalent amounts and in the identical fractions in both samples (Fig. 5A), the fractions from CCS$^{-/-}$ lysates was devoid of SOD1 oxidase and contained only apoSOD1 when analyzed following nondenaturing, nonreducing PAGE (data not shown), confirming that apoSOD1 from these cells had not acquired copper during the chromatography. As SOD1 was not detected in
any other elution fractions, these findings indicate that the conformational differences in holo and apoSOD1 observed above are not sufficient to result in separation of these two species during chromatography. Therefore, the identical elution pattern of SOD1 from CCS$^{+/+}$ and CCS$^{-/-}$ liver lysates, taken together with the $^{64}$Cu incorporation data (Fig. 2A), indicates that holo and apoSOD1 must exist as homodimers in vivo. The abundance of SOD1 appeared identical in liver lysates from CCS$^{+/+}$ and CCS$^{-/-}$ mice and consistent with this, pulse-chase analysis in embryonic fibroblasts derived from these animals revealed equivalent half-lives of SOD1, indicating that neither CCS nor copper incorporation has an essential role in the stability or turnover of SOD1 in vivo (Fig. 5B).
Discussion

The data in this study reveal a striking rapidity to the kinetics of copper incorporation into SOD1. Indeed, the time course of the pulse and pulse-chase experiments indicate that, following uptake, copper is immediately delivered to SOD1 with no equilibration into an intermediate pool of free copper (Fig. 1 A,B) and that this is entirely dependent upon the copper chaperone CCS (Fig. 2 A). Taken together, these findings suggest that a pool of free copper ions is not used in the physiological activation of cuproenzymes in mammalian cells. The requirement for CCS in this process supports the hypothesis that copper chaperones function to protect copper from intracellular scavenging while ensuring that this metal is available for specific intracellular proteins (3-6). Although the mechanisms of copper delivery to CCS are not known, recent studies indicate that high affinity copper uptake requires copper-stimulated endocytosis of plasma membrane Ctr1 (21). Given the kinetics observed in this current study, these observations suggest the possibility for direct interaction of Ctr1 and CCS with copper transfer within this endocytosed compartment.

The results shown here also indicate that, once incorporated into SOD1, copper is unavailable for cellular exchange, turning over with a half-life identical to that for the protein (Fig. 1D). These data are consistent with previous observations on ceruloplasmin (20) and suggest no role for SOD1 in the specific delivery or trafficking of copper to other intracellular sites or tissues. Intriguingly however, the data do reveal that despite the continuous incorporation of copper into nascent SOD1 protein, a significant portion of SOD1 exists as apoSOD1 that can be rapidly converted to holoprotein (Fig. 2). Given the abundance of this intracellular protein, the finding that this incorporated copper is not exchangeable raises the possibility that this apoSOD1 pool may serve in a buffering capacity in mammalian cells during periods of environmental or genetic.
copper toxicity. Indeed, previous studies in *Saccharomyces cerevisiae* have suggested that SOD1 may have such a function in the homeostasis of copper, as deletion of SOD1 in this organism confers an increased sensitivity toward this metal that is unrelated to superoxide dismutase activity (22). While it is unclear if copper is reutilized following SOD1 turnover or trafficked directly to pathways for cellular efflux, it is apparent that incorporated copper has no effect on the abundance or half-life of SOD1 (Fig. 5 A,B). Taken together with recent findings indicating that the abundance of CCS is inversely proportional to nutritional copper status (23), these observations indicate regulation at the level of copper delivery to SOD1, rather than following protein turnover, a finding consistent with a role for SOD1 in copper homeostasis under situations of copper overload.

The rapid and specific incorporation of copper into SOD1 observed in this study suggested that copper may activate a preexisting pool of apoSOD and the biochemical data directly demonstrate this pool, revealing that the abundance of apoSOD1 is inversely proportional to the intracellular copper content (Fig. 4). These findings are consistent with previous observations in copper-deficient rats and differentiating K562 cells that implied the presence of such an apoSOD1 pool (24,25) and with recent data indicating that CCS can activate SOD1 in yeast in the absence of new protein synthesis (12). Nevertheless, direct assessment of copper incorporation into SOD1 indicates that under copper replete conditions approximately 50% of newly arriving copper is incorporated into nascent SOD1 (Fig. 2) while under circumstances of copper deficiency copper is preferentially incorporated into the apoSOD1 pool (Fig. 3). These observations are consistent with previous work in human lymphocytes revealing that the incorporation of copper into SOD1 is partially inhibited by cycloheximide under conditions of increased intracellular copper content (26,27). The finding that copper incorporation into nascent
SOD1 is dependent upon CCS (Fig. 2A), suggests a potential role for this chaperone in protein folding during copper incorporation, consistent with the differences in apo and holoSOD1 revealed during electrophoresis (Fig. 4C) as well as recent evidence that CCS disulfide isomerase and oxidase activity are required for the mechanism of copper insertion into SOD1.

Taken together, the data in this study provide a cell biological model for the mechanisms of SOD1 synthesis in mammalian cells. In addition to copper, zinc ions play a critical role in the stability and function of active SOD1. While there are currently no similar methods to assess zinc incorporation into SOD1, recent studies indicate that CCS preferentially incorporates copper into the reduced, apo but zinc metallated form of SOD1. Given the kinetics of CCS-dependent copper incorporation observed in this current study, these observations suggest that the incorporation of zinc into SOD1 must occur with similarly rapid and specific mechanisms. The findings reported here also reveal another aspect of SOD1 synthesis that may be critical in the regulation of cellular antioxidant activity. Recent studies have shown that a fraction of active SOD1 localizes within the mitochondrial intermembrane space in both yeast and rodents, protecting this organelle from oxidative damage arising from any superoxide entering or produced in this compartment (28,29). Interestingly, both CCS and SOD1 are localized in the mitochondrial intermembrane space and the abundance of active SOD1 in this compartment is dependent upon this chaperone (28). Although neither CCS nor SOD1 contain typical N-terminal presequences for mitochondrial uptake, recent data indicate that it is the apo, reduced form of SOD1 that is taken up by mitochondria (30), indicating that the intracellular availability of apoSOD1 is a critical factor in antioxidant protection in this organelle. As mitochondria are the major source of superoxide in mammalian cells (29), understanding the mechanisms that determine the synthesis, abundance and localization of cellular apoSOD1 as well as the factors
effecting interaction with and activation by CCS will be critical in elucidation of the molecular and cellular mechanisms of antioxidant defense.

The findings reported here also have important implications for our understanding of the cellular pathogenesis of motor neuron degeneration in familial amyotrophic lateral sclerosis (FALS) due to autosomal dominantly inherited mutations in SOD1 (31). Previous studies have shown that while these SOD1 mutants vary considerably with respect to activity, polypeptide half-life and resistance to proteolysis these variables do not correlate with disease onset or progression (32). Furthermore, although CCS has been shown to directly interact with several of these mutants (9), recent data has shown that this metallochaperone plays no role in the pathogenesis of motor neuron disease in specific mouse models of FALS (33). Taken together, these observations suggest that the biology of the apoSOD1 pool observed in this current study for wild-type SOD1 is likely the predominant factor affected by these mutations. This concept is consistent with recent findings demonstrating that regardless of the specific mutation, oligomerization of mutant SOD1 appears to be important in the toxicity of these proteins (34) and with the observation that impaired mitochondrial uptake of mutant apoSOD1 results in aberrant utilization of heat shock proteins and cell death (35). Further study of the mechanisms of wild-type and mutant apoSOD1 accumulation, trafficking and turnover will likely bring useful information in devising novel therapeutic approaches in this disease.
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Footnotes

1. Abbreviations used in this paper include: SOD1, copper/zinc superoxide dismutase; CCS, copper chaperone for superoxide dismutase; MEFs, mouse embryonic fibroblasts; TEPA, tetraethylenepentamine
2. Tom O’Halloran, personal communication
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Figure Legends

Fig 1. Kinetics of copper incorporation and turnover in SOD1. A. SOD1^{+/+} and SOD1^{-/-} mice were injected with $^{64}$Cu and brain tissue lysates analyzed for $^{64}$Cu incorporation into protein (lanes 1, 2), SOD activity (lanes 3, 4) and SOD1 protein (lanes 5,6) following 4-20% Tris-HCl non-denaturing, non-reducing PAGE as described under “Experimental Procedures”. B. CCS^{+/+} fibroblasts were incubated in serum-free media with 100 µCi/ml $^{64}$Cu for 5 min and chased in media with excess non-radioactive copper for the times indicated. Lysates were analyzed on 4-20% Tris-HCl non-denaturing, non-reducing PAGE and $^{64}$Cu detected by exposure of gels to PhosphoImager. C. HepG2 cells were incubated in serum-free media with 100 µCi/ml $^{64}$Cu for 5 min and chased in media with excess copper for the times indicated. Lysates were analyzed on 4-20% Tris-HCl non-denaturing, non-reducing PAGE and SOD activity (lane 1), SOD1 protein (lane 2) and $^{64}$Cu –SOD1 detected as described under “Experimental Procedures”. D. CCS^{+/+} fibroblasts were incubated in either 200 µCi/ml $^{64}$Cu or 250 µCi/ml [^{35}S]cysteine for 1 h, followed by incubation in media with excess copper or cysteine and lysis at the specific times indicated. $^{64}$Cu-SOD1 was detected following 4-20% Tris-HCl non-denaturing, non-reducing PAGE and quantitated by PhosphoImager. $^{35}$S-SOD1 was immunoprecipitated from lysates, analyzed by 15% SDS-PAGE, and quantitated by densitometric analysis of autoradiographs. Percent remaining $^{64}$Cu-SOD1 or $^{35}$S-SOD1 were plotted versus time to determine half-life. Similar results were obtained in four independent experiments.

Figure 2. Effect of cycloheximide on $^{64}$Cu incorporation into SOD1. A. Indicated cell lines were incubated in serum-free media with or without 10 µg/ml cycloheximide (chx) for 3 hours and 100 µCi $^{64}$Cu/ml was included for the last hour. $^{64}$Cu-SOD1 and SOD1 activity was
determined following 4-20% Tris-HCl non-denaturing, non-reducing PAGE and SOD1 protein following 15% SDS-PAGE as described under “Experimental Procedures”. B. Indicated cell lines were incubated in serum-free media with or without 10 µg/ml cycloheximide (chx) for 3 hours, 100 µCi $^{64}$Cu/ml was included for the last hour and $^{64}$Cu/µg cell lysate quantitated as described under “Experimental Procedures”. Cpm/µg in chx-treated cell lysates expressed as a percentage of cpm/µg in untreated cell lysates. Results are shown as standard deviation of four individual experiments.

Fig 3. **Effect of cycloheximide on $^{64}$Cu incorporation into SOD1 following copper chelation.**

A. Indicated cell lines were pretreated with 50 µM TEPA for 5 days, then incubated in serum-free media with or without 10 µg/ml cycloheximide (chx) for 3 hours, 100 µCi $^{64}$Cu/ml was included for the last hour, followed by analysis of cell lysates on 4-20% Tris-HCl non-denaturing, non-reducing PAGE for $^{64}$Cu-SOD1 and SOD1 activity and 15% SDS-PAGE for SOD1 protein as described under “Experimental Procedures”. B. Indicated cell lines were pretreated with 50 µM TEPA for 5 days, then incubated in serum-free media with or without 10 µg/ml cycloheximide (chx) for 3 hours, 100 µCi $^{64}$Cu/ml was included for the last hour and $^{64}$Cu/µg cell lysate quantitated as described under “Experimental Procedures”. Cpm/µg in chx-treated cell lysates expressed as a percentage of cpm/µg in untreated cell lysates. Results are shown as standard deviation of four individual experiments. C. CCS$^{++}$ fibroblasts were preincubated with 50 µM CuCl$_2$ for four days, then incubated in serum-free media with (open circles) or without (filled circles) 10 µg/ml chx for 3 h, followed by incubation with 100 µCi/ml $^{64}$Cu for times indicated. Cell lysates were subjected to 4-20% Tris-HCl non-denaturing, non-reducing PAGE and $^{64}$Cu incorporation into SOD1 analyzed by PhosphoImager and quantitated following densitometric analysis as described under “Experimental Procedures”. Data are
expressed as a ratio to that observed at 0 min and plotted with respect to time. Similar results were obtained in four independent experiments.

Fig. 4. Detection of holo and apoSOD1. A. Lysates of liver from CCS+/+ and CCS−/− mice injected with 64Cu were analyzed for 64Cu incorporation into SOD1 (lanes 1, 2), SOD activity (lanes 3, 4) and SOD1 protein (lanes 5, 6) following 4-20% Tris-HCl non-denaturing, non-reducing PAGE and SOD1 protein following 15% SDS-PAGE (insert beneath lanes 5, 6) as described under “Experimental Procedures”. B. HepG2 cells, treated without (lane 1) or with (lane 2) 50 µM TEPA for 5 days, were incubated in serum-free media with 100 µCi 64Cu/ml for 3 hours, lysed and 64Cu-SOD1, SOD activity, and SOD1 protein determined following 4-20% Tris-HCl non-denaturing, non-reducing PAGE and SOD1 protein following 15% SDS-PAGE (SOD1 – bottom panel) as described under “Experimental Procedures”. C. Purified human erythrocyte SOD1 was incubated in 6 M guanidine at 4 ºC for 0 to 9 hours (lanes 1-4). Ethanol-precipitated samples were resuspended as described under “Experimental Procedures” and analyzed for SOD1 activity and SOD1 protein following 4-20% Tris-HCl non-denaturing, non-reducing PAGE, and SOD1 protein following 15% SDS-PAGE (SOD1 – bottom panel) as described under “Experimental Procedures”. HepG2 cells, treated without (lane 5) or with (lane 6) 50 µM TEPA for 5 days, were lysed and SOD1 activity and SOD1 protein determined following 4-20% Tris-HCl non-denaturing, non-reducing PAGE, and SOD1 protein following 15% SDS-PAGE (SOD1 – bottom panel) as described under “Experimental Procedures”.

Fig. 5. Biochemical analysis of SOD1 in vivo. A. 5 mg of liver lysate from CCS+/+ (filled circles, solid line), and CCS−/− (open circles, short dash line) mice and 100 µg of purified human SOD1 (filled squares, long dash line) were subjected to gel filtration chromatography on a Superose 12 column calibrated with molecular weight standards. 35 µL of each liver lysate
fraction was analyzed for SOD1 following 15% SDS-PAGE and 50 µl of each fraction containing SOD1 from CCS\(^{+/+}\) mice analyzed for SOD activity as described under “Experimental Procedures”. The percent maximal SOD1 immunoblot signal as measured by densitometric analysis is shown plotted with respect to elution volume. Results are representative of three independent experiments. B. CCS\(^{+/+}\) (filled circles) and CCS\(^{-/-}\) (open circles) mouse fibroblasts were incubated with 200 µCi \([^{35}S]\)cysteine for 3 h, chased in media containing excess non-radioactive cysteine for times indicated and SOD1 immunoprecipitated from cell lysates. Following 15% SDS-PAGE SOD1 was quantitated by densitometric analysis as described under “Experimental Procedures” and percent remaining SOD1 was plotted versus time to determine half-life. Similar results were obtained in four independent experiments.
A

|        | CCS^{+/+} | CCS^{-/-} | HepG2 | HeLa |
|--------|-----------|-----------|-------|------|
| Chx    | -         | +         | -     | +    |
| 64Cu SOD1 |           |           |       |      |
| SOD1 activity |       |           |       |      |
| SOD1   | 1         | 2         | 3     | 4    |

B

% 64Cu-SOD1 +chx/-chx

|        | CCS^{+/+} | HepG2 | HeLa |
|--------|-----------|-------|------|
|       | 100       | 110   | 70   |

C

relative 64Cu-SOD1 signal vs time (min)

- CCS^{+/+} (black dots)
- CCS^{-/-} (black circles)
- HepG2 (white circles)
- HeLa (white circles)
A

$kD: \quad 63.7 \quad 48.6 \quad 20 \quad 15.7$

% SOD1

elution volume (mL)

erythrocyte SOD1 ■

CCS+/+ liver ●

CCS−/− liver ○

SOD1

CCS+/+

CCS−/−

SOD1 activity

B

% remaining SOD1

% remaining SOD1

0 10 20 30 40 50

time (hours)

CCS+/+

CCS−/−

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