Mutations in Cu/Zn superoxide dismutase (SOD1) can cause amyotrophic lateral sclerosis (ALS). Although the precise copper donor in this case is not understood, the CCS-independent pathway shows a dependence on glutathione and is particularly sensitive to certain perturbations in SOD1 structure. Specifically, prolines at SOD1 positions 142 and 144 (based on S. cerevisiae and human SOD1) will block activation by the CCS-independent pathway, but not by CCS. S. cerevisiae SOD1 naturally contains these prolines and shows total dependence on CCS. SOD1 molecules from higher organisms generally lack prolines 142, 144, and can be activated independent of CCS. How CCS-independent activation is blocked by prolines 142, 144 is uncertain, but these residues have been proposed to disrupt the monomer-dimer equilibrium of apo-SOD1.

Although SOD1 is normally a protective enzyme, dominant mutations throughout the SOD1 polypeptide have been linked to the fatal motor neuron disease, amyotrophic lateral sclerosis, also known as ALS. The underlying mechanism is incompletely clear, but a well-accepted model...
Involves misfolding of SOD1 mutants and consequent accumulation of toxic SOD1 aggregates (9-11). The misfolding and instability of SOD1 mutants is curious, in that SOD1 is normally a highly stable enzyme (12). Studies with purified enzyme indicate that the bound metal co-factors as well as an intramolecular disulfide in SOD1 help stabilize the structure of the SOD1 homodimer, and this is true for both WT (wild type) and ALS mutant polypeptides (13-18).

During disease, loss of the intramolecular disulfide correlates with misfolding of mutant SOD1 (19), and formation of improper intermolecular disulfides helps aggregate the protein (20-22). As such, the cellular factors that promote faithful oxidation of the correct intrasubunit disulfide in SOD1 should promote SOD1 stability.

Very little is known regarding the cellular factors that impact on the disulfide of human SOD1. O’Halloran and colleagues found that in the case of yeast SOD1, Cu-CCS promotes oxidation of the disulfide (23). The role of the CCS-independent pathway in oxidizing the disulfide of metazoan SOD1 has not been previously addressed. Without copper activation, the disulfide cysteines may be reduced, as was shown for yeast SOD1 in vivo (23), however the thiol reductants that promote this process are unknown. The low redox potential of the cytosol should favor cysteine reduction. Additionally, the thiol oxidoreductases thioredoxin and glutaredoxin (GRX) are known to target a limited number of cysteines in polypeptides (24,25). GRX can resolve mixed disulfides between glutathione (GSH) and a polypeptide cysteine (a S-thionylated polypeptide) (24,26), and can also directly reduce intramolecular disulfides in proteins without a S-thionylated intermediate. Such reduction of intramolecular disulfides has been described for E. coli Grx1 (24,27,28). But to date, no intramolecular disulfide target has been described for the GRXs of eukaryotes. Could SOD1 represent such a target?

Herein, we describe cellular factors that control the status of the SOD1 disulfide in vivo. Using a yeast expression system, we find that in addition to CCS, the CCS-independent pathway for copper activation helps oxidize the disulfide of human SOD1. When both pathways are blocked, the SOD1 disulfide cysteines are reduced and in the case of ALS SOD1 mutants A4V, G93A and G37R, the protein is highly unstable and is subject to degradation. We also demonstrate for the first time that cytosolic glutaredoxins (GRX) can reduce the SOD1 disulfide cysteines and thereby affect stability of certain ALS SOD1 mutants.

**EXPERIMENTAL PROCEDURES**

**Yeast strains, growth conditions and plasmids** - The yeast strains in this study were derived from parental strains EG103 (MATα, leu2-3, 112, his3Δ1, GAL+, trp1::289a, ura3-52) (29), CY4 (MATα ura3-52 leu2-3 trp1-1 ade2-1 his3-11 can1-100) (30), or BY4741 (MATα, leu2Δ0, met15Δ0, ura3Δ0, his3Δ1). KS107 (sod1Δ::TRP1) (29), PS131 (ccs1Δ::TRP1) (6) and LS101 (sod1Δ::TRP1, ccs1Δ::URA3) were derived from EG103. MC108 (ccs1Δ::ADE2), MC119 (grx1Δ::LEU2 grx2Δ::HIS3 ccs1Δ::URA3) and MC120 (grx1Δ::LEU2 grx2Δ::HIS3 ccs1Δ::ADE2) were generated by introducing ccs1Δ::URA3 and ccs1Δ::ADE2 deletions with plasmids pPS005 and pPS003, in strains CY4 and in the Y117 (grx1Δ::LEU2 grx2Δ::HIS3) derivative of CY4 (30). Strains 614 and 4347 are ccs1Δ::kanMX4 and grx2:: kanMX4 isolates from BY4741 (Research Genetics, Huntsville, AL). MC105 (grx2::kanMX4 ccs1Δ::LEU2) was generated by disrupting the CCSI gene in 4347 using plasmid pLJ165 (6).

For most biochemical analyses, 50 mL yeast cells were propagated overnight at 30°C in either minimal synthetic dextrose (SD) selecting media (starting O.D.600 = 0.15) or in enriched YPD (yeast extract, peptone, dextrose) medium (starting O.D.600= 0.05). In studies with methionine repression, overnight cultures were diluted in a volume of 200 mL to an O.D.600= 0.6, and allowed to grow for an additional 2 hours to early log phase. 1 mM methionine was then added and aliquots of 50 mL were harvested at various time points for cell lysis.

Plasmids pLC1, pLC2 and pLC3 (2μ URA3) express WT, A4V and G41D human SOD1 under the control of S. cerevisiae PGK1 (31). The S142P/L144P variant of human SOD1 (6), as well as mutants C6S, C111S, C6S/C111S, C146S and C57S were obtained by site-directed mutagenesis of pLC1 (QuikChange kit, Stratagene). The S142P/L144P substitution was also introduced in
the LEU2 CEN plasmid pLS121 expressing human SOD1 under S. cerevisiae SOD1 (7), creating pMS001. pLS121 and pMS001 were used as templates to introduce ALS mutations A4V, G37R and G93A. Plasmids pLC41 and pLC42 (2µ TRP1) express WT and A4V human SOD1 under the methionine repressible S. cerevisiae MET25 promoter (31). Plasmid pPS015 (HIS3 CEN) harbors human CCS under the S. cerevisiae PGK1 promoter.

For expression of Grx2p in yeast, the S. cerevisiae GRX2 gene was amplified with primers that introduced BamHI and SalI sites at -962 and +987 and inserted at these same sites in either pRS413 (HIS3 CEN), creating pCO147 or pRS414 (TRP1 CEN), creating plasmid pVCO147. For expression in E. coli, GRX2 was amplified without the mitochondrial targeting signal (residues 1-34) (32) from pCO147 using primers that introduced an NdeI site at Met35, and a BamHI site 15 bp after the stop codon. The fragment was inserted at the Ndel and BamHI sites of pET21a (Novagen), creating pCO150.

**Purification of recombinant yeast Grx2p and human SOD1** - For production of recombinant Grx2p, E. coli strain BL21(DE3) (Novagen) transformed with plasmid pCO150 was grown in 5 L of LB media and induced for 2.5 hours with 1 mM isopropyl-β-D-thiogalactopyranoside when O.D.₆₀₀ reached 0.7. Harvested cells were stored at -80 ºC. A frozen cell paste equivalent to ~800 ml cell culture was lysed by freeze-thawing and resuspended in 50 mL of 50 mM Tris-HCl, pH 8.0, 5 mM DTT. Cell debris was removed by centrifugation, and proteins precipitated with 65% (NH₄)₂SO₄ followed by resuspension in 20 mM Tris-HCl, pH 8.0, 25 mM NaCl, 5 mM DTT to a final volume of ~ 3 mL. The protein solution was desalted using a Hi-Trap™ Desalting column (GE Healthcare), then loaded onto a Hi-Trap™ Q Sepharose XL column (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 8.0, 5 mM DTT. Recombinant Grx2p was eluted with 0-50 mM NaCl and concentrated to 37 mg/mL (3.1 mM). Protein purity was demonstrated by SDS PAGE and Coomassie staining (Supplemental Data, Fig. S3A). Protein was stored at -80ºC with 5% glycerol added to the buffer. This recombinant Grx2p was found to efficiently catalyze the reduction of 2-hydroxyethyl disulfide (HED)¹ by GSH with activity (~300 U/mg protein) comparable to E. coli Grx1 (33).

Recombinant human WT and ALS mutant SOD1 proteins were obtained from a S. cerevisiae expression system as previously described (18). Purity of these proteins was demonstrated by a single band on SDS-PAGE and are of the correct mass as demonstrated by electrospray ionization mass spectrometry using a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer (Thornhill, Canada). Highly purified SOD1 proteins purified in this manner have been used in single crystal X-ray diffraction analyses (15,17), differential scanning calorimetry analyses (34) and in vivo proteasomal digestion assays (35). The latter study further verified the purity of wild type and pathogenic SOD1 proteins isolated in this fashion using reverse phase HPLC immediately prior to mass spectrometric analysis. By inductively coupled plasma mass spectrometry, WT and A4V SOD1 contained equal amounts of copper and zinc in the copper binding site and only zinc in the zinc-binding site (36). Metal-free SOD1 proteins were generated by dialysis of this material at low pH in the presence of EDTA as described (18,37), resulting in apo-SOD1 containing < 0.05 equivalents of both copper and zinc per dimer (18,38), and an oxidized disulfide (as determined by AMS analysis; see Fig. 6 and in (35)).

**Biochemical Assays** - AMS¹ (4-acetamido-4'-maleimidyldisulfonic acid) was used to monitor the disulfide status of SOD1. Yeast cells from a 50 ml culture were washed两次 in deionized water and a 100 µl cell pellet was resuspended in 200 µl of a G4HCl buffer (6M Guanidine-HCl, 3mM EDTA, 0.5% Triton X-100, 50 mM Tris-HCl (pH 8.3)) that contained as needed, 15 mM AMS (Molecular Probes). 100 µL of glass beads (425-600µm; Sigma) were added and cells lysed by 3 cycles of vortexing at room temperature for 2 minutes, interspersed by 1 minute incubations on ice. Extracts were clarified by centrifugation at 10,000 x g for 5 minutes and the supernatant incubated at 37°C for 1 hr in the dark. A 50 µl aliquot was then applied to a MicroSpin G-25 gel filtration column (Amersham Biosciences) and 32µL of flow-through was prepared for SDS-PAGE by incubating with SDS-DTT loading buffer at room temp for 7 minutes, followed by quick clarification by centrifugation.
For AMS modification of SOD1 from fibroblasts, cells were plated at a density of $6.67 \times 10^6$ cells per 100mm tissue culture dish and cultured for 24 hours at 37°C. Media was aspirated, cells washed once in PBS, followed by cell lysis through addition of 200 µl of GnHCl buffer (see above) containing as needed, 15 mM AMS. The AMS reaction and subsequent gel filtration proceeded as above. 20 µl of column flow-through was boiled in SDS-DTT gel loading buffer and clarified by centrifugation prior to analysis by SDS-PAGE on 14% precast gels (Invitrogen).

Immunoblotting with fibroblasts used an antibody (1:1000 dilution) that only recognizes human SOD1 (6) while detection of SOD1 from yeast lysates generally employed a peptide-derived antibody (39) that recognizes both human and mouse SOD1. Cross reactivity with non-specific yeast products of ≈39 and ≈69 kDa was occasionally observed with early preparations of the antibody (e.g., see Supplemental Data, Fig S1A), but not with later preparations of higher titer (e.g., see Fig. 5A). Standard immunoblots (no AMS) and native gels for SOD1 activity used 30-50 µg yeast cell lysate protein. In non-reducing gels (as in Fig. 5A), the gel was pre-soaked in Tris(2-carboxyethyl) phosphine (TCEP) according to published methods (21) prior to electro-blotting. SOD1 activity was monitored by native gel electrophoresis on 12% precast gels and by nitroblue tetrazolium staining as described (40,41).

GRX activity of yeast cell lysates was monitored by the HED assay (33). Yeast cells grown to confluency in selecting media were subjected to glass bead lysis. Cell lysates were heated at 85°C to inactivate glutathione reductase and thioredoxin reductase (42). 15 µg cell lysate protein was added to a 1 ml quartz cuvette containing a 300 µl HED reaction mix (100 mM Tris-HCl, pH 8.0, 2.0 mM EDTA, 1 mM GSH, 0.4 mM NADPH, 6µg/mL Glutathione reductase and 0.7 mM HED. GRX-reduction of the HED substrate was measured by continuous monitoring of NADPH consumption at 340nm over a range of 2 minutes.

An in vitro assay for reduction of the human SOD1 disulfide by recombinant Grx2p was carried out in a 300 µl reaction containing the aforementioned GRX assay constituents with 0.2 mM NADPH rather than 0.4 mM NADPH and no HED. 2-3 µM purified human SOD1 with an oxidized disulfide was added as substrate, and following the addition of recombinant Grx2p to a concentration of 0.05 µM, the reaction incubated at 30°C. At specific time points, 10 µl aliquots were mixed with 60 µl of GnHCl buffer (see above) containing 15 mM AMS. Following incubation for 1 hour at 37°C, samples (≈100 ng SOD1) were subject to gel filtration and analysis of SOD1 by SDS-PAGE and immunoblot. It is noteworthy that in certain preparations of recombinant SOD1, thiol modification at C111 was incomplete. For example, Sigma purchased human WT SOD1 showed no AMS reactivity at C111 unless pre-treated with reducing agents. Apparent oxidation at C111 was also observed upon storage (> 2 weeks at 4°C or > 6 months - 70°C) of apo but not metallated A4V and G93A human SOD1. Similar problems with C111 have been reported elsewhere (35,43). As such, it is important to carry out analysis of the disulfide with freshly prepared samples of purified apo SOD1.

RESULTS

The disulfide in human SOD1 is oxidized by both the CCS-dependent and -independent pathways for copper loading - We sought to understand how copper trafficking pathways affect the disulfide of human SOD1. Disulfide status was probed by AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid), which forms stable thioether linkages with free polypeptide cysteines, but is non-reactive towards cysteines bridged in a disulfide. Unlike S. cerevisiae SOD1 and the Cu/Zn SOD of C. elegans that contain only 2 cysteines (7), human SOD1 contains four cysteines: the disulfide C57 and C146 cysteines, and C6 and C111. To discern between these, we introduced single and combined mutations at these cysteines. The corresponding SOD1 variants were expressed in yeast, and lysates prepared in 6N guanidinium were treated with AMS and analyzed by immunoblot. The goal of such a “redox western” is not to determine total protein levels, but to compare the level of oxidized versus reduced cysteines within a particular sample. Typically, the 15.9 kDa human SOD1
migrates on SDS gels at the position of \( \approx 21.5 \text{kDa} \) (Fig. 1A, lane 1) (44). When WT SOD1 from CCS1+ yeast is reacted with AMS, a major band corresponding to a shift in mobility of \( \approx 5.0 \text{kDa} \) is observed (thin arrow, lane 2). This reflects AMS modification at the non-disulfide C6 and C111 cysteines, because no mobility shift is observed with C6S/C111S SOD1 expressed in CCS1+ yeast (lane 5). The lack of AMS reactivity at C57 and C146 demonstrates that the disulfide is oxidized in CCS1+ yeast cells, as would be expected for active SOD1. It is noteworthy that AMS modification at C6 and C111 produces a larger shift in mobility than the 1.07 kDa expected for 2 AMS moieties. This is due to AMS modification at C111, since C6S SOD1, but not C111S, also produces an aberrantly large shift in mobility (Fig. 1B, also see Supplemental Data Fig. S1B and Tables S1, S2). Certain small modifications in SOD1 composition can affect anomalous mobility on SDS gels, such as the C57S mutation (Fig. 1A, lane 11) and several other substitutions reported for SOD1 (31,39,41,44). AMS modification at C111 likewise results in anomalous mobility on SDS-PAGE. A more detailed description of the effects of AMS modification at each of the four cysteines is presented in Supplemental Data Tables S1-S2.

When human SOD1 is expressed in ccs1Δ null yeast cells lacking the CCS1 gene, a second AMS product bearing increased mobility shift appears (Fig. 1A, heavy arrow, lane 3). This corresponds to additional AMS reactivity at the disulfide cysteines C57 and/or C146 because the same shift is seen with C6S/C111S SOD1 expressed in ccs1Δ cells (lane 6), but not with C146S and C57S SOD1 affecting the disulfide (Fig. 1A lanes 9 and 13). AMS reactivity at C57 and/or C146 indicates that the disulfide cysteines have been reduced in cells lacking CCS.

We also monitored the disulfide of human SOD1 expressed in mammalian cells. These studies employed skin fibroblasts derived from CCS+/+ and CCS−/− null homozygous mice that are transgenic for human WT SOD1 or the ALS SOD1 mutant G37R (6,45). When expressed in CCS+/+ fibroblasts, human WT SOD1 exhibits the identical AMS reactivity pattern seen with disulfide-oxidized SOD1 from CCS1+ yeast (Fig. 1C, compare lanes 1 and 3). In CCS−/− fibroblasts, two products are observed, representative of both oxidized and reduced states of the disulfide cysteines (lane 6). With the G37R ALS mutant SOD1, there is evidence for disulfide reduction even in CCS+/+ fibroblasts (Fig. 1C, lane 8), consistent with the notion that ALS mutants are more susceptible to disulfide reduction (46). As has been shown for the endogenous SOD1 of yeast (23), CCS helps oxidize the disulfide of human SOD1 expressed in yeast and mammalian cells.

We tested whether the CCS-independent pathway affects the human SOD1 disulfide (6). This auxiliary pathway for activating SOD1 is blocked by introducing prolines in SOD1 at positions corresponding to amino acids 142 and 144 (6,7). Human SOD1 contains ser and leu at these positions, and a S142P/L144P mutant of human SOD1 is only activated by CCS (6). When S142P/L144P human SOD1 is expressed in CCS1+ yeast cells, the enzyme is active (6), and the disulfide is oxidized (Fig. 2A lane 3). However, when expressed in ccs1Δ yeast cells, S142P/L144P human SOD1 cannot obtain copper by either pathway; the enzyme is inactive (6), and the disulfide cysteines are completely reduced (Fig. 2A, lane 4). Hence, both CCS-dependent and -independent pathways for activating SOD1 contribute to disulfide oxidation with human SOD1.

**The disulfide and stability of ALS mutant SOD1** - We explored how changes in copper loading *in vivo* affect ALS mutants of SOD1. Human SOD1 mutants G37R, G93A and A4V were expressed in yeast under conditions where copper activation by CCS and/or the CCS-independent pathway were blocked. CCS was inhibited by expression in a ccs1Δ null yeast strain and CCS-independent activation was blocked by introducing prolines 142 and 144 into SOD1. As seen in Fig. 2B lane 4, loss of the CCS-independent pathway alone through a S142P/L144P substitution resulted in some lowering of the steady state level of the ALS mutants, particularly A4V (also see Supplemental Data, Fig. S2). Moreover, when the S142P/L144P variants of G37R, G93A and A4V were expressed in ccs1Δ cells, no polypeptide could be recovered (Fig. 2B, lane 3). By comparison, the S142P/L144P variant of WT SOD1 stably accumulated in ccs1Δ cells (lane 3 top panel, also see Supplemental Data, Fig. S2). The ALS mutants, but not WT SOD1, appeared highly...
unstable and degraded when both the CCS-dependent and -independent pathways for copper loading and disulfide oxidation were eliminated.

Loss of CCS alone can also affect stability of certain ALS mutants expressed in yeast. For example, G41D accumulates to very low steady state levels in ccs1Δ yeast (Fig. 2C). A4V SOD1 also appears somewhat unstable in ccs1Δ cells, but to a lesser degree (Fig. 2C, also see Fig. 4C and 5B). It is noteworthy that this same pattern of instability has been observed in mammalian expression systems: G41D and A4V are more prone to degradation and aggregation than other ALS mutants (39,47). It is important to note that unlike mammalian expression systems where SOD1 misfolding can lead to both degradation and aggregation of the polypeptide, protein degradation is the primary end point of SOD1 misfolding in yeast. There is no evidence of SOD1 aggregation in yeast expression systems (see Discussion).

To monitor turnover of disulfide-reduced versus disulfide-oxidized polypeptides, SOD1 synthesis in yeast cells was controlled by the methionine repressible MET25 promoter. In this manner, A4V SOD1 is actively synthesized in yeast cells not treated with methionine, but is repressed upon addition of methionine to the growth medium, allowing us to monitor loss of the SOD1 polypeptides over time. In the absence of methionine, a good fraction of A4V SOD1 expressed in ccs1Δ cells is seen in the disulfide-reduced state, and the ratio of reduced to oxidized disulfide remains relatively constant over 3 hours (Fig. 3A lanes 1,2; also see Supplemental Data Fig. S2). But when A4V expression was repressed by methionine supplements, the disulfide-reduced form of A4V SOD1 was lost, whereas the disulfide-oxidized form was more stable (Fig. 3A lanes 4-6; also see Supplemental Data, Fig. S2). This result is consistent with in vitro studies with recombinant SOD1 showing that disulfide-reduced SOD1 is more prone to degradation by the proteosome than disulfide-oxidized SOD1 (35).

We similarly examined stability of WT human SOD1. In the experiment of Fig. 3B, the disulfide of WT SOD1 at steady state is more oxidized than that of A4V SOD1 examined in parallel (compare t=0 samples for A4V and WT SOD1). Following 3 hours of methionine repression, both the reduced and oxidized pools of WT SOD1 were retained, compared to A4V SOD1 that exhibited instability particularly with the disulfide-reduced fraction (Fig. 3B, also see Supplemental Data Fig. S2). The disulfide-reduced form of WT SOD1 is not subject to same dramatic turn over as the ALS mutant.

The role of cytosolic glutaredoxins in reducing the SOD1 disulfide and destabilizing ALS mutant polypeptides - In the absence of copper activation, what cellular factors favor reduction of the SOD1 disulfide cysteines? We tested the possible role of glutaredoxins (GRX), S. cerevisiae expresses two GRXs in the cytosol, namely Grx1p and Grx2p. Double grx1 grx2 null mutations do not alter cellular redox or GSH/GSSG ratios (38). We tested how loss of GRX affects the disulfide of SOD1 expressed in ccs1Δ null yeast strains, where the SOD1 is normally a mixed population of disulfide-reduced and -oxidized forms (Fig. 4A, lanes 2,5). Loss of GRX through grx1Δ grx2Δ null mutations shifted the disulfide of SOD1 towards the oxidized state and the effects were particularly pronounced with A4V SOD1 (Fig. 4A, lane 6).

Loss of yeast GRXs not only affected the SOD1 disulfide, but also stability of ALS mutants. In Fig.4B, A4V SOD1 stability was monitored through methionine repression. The disulfide-oxidized form of SOD1 that accumulates in grx1Δ grx2Δ ccs1Δ strains is quite stable over three hours of methionine repression (Fig. 4B, lanes 3,4). As such, the steady state levels of total A4V SOD1 increases, as do levels of G41D SOD1 expressed in grx1Δ grx2Δ ccs1Δ yeast (Fig. 4C, lanes 2,3).

Of the two cytosolic GRXs in yeast, Grx2p is the predominant form (38). Single grx2Δ mutations were sufficient to increase steady state levels of G41D SOD1 (Fig. 4C lane 5), while grx1Δ mutations were not (not shown). To examine the effects of Grx2p further, grx1Δ grx2Δ ccs1Δ cells were transformed with a low copy plasmid expressing GRX2 under its native promoter. Plasmid borne Grx2p was indeed enzymatically active, as monitored by the standard in vitro assay for GRX activity using 2-hydroxyethyl disulfide (HED) as substrate (33) (Fig. 4D, top). Expression of Grx2p in grx1Δ grx2Δ ccs1Δ yeast also correlated with reduction of the disulfide in A4V SOD1 (Fig. 4D, bottom).
Loss of GRX clearly effects oxidation of the intramolecular disulfide in SOD1, but what about non-native disulfides? Recently, ALS SOD1 mutants have been shown to oligomerize and form intermolecular disulfide cross-links that can be visualized by electrophoresis under non-reducing conditions (19-22,48). However, when analyzed under non-reducing conditions (“-DTT”), A4V SOD1 expressed in grx1Δ grx2Δ ccs1Δ yeast only exists as a monomer and there were no unique high molecular species consistent with intermolecular disulfides (Fig. 5A, right). Therefore, GRXs appear to only target only the chief intramolecular disulfide of SOD1.

We also examined the effects of GRX loss on SOD1 activity. As seen in Fig. 5B, there was no significant change in SOD1 activity with WT, G93A or A4V variants expressed in grx1Δ grx2Δ ccs1Δ cells compared to ccs1Δ single mutants. A4V SOD1 shows poor CCS-independent activity in ccs1Δ cells, and this does not change with additional grx1Δ grx2Δ mutations (Fig. 5B). In spite of oxidation of the A4V SOD1 disulfide in this strain (shown in Fig. 4A, B, D), the SOD1 remains largely inactive. Presumably, the disulfide-oxidized SOD1 is still copper deficient in grx1Δ grx2Δ ccs1Δ cells (see Discussion).

To more directly test whether GRX can reduce the SOD1 disulfide, we designed an in vitro assay using purified recombinant yeast Grx2p and purified human WT or A4V SOD1. In order to regenerate reduced GRX, the in vitro reactions also contained GSH. In the experiment of Fig. 6A, recombinant Grx2p at 50 nM was allowed to react with disulfide-oxidized A4V SOD1 that was apo for metals and present at a concentration of 2.0 µM. Within 1 hour, the disulfide was reduced (Fig. 6A lane 3, also see Fig. 6C, lane 6). Similar results were obtained with apo G93A SOD1 (not shown). GSH alone was not sufficient to reduce the disulfide (Fig. 6A, lane 6), but GSH was required for Grx2p-dependent reduction of the disulfide (Supplemental Data, Fig. S3B), as would be expected for GRX reactions (24). Compared to A4V SOD1, the disulfide of apo WT human SOD1 exhibited poor reactivity towards Grx2p and GSH (Fig. 6B) even after 2 hours of incubation (Fig. 6C, lane 3). At best, a $\approx 2.0\%$ conversion to the reduced form was seen in one experiment trial out of eight with apo WT SOD1 (See Supplemental Data, Fig. S3B).

We also tested Grx2p reactivity towards metallated SOD1. As seen in Fig. 6D, the disulfide of metallated A4V SOD1 containing both copper and zinc was refractory to reduction by Grx2p in vitro. The apo version is the preferred substrate for disulfide reduction by Grx2p.

### DISCUSSION

Herein we describe how post-translational modification factors for SOD1 can impact on the intramolecular disulfide and stability of ALS mutant SOD1. Two classes of intracellular factors are shown to work in opposite to control status of the disulfide. First, the CCS-dependent and -independent pathways for copper activation promote oxidation of the human SOD1 disulfide and enhance stability of ALS mutants A4V, G93A and G37R expressed in yeast. Without copper activation, intracellular reductants such as GRX promote disulfide reduction, contributing to SOD1 instability.

The ALS mutants seemed particularly vulnerable to loss of copper activation. While WT SOD1 stably accumulated in yeast cells without the copper co-factor or an oxidized disulfide, the three ALS mutants we examined (G93A, G37R and A4V) were degraded when both CCS-dependent and -independent pathways were blocked. In the yeast expression system, misfolded SOD1 mutants lacking metals and the disulfide are effectively cleared by protein degradation. There is no evidence of SOD1 aggregation in yeast by either formation of high molecular weight species on SDS gels (as in Fig. 5A and Supplemental Data, Fig. S4) or by formation of detergent insoluble precipitates (data not shown). In mammalian cells, the clearance of misfolded SOD1 may be incomplete, allowing for accumulation of misfolded aggregates. Regardless of whether the end point is degradation or aggregation, the initiating misfolding event in SOD1 can be promoted by absence of copper and the intramolecular disulfide. Copper loading of SOD1 is incomplete in various cells and tissues (5,49-51) and in the case of certain ALS mutants, this pool of immature SOD1 may very well seed formation of misfolded aggregates.
ALS mutants may also be more vulnerable to disulfide reduction by GRX. Yeast Grx2p was seen to promote reduction of the disulfide cysteines of A4V SOD1 both in vivo and in vitro while WT human SOD1 was less reactive. It is possible that misfolding of certain ALS mutants allows for greater access of the GRX molecule toward the disulfide. This increased reactivity with GRX, together with the high instability of the disulfide-reduced state makes the SOD1 mutant a prime target for protein misfolding and degradation.

To date, very few in vivo substrates have been documented for eukaryotic dithiol GRXs. Mammalian GRX can act as a dethionylase for actin, Hsp70 and Ras (26,52,53), but no substrates have been identified for S. cerevisiae Grx1p and Grx2p. Furthermore, there have been no reports of an intramolecular disulfide target for eukaryotic GRXs, only S-thionylated targets. We favor a model in which GRX acts on the intramolecular disulfide of SOD1 rather than an S-thionylated intermediate. If SOD1 were S-thionylated, such an intermediate would be detected by AMS modification and would hyper-accumulate in grx1Δ grx2Δ yeast mutants lacking dethionylase activity. To our knowledge, SOD1 represents the first reported intramolecular disulfide substrate for a eukaryotic GRX. It is quite possible that other polypeptide disulfides serve as targets, including those noted in the prion (54) and transthyretin redox sensitive proteins (55) implicated in disease.

Our studies strongly indicate that GRX preferentially acts on a SOD1 molecule that contains an oxidized disulfide, yet lacks copper. First, the in vivo effects of GRX on the SOD1 disulfide were only observed in yeast strains where copper-activation was low (e.g., in ccs1Δ strains). Loss of GRX correlated with disulfide oxidation, but the SOD1 enzyme remained largely inactive, indicative of no copper co-factor. Moreover, recombinant Grx2p could reduce the disulfide of apo, but not metallated A4V SOD1. If copper-deficient, disulfide-oxidized SOD1 is indeed the substrate for GRX, this would imply that SOD1 can obtain an oxidized disulfide in vivo without copper insertion. How the SOD1 disulfide is oxidized without copper is still unclear, but is the subject of current investigations. In any case, the GRX and/or GSH-mediated reduction of the disulfide in copper-deficient SOD1 would be beneficial to the cell, as it would provide additional substrate for CCS that is normally inert towards disulfide-oxidized SOD1 (23).

Although these studies on the human SOD1 disulfide were largely conducted in yeast, they are predicted to have important implications for SOD1 folding and stability in mammalian cells as well. First, the factors that control the SOD1 disulfide are well conserved in yeast and mammals, including CCS (2), the CCS-independent pathway (6) and the dithiol GRX molecules of the cytoplasm (38). Moreover, the relative instability observed with ALS mutants A4V and G41D expressed in yeast is remarkably similar to what has been reported in mammalian cells (39,47). Therefore, the effects of copper loading pathways and thiol-reductants on the disulfide and stability of SOD1 are expected to be conserved. In a previous transgenic mouse study, loss of CCS was reported to not affect motor neuron disease associated with expression of ALS mutants G93A, G37R or G85R (45). Based on our studies in yeast, these mutants are stable without CCS due to compensatory effects of the CCS-independent pathway. It is therefore important to consider the impact of both copper loading pathways, as well as thiol-reductants such as GRX on the fate of ALS mutants in motor neuron disease.

REFERENCES

1. McCord, J. M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055
2. Culotta, V. C., Klomp, L., Strain, J., Casareno, R., Krems, B., and Gitlin, J. D. (1997) J. Biol. Chem. 272, 23469-23472
3. Casareno, R. L., Waggoner, D., and Gitlin, J. D. (1998) J Biol Chem 273(37), 23625-23628
4. Lamb, A. L., Torres, A. S., O’Halloran, T. V., and Rosenzweig, A. C. (2001) Nature Struct Biol. 8
5. Brown, N. M., Torres, A. S., Doan, P. E., and O’Halloran, T. V. (2004) Proc Natl Acad Sci U S A 101, 5518-5523
6. Carroll, M. C., Giroud, J. B., Ulloa, J. L., Subramaniam, J. R., Wong, P. C., Valentine, J. S., and Culotta, V. C. (2004) *Proc Natl Acad Sci USA* 101(16), 5964-5969
7. Jensen, L. T., and Culotta, V. C. (2005) *J Biol Chem* 280, 41373-41379
8. Banci, L., Bertini, I., Cantini, F., D'Amelio, N., and Gaggelli, E. (2005) *J Biol Chem*
9. Cleveland, D. W., and Liu, J. (2000) *Nature Med.* 6, 1320-1321
10. Valentine, J. S., and Hart, P. J. (2003) *Proc. Natl. Acad. Sci. USA* 100, 3617-3622
11. Wang, J., Xu, G., Slunt, H. H., Gonzales, V., Coonfield, M., Fromholt, D., Copeland, N. G., Jenkins, N. A., and Borchelt, D. R. (2005) *Neurobiol Dis*
12. Forman, H. J., and Fridovich, I. (1973) *J. Biol. Chem.* 248, 2645-2649
13. Assfalg, M., Banci, L., Bertini, I., Turano, P., and Vasos, P. R. (2003) *J Mol Biol* 330(1), 145-158
14. Arnesano, F., Banci, L., Bertini, I., Martinelli, M., Furukawa, Y., and O'Halloran, T. V. (2004) *J Biol Chem* 279, 47998-48003
15. Elam, J. S., Taylor, A. B., Strange, R., Antonyuk, S., Doucette, P. A., Rodriguez, J. A., Hasnain, S. S., Hayward, L. J., Valentine, J. S., Yeates, T. O., and Hart, P. J. (2003) *Nat Struct Biol* 10(6), 461-467
16. Rodriguez, J. A., Valentine, J. S., Eggers, D. K., Roe, J. A., Tiwari, A., Brown, R. H., and Hayward, L. J. (2002) *277*, 15932 - 15937
17. Strange, R. W., Antonyuk, S., Hough, M. A., Doucette, P. A., Rodriguez, J. A., Hart, P. J., Hayward, L. J., Valentine, J. S., and Hasnain, S. S. (2003) *J Mol Biol* 328(4), 877-891
18. Doucette, P. A., Whitson, L. J., Cao, X., Schirf, V., Demeler, B., Valentine, J. S., Hansen, J. C., and Hart, P. J. (2004) *J. Biol. Chem.* 279, 54558-54566
19. Jonsson, P. A., Graffmo, K. S., Andersen, P. M., Brannstrom, T., Lindberg, M., Oliveberg, M., and Marklund, S. L. (2006) *Brain* 129(Pt 2), 451-464
20. Deng, H. X., Shi, Y., Furukawa, Y., Zhai, H., Fu, R., Liu, E., Gorrie, G. H., Khan, M. S., Hung, W. Y., Bigio, E. H., Lukas, T., Dal Canto, M. C., O'Halloran T, V., and Siddique, T. (2006) *Proc Natl Acad Sci USA* 103(18), 7142-7147
21. Furukawa, Y., Fu, R., Deng, H., Siddique, T., and O'Halloran, T. V. (2006) *Proc. Natl. Acad. Sci. USA* 103, 7148-7153
22. Wang, J., Xu, G., and Borchelt, D. R. (2006) *J Neurochem*
23. Furukawa, Y., Torres, A. S., and O'Halloran, T. V. (2004) *Embo J* 23(14), 2872-2881
24. Fernandes, A. P., and Holmgren, A. (2004) *Antioxid Redox Signal* 6(1), 63-74
25. Carmel-Harel, O., and Storz, G. (2000) *Annu Rev Microbiol* 54, 439-461
26. Shelton, M. D., Chock, P. B., and Mieyal, J. J. (2005) *Antioxid Redox Signal* 7(3-4), 348-366
27. Holmgren, A. (1976) *Proc Natl Acad Sci USA* 73(7), 2275-2279
28. Zheng, M., Aslund, F., and Storz, G. (1998) *Science* 279(5357), 1718-1721
29. Slekar, K. H., Kosman, D., and Culotta, V. C. (1996) *J. Biol. Chem.* 271, 28831-28836
30. Draulic, T., Dawes, I. W., and Grant, C. M. (2000) *Mol Microbiol* 36(5), 1167-1174
31. Corson, L. B., Strain, J., Culotta, V. C., and Cleveland, D. W. (1998) *Proc. Natl. Acad. Sci. USA* 95, 6361-6366
32. Pedrajitas, J. R., Porras, P., Martinez-Galisteo, E., Padilla, C. A., Miranda-Vizuete, A., and Barcena, J. A. (2002) *Biochem J* 364(Pt 3), 617-623
33. Holmgren, A., and Aslund, F. (1995) *Methods Enzymol* 252, 283-292
34. Antonyuk, S., Elam, J. S., Hough, M. A., Strange, R. W., Doucette, P. A., Rodriguez, J. A., Hayward, L. J., Valentine, J. S., Hart, P. J., and Hasnain, S. S. (2005) *Protein Sci* 14(5), 1201-1213
35. Di Noto, L., Whitson, L. J., Cao, X., Hart, P. J., and Levine, R. L. (2005) *J Biol Chem* 280(48), 39907-39913
36. Strange, R. W., Antonyuk, S. V., Hough, M. A., Doucette, P. A., Valentine, J. S., and Hasnain, S. S. (2006) *J Mol Biol* 356(5), 1152-1162
37. Valentine, J. S., and Pantoliano, M. W. (1981) Protein-metal ion interactions in cuprozinc protein (superoxide dismutase). In: Spiro, T. (ed). *Copper Proteins*, Wiley-Interscience, New York
38. Luikenhuis, S., Dawes, I. W., and C. M. Grant, C. M., 1081-1091. (1997) *Mol. Biol. Cell* 9, 1081-1091
39. Borchelt, D. R., Lee, M. K., Slunt, H. H., Guarnieri, M., Xu, Z., Wong, P. C., Brown, R. H., Price, D. L., Sisodia, S. S., and Cleveland, D. W. (1994) *Proc. Natl. Acad. Sci. USA* 91, 8292-8296
40. Flohe, L., and Otting, F. (1984) Superoxide dismutase assays. In: Packer, L. (ed). *Methods in enzymology: oxygen radicals in biological systems*, Academic press, New York
41. Schmidt, P. J., Ramos-Gomez, M., and Culotta, V. C. (1999) *J. Biol. Chem.* 274, 36952-36956
42. Rodriguez-Manzaneque, M. T., Ros, J., Cabisco, E., Sorribas, A., and Herrero, E. (1999) *Mol. Cell Biol.* 19(12), 8180-8190
43. Liu, H., Zhu, H., Eggers, D. K., Nersissian, A. M., Faull, K. F., Goto, J. J., Ai, J., Sanders-Loehr, J., Gralla, E. B., and Valentine, J. S. (2000) *Biochem J.* 36952-36956
44. Borchelt, D. R., Guarnieri, M., Wong, P. C., Lee, M. K., Slunt, H. S., Xu, Z., Sisodia, S. S., Price, D. L., and Cleveland, D. W. (1995) *J. Biol. Chem.* 270, 3234-3238
45. Subramaniam, J. R., Lyons, W. E., Liu, J., Bartnikas, T. B., Rothstein, J., Price, D. L., Cleveland, D. W., Gitlin, J. D., and Wong, P. C. (2002) *Nat. Neurosci.* 5, 301-307
46. Tiwari, A., and Hayward, L. J. (2003) *J. Biol. Chem.* 278, 5984-5992
47. Wang, J., Slunt, H., Gonzales, V., Fromholt, D., Coonfield, M., Copeland, N. G., Jenkins, N. A., and Borchelt, D. (2003) *Hum. Molec. Genet.* 12, 36952-36956
48. Furukawa, Y., and O'Halloran, T. V. (2005) *J Biol Chem* 280(17), 17266-17274
49. Petrovic, N., Comi, A., and Ettinger, M. J. (1996) *J. Biol. Chem.* 271, 28331-28334
50. Steinkuhler, C., Carri, M. T., Micheli, G., Knoepfel, L., Weser, U., and Rotilio, G. (1994) *Biochem. J.* 302, 687-694
51. Bartnikas, T. B., and Gitlin, J. D. (2003) *J. Biol. Chem.* 278, 33602-33608
52. Adachi, T., Pimentel, D. R., Heibeck, T., Hou, X., Lee, Y. J., Jiang, B., Ido, Y., and Cohen, R. A. (2004) *J Biol Chem* 279(28), 29857-29862
53. Hoppe, G., Chai, Y. C., Crabb, J. W., and Sears, J. (2004) *Exp Eye Res* 78(6), 1085-1092
54. Lee, S., and Eisenberg, D. (2003) *Nat Struct Biol* 10(9), 725-730
55. Zhang, Q., and Kelly, J. W. (2005) *Biochemistry* 44(25), 9079-9085
56. Choi, J., Rees, H. D., Weintraub, S. T., Levey, A. I., Chin, L. S., and Li, L. (2005) *J Biol Chem* 280(12), 11648-11655

**FOOTNOTES**

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1 The abbreviations used are: ALS, amyotrophic lateral sclerosis; SOD1, superoxide dismustase 1; CCS, copper chaperone for SOD1; GRX, glutaredoxin; WT, wild type; AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; TCEP, Tris(2-carboxyethyl) phosphine; HED, 2-hydroxyethyl disulfide

**FIGURE LEGENDS**
Fig. 1: AMS modification analysis of the disulfide of human SOD1 expressed in yeast cells and fibroblasts. Cells expressing either WT or the indicated mutant derivatives of human SOD1 were lysed in a 6N Guanidine HCl buffer also containing where indicated (AMS +), 15 mM AMS. AMS modification of the human SOD1 polypeptide was discerned by SDS-PAGE and immunoblotting. Heavy and light arrows indicate positions of disulfide-reduced and disulfide-oxidized SOD1, respectively. “31,17” = size in kDa of molecular weight markers run in parallel. (A, B, C lane 1) Human SOD1 expressed in yeast under control of the S. cerevisiae PGK1 promoter. (A) Strains utilized are “CCS1+” = KS107, wild type for CCS1 and “CCS1Δ” = the ccs1Δ null strain PS131. C57S SOD1 migrates aberrantly on SDS gels (compare lanes 10,11) as has been reported for other SOD1 mutants (31,39,41,44). (B, C lane 1) Human SOD1 expressed in the CCS1 + strain KS107. (C lanes 2-9) Analysis of immortalized fibroblasts from CCS +/+ and CCS −/− mice that were transgenic for either WT or G37R human SOD1 as described (6,45). “Y” = CCS1 + yeast expressing WT human SOD1; “M” = fibroblasts from CCS +/+ mice expressing WT human SOD1. It is noteworthy that with long exposures, disulfide-oxidized SOD1 often runs as a doublet, with a minor band ≈1.5 kDa smaller than the major disulfide-oxidized product (e.g., part A lane 2). Since this doublet is observed with WT, C146S, C57S and C6S, but not with C111S SOD1 (also see Supplemental Data, Fig. S1B), AMS modification at C111 might effect both ≈4.5 kDa (major) and ≈3.0 kDa (minor) shifts in apparent molecular weight. However, we cannot exclude other SOD1 modifications, such as oxidative products and retention of some metal binding during electrophoresis as has been previously reported to cause multiple SOD1 isoforms during electrophoresis (23,56). While AMS reactivity generally goes to completion, there occasionally is some low level of incomplete modification as is seen in lanes 8,9 part B.

Fig. 2: The effects of copper activation on the disulfide and steady state levels of human SOD1 variants expressed in yeast. Yeast strains expressing WT human SOD1 or the indicated mutant variants were analyzed for (A) AMS modification of SOD1 cysteines as in Fig. 1, or for (B-C) total human SOD1 protein by immunoblot. (A) Strains as described in Fig. 1A expressed either WT or S142P/L144P human SOD1 under control of the S. cerevisiae PGK1 promoter. Heavy and light arrows indicate disulfide-reduced and disulfide-oxidized SOD1. (B) The ccs1Δ sod1Δ strain LS101 was transformed where indicated (CCS+) with the human CCS expressing plasmid pPS015 and also with the indicated variants of human SOD1 under control of the S. cerevisiae SOD1 promoter. In lanes 3-4, the S142P/L144P substitution was introduced in WT, G37R, G93A and A4V human SOD1. Quantification of the levels of SOD1 can be found in Supplemental Data Fig. S2. (C) Human WT, G41D and A4V SOD1 under control of the PGK1 promoter were expressed in strains described in Fig. 1A.

Fig. 3: Time course for loss of the disulfide-reduced form of A4V SOD1. The ccs1Δ strain MC108 expressing either A4V SOD1 (part A and part B where indicated) or WT human SOD1 (part B where indicated) under control of the MET25 methionine repressible promoter was cultured in the absence of methionine. Where designated (part A, +Met and part B), 1.0 mM methionine was added to fully repress SOD1 expression (31) and aliquots were removed at the indicated time points for AMS modification and immunoblot analysis. Heavy and light arrows indicate positions of disulfide-reduced and disulfide-oxidized forms of SOD1. “31,17” = size in kDa of molecular weight markers run in parallel. Two different exposures of the results obtained with WT human SOD1 are provided to visualize both the disulfide-reduced and disulfide-oxidized forms. Quantification of these results can be found in Supplemental Data, Fig. S2.

Fig. 4: Effects of cytosolic glutaredoxins on the SOD1 disulfide. (A) The indicated yeast strains expressing WT or A4V human SOD1 under control of the PGK1 promoter were subject to AMS analysis of the disulfide as in Fig. 1A. (B) The indicated strains expressing A4V SOD1 under the control of MET25 were subject to methionine repression for 3 hours where indicated prior to AMS analysis of the disulfide. Heavy and light arrows indicate disulfide-reduced and disulfide-oxidized SOD1. “31,17” = size in kDa of molecular weight markers run in parallel. Strains utilized: GRX1/GRX2 + = the ccs1Δ
strain MC108; GRX1/GRX2 Δ = the grx1Δ grx2Δ ccs1Δ strain MC119. (C) The indicated strains expressing either G41D or A4V human SOD1 under PGK1 were monitored for steady state levels of SOD1 by immunoblot. Strains utilized: lane 1, parental strain CY4; lane 2, the ccs1Δ strain MC108; lane 3, the grx1Δ grx2Δ ccs1Δ strain MC120; lane 4, the ccs1Δ strain 614; lane 5, the grx2Δ ccs1Δ derivative of 614, MC105. (D) The grx1Δ grx2Δ ccs1Δ strain MC120 (identical to MC119 except ccs1Δ::ADE2 rather than ccs1Δ::URA3) also expressing A4V SOD1 under PGK1 was transformed where indicated (GRX2), with the pVC0147 plasmid for expressing S. cerevisiae Grx2p or with empty vector pRS313 (V).

Fig. 5 Loss of glutaredoxins in yeast does not effect SOD1 oligomerization or alter SOD1 activity. (A) Lysates from the indicated yeast strains expressing A4V SOD1 under PGK1 were subjected to denaturing gel electrophoresis and immunoblot analysis for steady state levels of SOD1. Prior to electrophoresis, samples containing 30 µg total extract protein were heated at 95 °C in SDS-buffer that either contained (+DTT) or lacked (-DTT) 10 mM DTT as a reducing agent. Gels were soaked in TCEP to help reduce polypeptides cysteines according to published methods (21) prior to immunoblotting. Vertical numbers indicate size of molecular weight markers run in parallel. (B) Lysates from the indicated yeast strains expressing WT, A4V or G93A SOD1 under PGK1 were subjected to either non-denaturing gel electrophoresis and NBT staining for SOD1 activity (40,41) (Top), or to SDS-PAGE and immunoblot for human SOD1 protein (Bottom). Strains utilized: GRX1/GRX2+ CCS1+, CY4; GRX1/GRX2+ CCS1Δ, the ccs1Δ strain MC108; GRX1/GRX2Δ CCS1Δ, the grx1Δ grx2Δ ccs1Δ strain MC120. This particular strain background (CY4) shows lower levels of CCS-independent SOD1 activity than other strains (e.g., BY4741 or EG103), perhaps due to lower abundance of intracellular GSH needed for efficient CCS-independent activation (6).

Fig. 6. Reduction of the ALS mutant disulfide by recombinant GRX. An in vitro assay for reduction of the SOD1 disulfide contained 2-3 µM concentrations of purified human SOD1 incubated (unless indicated otherwise) with 0.05 µM concentrations of recombinant S. cerevisiae Grx2p. Following the designated time points in minutes, the SOD1 disulfide was analyzed by AMS modification and immunoblotting. Heavy and light arrows mark disulfide-reduced and disulfide-oxidized SOD1, respectively. (A) Apo A4V SOD1 reacted in the presence or absence of Grx2p as indicated. (B) Apo WT SOD1 reacted as in A. (C) Comparison of apo WT and apo A4V SOD1 over 2 hours of incubation. (D) Comparison of apo and metallated (Cu/Zn) forms of A4V SOD1.
Fig. 3

A

- MET

+ MET

T = 0 3hr

B

WT

A4V

T = 0 0.5 1 2 3hr
Fig. 4

A

| WT SOD1 | A4V |
|---------|-----|
| GRX1/GRX2: | + + Δ | + + Δ |
| AMS: | - + + | - + + |

B

| GRX1/GRX2: | 0 | 3hrs |
| AMS: | + | + |

C

| GRX2: | + + Δ | + Δ |
| GRX1: | + + Δ | + Δ |
| CCS1: | + Δ | + Δ |

D

NADPH consumed (μmol/min)

V GRX2
The effects of glutaredoxin and copper activation pathways on the disulfide and stability of Cu/Zn superoxide dismutase

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