Activation of CuZn Superoxide Dismutases from Caenorhabditis elegans Does Not Require the Copper Chaperone CCS*

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Reactive oxygen species are produced as the direct result of aerobic metabolism and can cause damage to DNA, proteins, and lipids. A principal defense against reactive oxygen species involves the superoxide dismutases (SOD) that act to detoxify superoxide anions. Activation of CuZn-SODs in eukaryotic cells occurs post-translationally and is generally dependent on the copper chaperone for SOD1 (CCS), which inserts the catalytic copper cofactor and catalyzes the oxidation of a conserved disulfide bond that is essential for activity. In contrast to other eukaryotes, the nematode Caenorhabditis elegans does not contain an obvious CCS homologue, and we have found that the C. elegans intracellular CuZn-SODs (wSOD-1 and wSOD-5) are not dependent on CCS for activation when expressed in Saccharomyces cerevisiae. CCS-independent activation of CuZn-SODs is not unique to C. elegans; however, this is the first organism identified that appears to exclusively use this alternative pathway. As was found for mammalian SOD1, wSOD-1 exhibits a requirement for reduced glutathione in CCS-independent activation. Unexpectedly, wSOD-1 was inactive even in the presence of CCS when glutathione was depleted. Our investigation of the cysteine residues that form the disulfide bond in wSOD-1 suggests that the ability of wSODs to readily form this disulfide bond may be the key to obtaining high levels of activation through the CCS-independent pathway. Overall, these studies demonstrate that the CuZn-SODs of C. elegans have uniquely evolved to acquire copper without the copper chaperone and this may reflect the lifestyle of this organism.

Although oxygen is essential for aerobic organisms, reactive byproducts of oxygen metabolism such as superoxide anion can be damaging to DNA, proteins, and lipids (1–5). A primary defense against reactive oxygen species involves the superoxide dismutase (SOD) enzymes that act to catalytically remove superoxide ions (6). Eukaryotic cells typically harbor two types of intracellular SODs: a manganese-containing enzyme present in the mitochondrial matrix (7, 8) and a single copper and zinc-containing enzyme that largely localizes in the cytosol (9) but is also found in the intermembrane space of mitochondria (8, 10). In addition, a separate CuZn-SOD that is secreted into the extracellular space is expressed in some organisms (11, 12). In all cases, enzyme catalysis involves redox cycling of the manganese or copper ion at the active site.

The intracellular CuZn-SOD enzyme (SOD1) is typically the most abundant of the SODs (9, 13) and based on studies in yeast and mammals, the activity of this enzyme in vivo is largely controlled by post-translational insertion of the copper cofactor. The copper chaperone for SOD1 (CCS) is the primary source of copper for yeast and mammalian SOD1 (14–16) and physically interacts with SOD1 to insert copper into the active site (17–21) in an oxygen-dependent manner (22). In addition to its role in copper delivery, CCS also catalyzes the oxidation of a conserved disulfide that is essential for activity (23).

CCS homologues have been characterized in several organisms (24–28) and inspection of the genome sequences from fungi, plants, and animal species indicates that CCS has been widely conserved throughout evolution. Saccharomyces cerevisiae shows an absolute requirement for CCS in activation of SOD1 (14). However, mammalian SOD1 can become partially activated in the absence of CCS (16, 29, 30) indicating that an activation pathway independent of CCS is present. Activation through this alternative pathway is sensitive to sequences near the C terminus of SOD1. Specifically, the presence of two proline residues in this region, as are found in S. cerevisiae SOD1, inhibits CCS-independent activation (30). The CCS-independent pathway is known to rely on glutathione for activity (30); however the copper carrier for this pathway has not been identified and how disulfide bond oxidation is occurring remains unknown.

The CCS-independent pathway may play an important role in the activation of CuZn-SODs in organisms other than mammals. However, this alternative activation pathway has not been investigated in other species. An interesting model organism in which to investigate SOD enzymes is the nematode Caenorhabditis elegans. Unlike most eukaryotes that express only two or three SOD molecules, at least four SOD encoding genes have been documented thus far for C. elegans. A CuZn enzyme encoded by sod-1 is predicted to be cytosolic (31), whereas sod-2 and sod-3 encode a pair of manganese-containing enzymes predicted to be mitochondrial (32, 33). A fourth SOD gene, sod-4, is thought to encode the extracellular CuZn enzyme (34). However, nothing is known regarding how the various SOD isoforms from C. elegans are activated in vivo.

In the current study, we investigated the requirements for activation of the intracellular CuZn-SODs from C. elegans by expressing these enzymes in a heterologous system, namely the baker’s yeast S. cerevisiae. In contrast to other eukaryotic CuZn-SODs, the enzymes from C. elegans are efficiently activated in the absence of the CCS copper chaperone and apparently utilize the same glutathione-requiring pathway recently shown to partially activate human SOD1 in cells lacking CCS (30). The ability of C. elegans CuZn-SODs to readily form a critical

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The abbreviations used are: SOD, superoxide dismutase; CCS, copper chaperone for SOD1; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; GSH, glutathione; hSOD1, human SOD1.
disulfide bond may be the key to obtaining high levels of activation in the absence of CCS.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—Yeast strains used in this study were derived from EG103 (MATa, leu2-3,112, his3Δ1, trp1-289, ura3-52) (35) and include KS107 (sod1Δ) (36), LS101 (sod1Δ ccs1Δ) (10), MC107 (sod1Δ gsh1Δ) (30), and LJ349 (sod1Δ ccs1Δ gsh1Δ). Disruption of CCS1 was generated with plasmid pPS003 as described previously (37).

Yeast transformations were performed using the lithium acetate procedure (38). Cells were propagated at 30 °C either in enriched yeast extract, peptone-based medium supplemented with 2% glucose (YPD) or in minimal SD (synthetic dextrose) medium (39).

The wild type C. elegans strain N2 used in this work was provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). C. elegans deletion mutants, sod-1(tm776) and sod-5(tm1146) were provided by Shohei Mitani, Tokyo Women’s Medical University School of Medicine, Japan. Nematodes were propagated at 20 °C on NGM plates or in S medium (40) supplied in each case with the bacterial strain OP50 as a food source.

**Plasmids**—Yeast expression plasmids for the intracellular C. elegans CuZn-SODs were generated using cDNA clones generously provided by Pamela Larsen (sod-1) and the National Institute of Genetics, Japan (sod-5). Both CuZn-SODs from C. elegans with or without sequences coding for the N-terminal extension were PCR-amplified introducing 5’-BamHI and NdeI sites and a 3’-SnaBI site. The PCR products were digested BamHI and SnaBI then ligated into pLS108 (10) cut with the same enzymes allowing for expression under control of S. cerevisiae SOD1 regulatory sequences. sod-1 plasmids pLJ255 and pLJ256 and sod-5 plasmids pLJ257 and pLJ274 encode for the wSODs with (L) or without (S) the N-terminal extension respectively. Mutations in wSOD-1S and ySOD1 were generated using the QuikChange mutagenesis kit (Stratagene). Worm SOD-1 plasmids pLJ279 (A145,147P) and pLJ330 (Y50,S1EE) were derived from pLJ256 and the S. cerevisiae SOD1 (referred throughout as ySOD1) plasmid pLJ300 (P142,144A) was derived from pLS108.

To generate plasmids for expression of recombinant C. elegans SOD molecules, wSOD-1 (L and S) and wSOD-5L were removed from pLJ255, pLJ256, and pLJ257 by digestion with NdeI and SnaBI. The S. cerevisiae CCS1 coding sequence was removed from pLS110 (10) with NdeI and BstZ17I. Digestion with either SnaBI or BstZ17I produces a 5’-BamHI and NdeI sites and a 3’-SnaBI site. The PCR products were digested BamHI and SnaBI then ligated into pLS108 (10) cut with the same enzymes allowing for expression under control of S. cerevisiae SOD1 regulatory sequences. sod-1 plasmids pLJ255 and pLJ256 and sod-5 plasmids pLJ257 and pLJ274 encode for the wSODs with (L) or without (S) the N-terminal extension respectively. Mutations in wSOD-1S and ySOD1 were generated using the QuikChange mutagenesis kit (Stratagene). Worm SOD-1 plasmids pLJ279 (A145,147P) and pLJ330 (Y50,S1EE) were derived from pLJ256 and the S. cerevisiae SOD1 (referred throughout as ySOD1) plasmid pLJ300 (P142,144A) was derived from pLS108.

**Formation of monomer versus dimer in wild type and mutant strains**—Yeast cells expressing various SODs were lysed in a buffer containing 6 M guanidine-HCl, 3 M EDTA, 0.5% Triton X-100, 50 mM Tris-HCl, pH 8.3, alone or in the presence of 15 mM AMS. Lysates were centrifuged to remove cellular debris and then incubated for one h at 25 °C. Unreacted AMS was then removed by gel filtration using G-25 microspin columns (Amersham Pharmacia Biotech). Samples were separated on 14% SDS gels and CuZn-SODs were detected with immunoblots.

**Protein Isolation**—Proteins were expressed in Escherichia coli strain BL21(DE3) transformed with the appropriate plasmids. For the preparation of antibodies, wSOD-1 and wSOD-5 containing the N-terminal extension were initially isolated. Both wSOD-1L and wSOD-5L were present in inclusion bodies, and after repeated washing of the postsonication pellet with 1 M urea, 1% Triton X-100, 10 mM Tris-HCl, pH 8, to remove contaminating proteins the inclusion bodies were solubilized with 8 M urea. The urea was removed by repeated dialysis against phosphate-buffered saline, and antibody production was performed by Cocalico Biologicals, Reamstown, PA. The antibody generated against wSOD-1L (JH766) displayed strong reactivity with CuZn-SODs from C. elegans as well as SOD1 from S. cerevisiae, mice, and humans and was used at a 1:5000 dilution in all immunoblots.

For copper transfer experiments wSOD-1 without the N-terminal extension was purified. When co-expressed with bacterial thioredoxin (42) and grown at 30 °C wSOD-1S was present primarily in the soluble fraction and was isolated using anion exchange chromatography following the procedure for yeast SOD1 (18). Yeast CCS1 was isolated from bacteria using anion exchange and size exclusion chromatography and reconstituted with copper as described by Rae et al. (18). A single band on SDS-PAGE was observed for the isolated proteins.

**Biochemical Assays**—SOD enzymatic activity was assayed from yeast grown shaking in selecting SD medium to an A600 of 1.5. Nematode cultures were grown in S medium for 5 days and contained predominantly L3, L4, and adult animals and were separated from the bacterial food source by flotation on sucrose (40). Yeast extracts were generated using the glass bead lysis protocol (43), and a modification of this procedure was used to generate nematode lysates. Nematode samples were resuspended in twice the volume of 0.6 M sorbitol, 10 mM HEPES, pH 7.5, containing protease inhibitors, and 425–600-μm glass beads were added equal to the volume of worms in each sample. Samples were then vortexed for 6 min, and debris was removed by centrifugation at 12,000 × g. Extracts were then filtered through a 0.45-μm membrane, and the protein content was measured by the method of Bradford (44). Sorbitol was included in the extraction buffer for both yeast and nematode samples to prevent the release of SOD2 from the mitochondria matrix. Mitochondrial and postmitochondrial supernatant fractions were isolated from yeast cells grown in YPD medium as described previously (10), and the relative purity of each fraction was monitored using antibodies against the cytosolic protein Pgk1 (Molecular Probes) and the mitochondrial protein Mas2 (a generous gift from Robert Jensen) and were used at a 1:10,000 dilution. An analysis of SOD activity by non-denaturing gel electrophoresis and staining with nitro blue tetrazolium, and the immuno blot analysis of SOD levels was performed as described previously (45) except that 10 mM EDTA was added to the native running buffer to prevent copper incorporation during electrophoresis.

To monitor disulfide bond formation in SODs the reactivity of cysteine residues to AMS (4-acetamido-4’-maleimidylstilbene-2,2’-disulfonic acid) was used. AMS forms a stable 0.5-kDa thiol-ether linkage with free cysteines, resulting in polypeptide mobility shifts (23). AMS modification of CuZn-SODs from yeast lysates was performed according to the method of Carroll et al. (23) Yeast cells expressing various SODs were lysed in a buffer containing 6 M guanidine-HCl, 3 M EDTA, 0.5% Triton X-100, 50 mM Tris-HCl, pH 8.3, alone or in the presence of 15 mM AMS. Lysates were centrifuged to remove cellular debris and then incubated for one h at 25 °C. Unreacted AMS was then removed by gel filtration using G-25 microspin columns (Amersham Pharmacia Biotech). Samples were separated on 14% SDS gels and CuZn-SODs were detected with immunoblots.

Formation of monomer versus dimer in wild type and mutant wSOD-1 molecules was monitored in yeast lysates using a 90 cm G-75 (Pharmacia Corporation) gel filtration column equilibrated with phosphate-buffered saline and 1 mM dithiothreitol. Fractions were collected and assayed for the presence of wSOD-1 using immunoblots after separation on 14% SDS gels. The column was calibrated for the estimation of molecular mass using bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12 kDa) as protein standards.

Worm SOD-1 for copper transfer experiments was prepared using a modification of the procedure for Furukawa et al. (23). The isolated wSOD-1S protein was treated with 200 mM dithiothreitol at 37 °C for 2 M. Carroll and V. Culotta, unpublished observations.
1 h to reduce the disulfide bond followed by acidification with 0.4% trifluoroacetic acid. Metal chelators (20 mM EDTA and 2 mM bathocuproine sulfonate) and organic solvents (15% CH$_3$CN and 10% CH$_3$OH) were then added, and the sample was incubated at 25 °C for 2 h. The wSOD-1 protein was then precipitated with 10% trichloroacetic acid and washed with acetone. The pellet was dissolved in 0.1% trifluoroacetic acid, and fractions containing wSOD-1 were collected. Activation assays were performed essentially as described previously (18) and contained 0.4 mM apo-wSOD-1, 1 mM GSH, and 4 mM ZnCl$_2$ in 50 mM sodium phosphate buffer, pH 7.8. As indicated 2 mM copper as Cu-GSH or Cu-$\gamma$CCS was included with or without the addition of 40 mM bathocuproine sulfonate. Samples were incubated for 1 h in air at 25 °C, and SOD activity was assayed using native activity gels.

RESULTS

C. elegans Contains Two Intracellular CuZn-SODs—A single intracellular CuZn-SOD gene, sod-1, has been reported in C. elegans (31). However, an inspection of the data base revealed a second CuZn isoform encoded by ZK430.3, which we have designated sod-5. For the purposes of this study we will refer to the C. elegans CuZn-SOD enzymes as wSOD-1 and wSOD-5 and as can be seen in Fig. 1, wSOD-1 and wSOD-5 from C. elegans are highly homologous to SOD1 from yeast and humans. It is notable that the protein sequence of wSOD-5 contains an N-terminal extension not seen in other CuZn molecules. Although the start codon for wSOD-1 was identified based on homology with other CuZn-SODs (31), our inspection of the available wSOD-1 sequences revealed a sequence similar to the wSOD-5 N-terminal extension containing another potential start site (Fig. 1). The presence of this sequence upstream of the reported wSOD-1 start site suggested that wSOD-1 could also have an N-terminal extension, although the actual start codon is still unknown.

To address whether sod-5 (as well as sod-1) encoded a functional superoxide dismutase activity, we chose to express the C. elegans CuZn-SODs in a yeast sod1Δ mutant lacking endogenous CuZn-SOD1. For these studies, we expressed both the long and short forms of wSOD-1 and wSOD-5 that contain or lack the putative N-terminal extension (see Fig. 1). Superoxide dismutase activity was assayed in soluble extracts prepared from yeast expressing each of the four types of C. elegans CuZn-SOD. Activity was apparent in extracts from cells expressing C. elegans SOD molecules (Fig. 2, lanes 2–5) but not in control cells (lane 1). Our yeast expression studies demonstrate that the long and short forms of wSOD-1 and wSOD-5 are active and exhibit very different mobility on native gels allowing us to easily distinguish between these enzymes.

We also tested for endogenous wSOD-1 and wSOD-5 in lysates from C. elegans. SOD activity was detected in soluble extracts prepared from cultures containing a mixed population of worms (Fig. 2, lane 6). C. elegans strains containing loss of function deletions in sod-1 and sod-5 were assayed for SOD activity, and this analysis revealed that the primary CuZn-SOD in actively growing worms is wSOD-1 (Fig. 2, lanes 7 and 8). wSOD-1 from worm lysates migrates to the same position as the short version of wSOD-1 expressed in yeast, indicating that if the N-terminal extension is part of the polypeptide, this sequence is absent in the mature enzyme (Fig. 2, compare lanes 2 and 3 with lane 6). Although wSOD-5 activity was not observed from a mixed population of worms, this SOD may be expressed at a specific growth stage. Microarray analysis does indicate that expression of sod-5 is increased in dauer larva (46).

C. elegans CuZn-SODs Are Extremely Active in the Absence of CCS—Eukaryotic CuZn-SOD molecules are generally believed to be posttranslationally activated by the CCS copper chaperone. CCS homologues have been identified by the inspection of genome sequences in organisms ranging from fungi and plants to various vertebrates and invertebrates; however, upon a detailed inspection of the C. elegans genome, we failed to detect an obvious CCS homologue in this organism. We therefore addressed whether the CuZn-SODs from C. elegans required CCS for activity when expressed in the heterologous S. cerevisiae system. In each case, the SODs were placed under the control of the S. cerevisiae sod1Δ promoter. C. elegans CuZn-SODs as well as human and S. cerevisiae SOD1 were expressed in yeast sod1Δ and sod1Δ ccs1Δ mutants lacking endogenous CuZn-SOD1 and CCS. As seen in Fig. 3, the control yeast SOD1 exhibits no activity in cells lacking the CCS copper chaperone, as this SOD is known to be fully dependent on CCS for activity (14) and human SOD1 retains only partial activity without CCS (29, 30). Interestingly, wSOD-1S and wSOD-5L expressed in yeast display no loss of activity in the absence of CCS.
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FIGURE 2. wSOD-1 and wSOD-5 are both active CuZn-SODs. Lysates were prepared from sod1Δ yeast expressing a control vector (V) or variants of wSOD-1 and wSOD-5 with (L) or without (S) the N-terminal extension (lanes 1–5). Lanes 6–8 contain lysates from wild type C. elegans (N2) or strains lacking wSOD-1 (sod-1(tm776)) and wSOD-5 (sod-5(tm1146)). SOD activity was analyzed using native gels followed by staining with nitro blue tetrazolium.

FIGURE 3. CuZn-SODs from C. elegans are efficiently activated in the absence of CCS. S. cerevisiae (yeast), human, and C. elegans CuZn-SODs were expressed in sod1Δ yeast with or without the CCS copper chaperone. SOD activity was monitored as in Fig. 2 (top panel), and protein levels were determined using immunoblots (bottom panel).

CCS (Fig. 3, top panel) indicating that these enzymes are efficiently utilizing a CCS-independent copper delivery pathway. Similar results were obtained with variants of wSOD-1 and wSOD-5 containing or lacking the N-terminal extension (data not shown), and wSOD-1 without the N-terminal extension was used in the remainder of this study. A polyclonal antibody generated against wSOD-1L was found to strongly react with C. elegans SODs as well as yeast and human SOD1 and was used for immunoblots from yeast lysates. Similar wSOD levels in the presence or absence of CCS (Fig. 3, lower panel) were observed indicating that the activity seen with the wSODs in strains lacking CCS is not because of increased accumulation of these proteins.

wSOD-1 Does Not Accumulate within Yeast Mitochondria—A fraction of SOD1 and CCS have been found within the mitochondrial intermembrane space in yeast (8, 10) and mammalian cells (47). Apo-ySOD1 with the disulfide bond reduced is imported into mitochondria (48), and its retention within the intermembrane space requires CCS (10, 48). This raises the question as to whether wSODs that do not rely on CCS for activation would accumulate within the mitochondria. Monitoring the mitochondrial levels of wSOD-1 revealed that compared with ySOD1 the accumulation of wSOD-1 in S. cerevisiae mitochondria was quite low. Furthermore, the presence of CCS had no effect on the mitochondrial accumulation of wSOD-1 (Fig. 4, compare lane 6 with 8). In fact, the amount of wSOD-1 in mitochondrial fractions was similar to that seen for ySOD1 in the absence of CCS (Fig. 4, lanes 4, 6, and 8). A similar pattern of localization was observed for wSOD-5 (data not shown). Therefore, CCS not only fails to activate C. elegans CuZn-SOD1 but also plays no role in mitochondrial accumulation of the polypeptide.

GSH Is Required for CCS-independent Activation of wSOD-1—The CCS-independent activation of human SOD1 (hSOD1) was previously shown to be sensitive to changes in intracellular glutathione, whereas the CCS-mediated pathway was not (30). Specifically, depletion of glutathione through a gsh1Δ mutation inhibited the CCS-independent activation of hSOD1 (Ref. 30 and Fig. 5). We tested whether activation of wSOD-1 is similarly sensitive to changes in glutathione. The wild type strain was used as a control in this experiment instead of supplementing the gsh1Δ strain with glutathione because of the poor import of glutathione into yeast cells (30). As is seen with hSOD1, the activation of wSOD-1 is inhibited by depletion of glutathione through gsh1Δ mutations (Fig. 5). However, unlike hSOD1, a gsh1Δ mutation also reduced activation of wSOD-1 in cells expressing CCS (Fig. 5, compare lane 3 with 7). Because wSOD-1 remains dependent on glutathione in the presence of CCS, the CCS copper chaperone may not participate at all in the activation of wSOD-1 in vivo. To address this further, we employed mutants of SOD1 known to block the glutathione-dependent copper delivery pathway.

wSOD-1 Exclusively Utilizes the CCS-independent Pathway in Vivo—Previously, we showed that prolines at positions 142 and 144 (as are naturally found in S. cerevisiae SOD1) will block CCS-independent activation and only allow activation by CCS, whereas non-proline residues at these positions (e.g. serine and leucine as is found in hSOD1) allow both CCS-dependent and CCS-independent activation (30). As expected, based on the activation in the absence of CCS, the amino acid sequences of wSOD-1 and wSOD-5 contain non-proline residues at positions corresponding to 142 and 144 in yeast SOD1 (see Fig. 1).

Substitution of these residues with proline in wSOD-1 (A145,147P) was found to not only block activation through the CCS-independent pathway but this mutant wSOD-1 is also inactive in the presence of CCS (Fig. 6 lanes 7 and 8). This finding is consistent with the results obtained with glutathione depletion (Fig. 5, lanes 7 and 8) that indicate wSOD-1 only obtains copper through the CCS-independent, glutathione-requiring pathway. When the CCS-independent pathway is blocked through either insertion of prolines at 145 and 147 or depletion of glutathione, activation of wSOD-1 is lost.

Disulfide Formation Occurs in wSOD-1 when Copper Insertion Is Blocked—Because both copper insertion and disulfide bond formation are necessary for activation of CuZn-SODs (14, 18, 23) we tested whether the reactivity of the disulfide bond in wSOD-1 was influencing its activation. The status of the disulfide bond in wSOD-1 was evaluated through the use of the thiol modification reagent AMS, which causes a shift in mobility observable on denaturing gels and has been used previously with ySOD1 (23). As expected, the copper loaded active SODs from either yeast or C. elegans (ySOD1/CSS+ and WT wSOD-1/CCS−) are present with the disulfide bond oxidized (AMS unreactive).
Disulfide formation occurs in wSOD-1 when copper insertion is blocked. Yeast cells expressing the indicated SODs were lysed in buffer alone or in the presence of 15 mM AMS. After modification unreacted AMS was removed, samples were separated on SDS gel, and SODs were detected with immunoblots as in Fig. 3. Arrows indicate A, disulfide-reduced (AMS reactive), and B, disulfide-oxidized (AMS unreactive) SODs.

In vitro activation and efficient utilization of alternative, CCS-independent pathway (Fig. 7, compare lane 4 with 8). Even in the absence of copper loading through the CCS-independent pathway the disulfide bond in wSOD-1 can be readily formed.

The disulfide in CuZn-SOD molecules is known to play a critical role in stabilizing the SOD1 homodimer (49, 50). In the case of ySOD1, disulfide bond formation has been shown to be sufficient to promote dimerization in vitro even in the absence of copper loading (23). Indeed we find that wSOD-1 molecules that lack copper because of inhibition of the CCS-independent pathway (e.g. through either depletion of glutathione or an A145P,147P mutation) remain dimers (Fig. 8). The ability of wSOD-1 to dimerize in the absence of copper activation is consistent with its high propensity to oxidize the disulfide.

Apo-reduced wSOD-1 is a Substrate for Cu-CCS in Vitro—The high tendency of wSOD-1 to form an oxidized disulfide in vivo may explain the inability to obtain copper via CCS. Furukawa et al. (23) have shown that ySOD1 can only be activated by CCS when the disulfide bond is reduced, and the high degree of disulfide formation seen with wSOD-1 in vivo may be inhibiting CCS-mediated activation. Alternatively, the lack of CCS reactivity with wSOD-1 in vivo may reflect an inherent inability of wSOD-1 to dock with this copper chaperone. To discern between these possibilities, we tested whether recombinant CCS could activate wSOD-1 present with the disulfide bond reduced in an in vitro reconstitution assay. As seen in Fig. 9, apo-reduced recombinant wSOD-1 can be activated by Cu-GSH and Cu-CCS (lanes 2 and 4). Although the addition of the copper chelator bathocuproine sulfonate was sufficient to inhibit activation of wSOD-1 by Cu-GSH this same treatment did not inhibit activation by Cu-CCS (Fig. 9, lanes 3 and 5). This pattern of activation is identical to that observed by Rae et al. (18) for ySOD1 and indicates a direct transfer between Cu-CCS and wSOD-1, as copper released from CCS would be rapidly bound by the excess bathocuproine sulfonate in the reaction. Clearly wSOD-1 does have the capacity to acquire copper from CCS when the disulfide bond is reduced. However, the fraction of apo-wSOD-1 that is present in the disulfide reduced form in vivo appears to be extremely small.

**DISCUSSION**

Copper chaperones are required not only for the proper delivery of copper within cells but also to minimize the toxic effects of copper ions. The primary route for incorporation of copper into CuZn-SODs in yeast and mammalian cells is the CCS copper chaperone (14–16); however C. elegans appears distinct from other eukaryotes as an obvious CCS homologue is not present in its genome. Herein, we demonstrated that CuZn-SOD molecules from C. elegans do not require CCS for activation and efficiently utilize an alternative, CCS-independent pathway.
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Figure 9. Apo-reduced wSOD-1 is a substrate for CCS in vitro. Apo reduced wSOD-1 was incubated with Cu-GSH or Cu-yCCS for 1 h in air with or without the addition of the copper chelator bathocuproine sulfonate (BCS). SOD activity was assayed using native activity gels as described in Fig. 2. 160 ng of wSOD-1 was present in each lane.

that requires glutathione. In contrast to ySOD1, the intramolecular disulfide bond in CuZn-SODs from C. elegans is readily oxidized in vivo, even in the absence of copper loading. The propensity to efficiently oxidize the disulfide bond may be the key difference between SODs from C. elegans and other eukaryotes that assist in the utilization of alternative copper sources for activation of this enzyme.

To date the only CuZn-SODs that are known to show no dependence on CCS are those from C. elegans. By comparison, human SOD1 from cells lacking CCS display only partial activity (16, 29, 30), and S. cerevisiae SOD1 is completely dependent on CCS for activity (14). The characterization of the C. elegans CuZn-SODs in this study was performed using the heterologous S. cerevisiae system, and biochemical differences between S. cerevisiae and C. elegans could affect the activation of the worm CuZn-SODs. However, the activation of human SOD1 through the CCS-independent pathway appears to be quite similar when expressed in yeast or in human cells (30) suggesting that the S. cerevisiae system is suitable for studying this alternative route for activation of CuZn-SODs.

Sequence differences between yeast and human SOD1 have been shown to influence CCS-independent activation (30), specifically the presence of non-proline residues at positions 142 and 144 (relative to hSOD1) was found to be necessary for CCS-independent activation of CuZn-SODs. However, hSOD1, which has non-proline residues at these positions, is only partially active in the absence of CCS indicating that other sequences are necessary for the efficient activation through the CCS-independent pathway that is seen with C. elegans CuZn-SODs. Although these precise sequences are not yet known, the two amino acid sequence insertions that are unique to C. elegans CuZn-SODs (segments "11" and "12"; see Fig. 1) are not adequate to promote efficient activation in the absence of CCS, although these sequences are important for maximal activity of CuZn-SODs (data not shown). The N-terminal extensions in CuZn-SODs also do not have any impact on activation, and these residues are not present in the mature CuZn-SOD-1 found in extracts from C. elegans. Other less obvious sequences specific to CuZn-SODs appear to be needed to confer the high level of CCS-independent activity observed. To fully understand the means by which C. elegans SODs preferentially employ the CCS-independent pathway structural information may be required. To this end, the three-dimensional structure of C. elegans CuZn-SOD is currently under investigation.

The factors involved in the CCS-independent activation of CuZn-SODs remain largely unknown; however the partial activation of hSOD1 in the absence of CCS requires reduced glutathione (30). A requirement for glutathione was also observed for the activation of CuZn-SOD-1 suggesting that although CuZn-SOD-1 is activated more efficiently in the absence of CCS than hSOD1, both of these enzymes utilize the same glutathione-dependent activation pathway. Although Cu(I)-GSH complexes can donate copper to SOD1 in vitro (51), GSH may not be acting directly as a copper carrier in CCS-independent activation. Disruption of glutathione reductase (GLR1) is known to alter the cellular redox potential without a substantial decrease in the concentration of reduced glutathione (52) as well as inhibiting the CCS-independent activation of hSOD1 (30). Taken together these findings suggest a redox role for GSH in the CCS-independent pathway. However, it seems unlikely that copper is present free in the cytosol (18) because of the ability of copper ions to cause toxicity, suggesting that an unidentified copper carrier may be involved in CCS-independent activation.

In addition to insertion of the copper cofactor, a critical step in the activation of CuZn-SODs is the oxidation of an unusual cytosolic disulfide bond (23, 49). Considering the reducing environment present in the cytosol it is not surprising that the disulfide bond in ySOD1 is present predominantly in the reduced form unless oxidized by CCS (23). However, we observed that unlike ySOD1 the disulfide bond in wSOD-1 exists primarily in the oxidized form when copper insertion is blocked. How the oxidation of the disulfide bond in C. elegans CuZn-SOD molecules is occurring is unclear; however, sequence and/or structural differences in the wSODs compared with other SOD1 molecules may increase the reactivity of the cysteines that form the disulfide bond. The enhanced oxidation of the disulfide bond in wSOD-1 may also explain the low mitochondrial accumulation that we observed in the yeast expression system. Previous studies demonstrated that only SOD1 molecules with the disulfide bond reduced are efficiently imported into mitochondria (47, 48). If wSOD-1 forms the disulfide prior to association with the mitochondria, this would preclude mitochondrial uptake. In any case, the ready ability of wSOD-1 to oxidize the disulfide bond would eliminate the need for the disulfide oxidase function of CCS (23) and may allow the C. elegans CuZn-SODs to utilize non-CCS copper sources. Although CCS can only activate SOD1 molecules with a reduced disulfide (23), it is possible that both reduced and oxidized forms of wSODs are substrates for the CCS-independent pathway.

It is puzzling as to why C. elegans has evolved to efficiently activate CuZn-SODs without the aid of the CCS copper chaperone. C. elegans has been characterized in fungi, animals, and plants (14, 16, 24, 53–55) indicating that this gene was present prior to the evolutionary divergence of eukaryotes and was subsequently lost in other eukaryotes that assist in the utilization of alternative copper sources for activation of CuZn-SODs.
ical antioxidant enzyme. Complex organisms, such as mammals, contain many cellular environments that may necessitate the use of multiple copper delivery pathways. In fact, incorporation of copper through the CCS-independent pathway has been suggested to alter the stability of mutant hSOD1 proteins involved in amyotrophic lateral sclerosis (ALS) also known as Lou Gehrig’s disease (62). Further characterization of the CCS-independent activation pathway used by wSODs should aid in our understanding of copper metabolism not only in C. elegans but also in other organisms such as humans that utilize this alternative activation pathway.

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