Instability of Superoxide Dismutase 1 of Drosophila in Mutants Deficient for Its Cognate Copper Chaperone

Received for publication, September 15, 2008, and in revised form, October 22, 2008. Published, JBC Papers in Press, October 23, 2008, DOI 10.1074/jbc.M807131200

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Copper,zinc superoxide dismutase (SOD1) in mammals is activated principally via a copper chaperone (CCS) and to a lesser degree by a CCS-independent pathway of unknown nature. In this study, we have characterized the requirement for CCS in activating SOD1 from Drosophila. A CCS-null mutant (ccs29E) of Drosophila was created and found to phenotypically resemble Drosophila SOD1-null mutants in terms of reduced adult life span, hypersensitivity to oxidative stress, and loss of cytosolic aconitase activity. However, the phenotypes of CCS-null flies were less severe, consistent with some CCS-independent activation of Drosophila SOD1 (dSOD1). Yet SOD1 activity was not detectable in Ccsc28E flies, due largely to a striking loss of SOD1 protein. In contrast, human SOD1 expressed in CCS-null flies is robustly active and rescues the deficits in adult life span and sensitivity to oxidative stress. The dependence of dSOD1 on CCS was also observed in a yeast expression system where the dsOD1 polypeptide exhibited unusual instability in CCS-null (ccs1Δ) yeast. The residual dsOD1 polypeptide in ccs1Δ yeast was nevertheless active, consistent with CCS-independent activation. Stability of dsOD1 in ccs1Δ cells was readily restored by expression of either yeast or Drosophila CCS, and this required copper insertion into the enzyme. The yeast expression system also revealed some species specificity for CCS. Yeast SOD1 exhibits preference for yeast CCS over Drosophila CCS, whereas dsOD1 is fully activated with either CCS molecule. Such variation in mechanisms of copper activation of SOD1 could reflect evolutionary responses to unique oxygen and/or copper environments faced by divergent species.

†This work was supported, in whole or in part, by National Institutes of Health Grant GM50016 (to V. C. C.) and NIEHS (Johns Hopkins University). This work was also supported by Natural Sciences and Engineering Research Council of Canada (to J. P. P.) and the Canadian Institutes of Health Research (to J. P. P. and A. J. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: SOD1, Cu,Zn superoxide dismutase; SOD2, Mn superoxide dismutase; cACON, cytoplasmic aconitase; CCS, copper chaperone for Cu,Zn superoxide dismutase; dSOD1, Drosophila SOD1; dCCS, Drosophila CCS; hSOD1, human SOD1; mACON, mitochondrial aconitase; TEMED, tetramethylethylenediamine; yCCS, yeast CCS; 6-HD, 6-hydroxydopamine.

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Drosophila CCS

opment, survival, and aging. Mutant Drosophila lacking SOD1 survive well as embryos and larvae but experience high rates of mortality as late pupae and early adults. Surviving adults exhibit a median life span about 10% of normal, severely reduced fertility, hypersensitivity to a variety of oxidative stress conditions (10), retinal degeneration (11), and increased rates of spontaneous somatic and germ line mutation (12). The high rate of early mortality in SOD1-null mutants is related to a general pattern of premature aging as reflected by the precocious onset and rapid progression of patterns of marker gene expression that typify normal senescence (13). Clearly, SOD1 plays a critical role in the biology and life history of Drosophila, but the biochemistry of the maturation process for this critical enzyme has not been explored. Here we describe a single CCS-encoding gene from Drosophila and the biological and biochemical consequences of losing the copper chaperone for SOD1.

EXPERIMENTAL PROCEDURES

Drosophila Stocks, Culture Conditions—The Sod1\(^{108}\) and ry\(^{+5}\) strains are described in Ref. 10. hSOD1 was expressed using lines carrying UAS-hSod1 and the GAL4 driver line, daGAL4\(^{G32}\) as described (14). Stocks were maintained at 25 °C on standard cornmeal and agar medium unless otherwise stated.

The copper chaperone coding region was amplified by PCR (primers CCS5 + CCS7) using DNA extracted from a Drosophila Canton S adult cDNA library (Stratagene catalog number 936603). The 930-bp PCR product was blunt-ended and subcloned into the Smal site of pBluescriptIISK (Stratagene) to generate the vector pBCC. The transformation vector pUCC was constructed by subcloning the EcoRI-NotI fragment of plasmid pHAL-413 (4) resulting in plasmid pLJ373. The digested dCCS PCR product was inserted into pLJ366 (a derivative of pLS113 (18) with an MluI site just prior to the yCCS1 start codon and an RsrII site immediately after the stop codon), replacing the yeast SOD1 coding sequence with that of dCCS, generating plasmid pLJ375. Both dSOD1 and dCCS were under control of their corresponding S. cerevisiae regulatory sequences. The sequence integrity of these plasmids was ensured by double-stranded DNA sequencing (DNA Analysis Facility, The Johns Hopkins University). Yeast CCS1 plasmids pHAL-413, pLS008 (C229S, C231S), and pLS010 (K136E, G137E) have been described previously (18). Domain I mutation in yCCS1 (C175, C205S) was introduced using the QuikChange mutagenesis kit (Stratagene) using pHAL-413 as a template (4) resulting in plasmid pPS031.

SOD1 In-gel Activity Assay—To analyze SOD1 activity from Drosophila, five males were homogenized in 50 μl of 1% Triton X-100. 10 μl of supernatant was transferred to a tube containing an equal volume of loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 0.01% bromphenol blue). 20 μl was run on a 10% polyacrylamide (4% stacking) gel. The gel was incubated in nitro blue tetrazolium (2 mg/ml) for 5 min followed by incubation in 36 mM K3HPO4, 0.035 mM riboflavin, 0.3% TEMED for 7 min and then washed several times in H2O as described (20).

SOD enzymatic activity was assayed from yeast grown shaking in selected SC medium to an A\(_{600}\) of 1.5. Yeast lysates were generated by glass bead homogenization in a buffer containing 0.6 M sorbitol, 10 mM HEPES, pH 7.2, and protease inhibitors. Analysis of SOD activity by non-denaturing gel electrophoresis using 12% pre-cast gel (Invitrogen) and staining with nitro blue tetrazolium was performed as described previously (21) except that 10 mM EDTA was added to the native running buffer.

SOD1 Spectrophotometric Assay—24–48-h-old males were homogenized in B1 (50 mM sodium phosphate, pH 7.4, 0.1 mM EDTA). The supernatant was extracted twice with chloroform (0.15 V), ethanol (0.25 V) with a 15-min incubation time for each extraction. SOD1 activity was determined spectrophotometrically by monitoring the autoxidation of 6-hydroxydopamine (6-HD) at 490 nm at 37 °C in 500 μl of B1 containing 0.1 mM 6-HD (22). Protein concentration was determined by using the Bio-Rad protein assay.

Aconitase Activity Assay—30 adult males were homogenized in 120 μl of extraction buffer (0.6 mM MnCl2, 2 mM citric acid, 50 mM Tris-HCl, pH 8.0) and centrifuged at 13,000 × g. Samples were electrophoresed on Sephraphore III membranes (Pall Corp.). Aconitase was detected by incubation of the membrane in 100 mM potassium phosphate, pH 6.5, 1 mM NADPH, 2 mM cis-aconitic acid, 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide, 0.3 mM phenazine methosulfate, 25 mM MgCl2, and 5 units/ml isocitrate dehydrogenase (23, 24).

Western Blotting—For analysis of SOD1 from Drosophila, males were homogenized in 1% Triton X-100. The extracts were centrifuged at 13,000 × g for 5 min at 4 °C, and the supernatant was transferred to a tube containing an equal volume of loading buffer (125 mM Tris-HCl, pH 7.2, 4% SDS, 10% mercaptoethanol, 20% glycerol, 0.01% bromphenol blue). Samples were boiled for 5 min, and 10 μg of total protein was separated on a SDS-polyacrylamide gel (4% stacking, 15% separating). The protein was transferred to nitrocellulose membrane (ECL) and probed with sheep anti-human Cu,Zn superoxide dismutase (Cedarlane catalog number K9007C) and rabbit anti-sheep IgG horseradish peroxidase conjugate (Chemicon catalog number AP147P). The ECL Western blotting detection reagents (RPN 2109, Amersham Biosciences) was used for detection.

Immunoblot analysis of Cu,Zn-SOD expressed in yeast was carried out with cell lysates prepared as described above.
and a polyclonal antibody that displays strong reactivity with Cu,Zn-SODs from several species (JH765) at a 1:5000 dilution (8). Detection utilized an Alexa Fluor 680 goat anti-rabbit secondary antibody (Invitrogen), and immunoblots were visualized using the Odyssey infrared imagining system (Lior Biosciences).

**Drosophila Life Span Determination** — 0–24-h-old males were collected, 25 males per vial, and kept on cornmeal food containing tegosept. 200 males of each line were used. The vials were kept at 25 °C. Flies were transferred to fresh food every 2–3 days.

**Drosophila Paraquat Resistance** — 0–24-h-old males were collected and kept on cornmeal food for 24 h, 10 males in each vial. They were then transferred to vials containing 3-mm paper filter disks saturated with 250 μl of 1% sucrose, 1% sucrose containing 2 mM paraquat (Sigma) or 1% sucrose and 5 mM paraquat. The vials were stored at 25 °C in the dark, and flies were enumerated after 24 h as originally described (10).

**RESULTS**

dCcs, the Copper Chaperone of SOD1 Gene of Drosophila — The Drosophila genomic homologue of yeast and mammalian Ccs was initially identified by isolation of a SOD1-like sequence from a Drosophila genomic library, later verified by sequence annotation in Flybase (CG17753, FBgn0010531), and confirmed by amplification of a Ccs cDNA from a D. melanogaster cDNA library (Fig. 1A). The architecture of the native Drosophila gene includes a 795-bp coding sequence interrupted by two small introns of 51 and 61 bp, respectively. The nearest open reading frame (CG11867) is ~5 kb upstream from the 5′-transcription start site. The encoded sequence contains the expected region of high homology to SOD1 (2) in the central domain II of CCS; the domain III CXC is conserved. Unexpectedly, the domain I CXXC copper-binding motif is absent (Fig. 1B). A comparison to all known CCS-like molecules in the data base revealed that the absence of a CXXC motif in Drosophila CCS is unique. Of 20 CCS sequences analyzed from fungi to humans, only D. melanogaster and Anopheles gambiae lack the MXCXC copper-binding site (Table 1). However, all 20 CCS molecules retain the CXC copper site in domain III.

**Ccs Deletion Mutation** — A loss of function mutation of Ccs (Ccs29E), generated by an apparent imprecise excision of a P element located near Ccs in the autosomal enhancer-detector parent strain, H340 (25), was identified by recovery of a strain, 29E, with reduced SOD1 activity and a set of phenotypes exhibited by known SOD1 deficiency mutants (see below). Sequence analysis of Ccs, restriction analysis by Southern hybridization, and SOD1 activity all indicated that Ccs is functionally and structurally intact in H340. The genomic deletion in Ccs29E includes 1617 bp of 5′-upstream region and 1907 bp downstream from the transcription start site, including all of the first exon, the first intron, and 124 bp of the second exon (Fig. 1A). Ccs transcripts are relatively rare throughout normal develop-
ment in H340 and are undetectable by Northern blot analysis in 29E (data not shown).

Loss of SOD1 Enzymatic Activity in CCS-null Flies—Because the only known assay for CCS function is via its role in the activation of SOD1, we assayed SOD1 activity in extracts of H340 and 29E using two functionally different methods, the in-gel with nitro blue tetrazolium reduction interference assay and the spectrophotometric 6-hydroxydopamine reduction interference assay (Fig. 2, A and B). Using both methods, we were unable to detect significant SOD1 activity in 29E. Loss of CCS in 29E appears to have no effect on the activity of the mitochondrial manganese-containing SOD2 (see Fig. 4A). These results by themselves would suggest that CCS in Drosophila, as in yeast and mammals, is specifically required for activating SOD1.

However, analysis of SOD1 protein levels revealed that the steady state level of SOD1 polypeptide in 29E is reduced to about one-quarter of its normal level in H340 (Fig. 2C). Thus, the loss of SOD1 activity in 29E probably arises primarily from the reduced level of SOD1 protein in the absence of CCS. Further analysis confirmed that the lack of SOD1 activity in 29E is not because of a mutation in the Sod1 gene.4

4 The possibility remained that the lack of SOD1 activity in the Css deletion strain, 29E, was actually caused by a cryptic loss-of-function mutation in the Sod1 gene itself. We tested this possibility by examining the SOD1 dimeric isoforms present in hybrids generated by crossing 29E to a strain bearing Sod1*, a mutant allele that encodes an electrophoretically slow but enzymatically active variant of SOD1. The capacity of the Sod1 gene in 29E to produce a functionally active SOD1 apoprotein can be diagnosed by the presence of a novel SOD1*/SOD15 dimeric protein in the 29E/Sod15 hybrid. The data in supplemental Fig. S1, showing the presence of an enzymatically active SOD1*/SOD15 hybrid, provides conclusive evidence that the lack of SOD1 activity in 29E is not because of a mutation in the Sod1 gene. We are therefore able to conclude with confidence that the lack of SOD1 activity and SOD1 protein in 29E is the indirect consequence of the loss of CCS and not because of a loss-of-function mutation in the Sod1 gene.

Deficiency of CCS Reduces the Functional Level of Specific Iron-Sulfur Protein Sentinels—The cytosolic and mitochondrial aconitases both contain cubane [4Fe-4S] centers that are sensitive targets of inactivation by superoxide (23, 26). Steady state levels of these activities in Drosophila are significantly reduced in the absence of SOD1 and SOD2, respectively (24). Assay of cACON and mACON activities in 29E shows that cACON activity is selectively depleted by about 50% (H340−1.6×29E), with no detectable effect on the activity of mACON (Fig. 2D). Taken together, these data indicate that at the biochemical level, the absence of CCS is equivalent to a deficiency in SOD1. The important question then arises, does this parallel extend to whole organism phenotypes?

Genetic Ablation of CCS Elicits Broad Phenotypic Consequences—At a minimum, the mutational loss of CCS should generate a set of phenotypes predicted by the loss of SOD1, including reduced adult life span with little or no overt effect on pre-adult development and hypersensitivity to applied oxidative stress. Both of these expected phenotypes are realized. Fig. 3A shows that the absence of CCS confers early onset adult mortality with an ∼30% reduction in the median adult life span. In contrast, 29E displays the extreme toxic hypersensitivity to the redox cycling agent, paraquat, exhibited by Sod1-null (Fig. 3B). In these respects, 29E appears to phenocopy a weakly expressing SOD1 hypomorphic mutant. Based on previous studies with RNA interference-mediated knockdown of SOD1 (27), and transgenic rescue of SOD1-null mutants (28), which show that paraquat toxicity is a more sensitive indicator of SOD1 deficiency than life span, the phenotype of 29E is consistent with the presence in 29E of a very small residual level of SOD1 activity which, although below the level of detection by conventional assays of whole-fly extracts, is sufficient to extend median adult life span (∼30 days) beyond the 10-day median life span of SOD1-null mutants. Such activity, if it were to occur, would have to arise via CCS-independent activation of apo-SOD1 (see below).

Human SOD1 Is Active in CCS-null Drosophila and Restores Their Adult Life Span—In earlier work (14), we described the functional expression of human SOD1 (hSOD1) in Drosophila in a Ccs−/− genetic background. Since then, the CCS-independent activation of hSOD1 has been described (7, 9). Here we investigated the activation of hSOD1 in flies in the absence of functional dCCS. As would be expected from this SOD1 capable of CCS independent activation, hSOD1 is quite active in CCS-null flies (Fig. 4A). Moreover, hSOD1 functions biologically to rescue the early mortality and reduced median life span of CCS-null flies (Fig. 4B). These results demonstrate the presence in Drosophila of a CCS-independent pathway capable of robust activation of heterologous hSOD1 while giving only weak activation of homologous dSOD1.

CCS-independent Activation of Drosophila SOD1 Revealed in Yeast Expression System—Based on amino acid sequence alone, dSOD1 is predicted to acquire copper independently of CCS. Studies with yeast, human, and C. elegans SOD1 have identified a pair of prolines near the C terminus that can prohibit CCS-

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**TABLE 1**

Drosophila CCS lacks domain 1 MCTXXC

| Organism                        | Domain 1 MCTXXC | Domain 3 CXXC |
|---------------------------------|-----------------|---------------|
| Saccharomyces cerevisiae        | MxCTXM         | MxxCxxC       |
| Candida albbrata                | MxCTXM         | MxxCxxC       |
| Aspergillus fumigatus           | MxCTXM         | MxxCxxC       |
| Neurospora crassa               | MxCTXM         | MxxCxxC       |
| Oryza sativa                    | MxCTXM         | MxxCxxC       |
| Solanum lycopersicum            | MxCTXM         | MxxCxxC       |
| Glycine max                     | MxCTXM         | MxxCxxC       |
| Solanum tuberosum               | MxCTXM         | MxxCxxC       |
| Arabidopsis thaliana            | MxCTXM         | MxxCxxC       |
| Anopheles gambiae               | MxCTXM         | MxxCxxC       |
| Nasonia vitripennis             | MxCTXM         | MxxCxxC       |
| Tribolium castaneum             | MxCTXM         | MxxCxxC       |
| Drosophila melanogaster         | MxCTXM         | MxxCxxC       |
| Strongylocentrus purpuratus     | MxCTXM         | MxxCxxC       |
| Xenopus tropicalis              | MxCTXM         | MxxCxxC       |
| Rattus norvegicus               | MxCTXM         | MxxCxxC       |
| Mus musculus                    | MxCTXM         | MxxCxxC       |
| Canis lupus familiaris          | MxCTXM         | MxxCxxC       |
| Sus scrofa                      | MxCTXM         | MxxCxxC       |
| Homo sapiens                    | MxCTXM         | MxxCxxC       |

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Independent activation (7, 8). Yeast SOD1 naturally contains these prolines and is incapable of CCS-independent activation, whereas mammalian and C. elegans SOD1 with nonproline residues at these positions can be activated independently of CCS. Drosophila SOD1 also lacks these prolines (Fig. 5A) leading us to predict that dSOD1 should not exhibit dependence on CCS for activity. And although from the phenotype of dCCS-nulls we inferred the presence of a residual level of SOD1 activity, the instability of dSOD1 in the absence of CCS obscures any measurable CCS-independent activation of dSOD1 that might occur in the Drosophila model. We therefore turned to a yeast expression system to further examine Drosophila CCS and SOD1.

To test the comparative functionality of dSOD1 in the absence of CCS, the coding sequences for human, yeast, and Drosophila SOD1 were all placed under control of the S. cerevisiae SOD1 gene promoter and analyzed for SOD1 activity and protein levels in CCS1+ versus ccs1Δ yeast. As in CCS-null flies (Fig. 2C), dSOD1 appears highly unstable in ccs1Δ yeast (Fig. 5B), and dSOD1 activity was initially difficult to discern (Fig. 5B, lane 6). Yet when increasing levels of cell lysate were analyzed, the dSOD1 polypeptide was readily detected, and CCS-independent activation of dSOD1 became apparent (Fig. 5B, lane 9), similar to levels seen with human SOD1 (lane 4). Hence, dSOD1 does have the capacity to be activated by the CCS-independent pathway, ostensibly explaining the less severe phenotypes of a CCS-null fly compared with a SOD1-null mutant.

Role of CCS in Helping to Stabilize dSOD1—The dramatic loss of the dSOD1 polypeptide in both ccs1Δ null yeast (Fig. 5B) and in CCS null flies (Fig. 2C) is atypical, and it is not observed with yeast and human SOD1 analyzed in parallel (Fig. 5B, lanes 2 and 4). This loss in dSOD1 can be rescued by expressing either yeast CCS or Drosophila CCS in the ccs1Δ yeast (Fig. 6C, lanes 8 and 9). This apparent stabilization of dSOD1 requires physical interactions between CCS and SOD1, as a K136E, G137E derivative of yeast CCS that cannot dock with SOD1 (29) fails to increase dSOD1 levels (Fig. 6A, lane 4). To test whether SOD1-CCS interactions are by themselves sufficient, we employed a C229S/C231S mutant of yeast CCS. The highly conserved cysteines Cys-229 and Cys-231 in domain III of CCS are needed for copper transfer and disulfide oxidation in SOD1, and a C229S/C231S mutant of CCS can dock with SOD1 but cannot activate the enzyme (29, 30). As seen in Fig. 6A, lane 3, C229S/C231S yCCS failed to stabilize dSOD1, indicating that copper transfer and/or disulfide oxidation are required. To directly test the requirement for copper, yeast cells were starved for copper by treatment with the Cu(I) chelator, bathocuproine sulfonate (31). Under such copper-limiting conditions, levels of the dSOD1 polypeptide were lowered even in cells expressing wild type CCS from yeast or Drosophila (Fig. 6B). Together, these studies indicate that apo-dSOD1 is unusually unstable and that CCS affords stability to dSOD1 by activating the enzyme through copper insertion and/or disulfide oxidation.

Unique N Terminus of Drosophila CCS—As mentioned above, Drosophila CCS lacks the MXCXXC copper-binding motif that is well conserved in CCS molecules from phylo-
Drosophila CCS

FIGURE 3. CCS deficiency confers early adult mortality and enhanced sensitivity to the redox cycling agent, paraquat. A, early mortality of CCS-null adults. Survival of at least 125 males of each genotype on standard cornmeal food was followed at 25 °C with enumeration and transfer of survivors to fresh bottles every 2–3 days. Ccs− is H340, the Ccs+ parent stock from which Ccs−/Ccs− was derived. Ccs−/Ccs− is Ccs29E/Ccs29E. Both stocks are homozygous for Sod1−. Inset, early mortality of SOD1-null adults. Sod1− is Sod11108/Sod11108, Sod1+ is the wild type stock, ny−/+. Both stocks are homozygous for Ccs+. B, paraquat sensitivity of CCS-null adults. Sod1− is Sod11108/Sod11108. Sod1+ is the wild type stock, ny−/+. Both stocks are homozygous for Ccs+ . Young adult males (at least 200 flies per genotype, 10 flies per vial) were exposed to 2 mM paraquat. Survivors were enumerated after 24 h. Paraquat data represent the mean ± S.D. of at least three independent determinations.

FIGURE 4. Expression of hSOD1 in CCS-null Drosophila. A, hSOD1 expressed in Drosophila is enzymatically active in the absence of CCS. In-gel activity assay of SOD used extracts of young adult males. hSOD1 was expressed in Ccs−/− and Ccs−/− flies using the GAL4/UAS system. Genotypes (by lane) are as follows: lane 1, Ccs+/Ccs−; dSod1+/dSod1−, daGAL445; lane 2, Ccs+/Ccs−; dSod1+/dSod1−; lane 3, Ccs+/Ccs−; dSod1−/dSod1−; lane 4, Ccs+/Ccs−; UAS-hSod1; dSod1+/dSod1−; lane 5, Ccs+/Ccs−; UAS-hSod1; dSod1+/dSod1−; lane 6, Ccs+/Ccs−; UAS-hSod1; dSod1+/dSod1−; lane 7, Ccs+/Ccs−; UAS-hSod1; dSod1−/dSod1−, daGAL4452; lane 8, Ccs+/Ccs−; UAS-hSod1; dSod1+/dSod1−, daGAL4456. The UAS-hSod1 transgene is on recombinant 2nd chromosomes carrying Ccs− or Ccs+. Ccs−/Ccs−B, human SOD1 rescues flies deficient in CCS. Genotypes are as follows: Ccs−/−; Ccs−/−; dSod1−/dSod1−, daGAL445; Ccs−/−; Ccs−/−; dSod1−/dSod1−; daGAL4452; Ccs−/−; Ccs−/−; dSod1−/dSod1−; daGAL4456; Ccs−/−; Ccs−/−; dSod1−/dSod1−, UAS-hSod1; dSod1−/dSod1−, daGAL4452; Ccs−/−/Ccs−/−; UAS-hSod1(a); dSod1+/dSod1−, daGAL4452; Ccs−/−; Ccs−/−; dSod1−/dSod1−, daGAL4456; Ccs−/−; Ccs−/−; dSod1−/dSod1−, UAS-hSod1(a); dSod1+/dSod1−, daGAL4456; Ccs−/−; Ccs−/−; dSod1−/dSod1−, daGAL4452; Ccs−/−/Ccs−/−; UAS-hSod1(b); dSod1+/dSod1−, daGAL4452; Ccs−/−; Ccs−/−; dSod1−/dSod1−, daGAL4456; Ccs−/−; Ccs−/−; dSod1−/dSod1−, UAS-hSod1(b); dSod1+/dSod1−, daGAL4456. Note that all strains are homozygous for dSod1− and hemizygous for daGAL4452, the latter of which is known to impose a mild dominant reduction of adult life span (compare with Fig. 3A). hSod1(a), hSod1(b) are independently selected recombinant 2nd chromosomes carrying both Ccs−/− and UAS-hSod1. Note that hSod1 appear to supers-rescue in the absence of CCS.

Drosophila CCS data represent the mean Ccsn Ccsn Ccsn the effects of a C17S,C20S substitution in yeast CCS. As seen in Yeast and mosquito CCS, all CCS molecules identified to date are derived. Drosophila CCS sensitivity to the redox cycling agent, paraquat. Sod1 is activated by yeast CCS, and yeast SOD1 preference for yeast CCS is encoded by a single genomic gene, CCS. In any case, SOD1 molecules from phylogenetically distant organisms exhibit unique preferences in copper activation pathways.

DISCUSSION

CCS in Drosophila is encoded by a single genomic gene, Ccs. Mutational inactivation of Ccs generates a set of phenotypes that are equivalent to those from a weakly expressing SOD1 hypomorphic mutation, and are consistent with the notion that CCS appears to have a single function in Drosophila, which is to

netically distant taxa. In fact, an inspection of CCS molecules across diverse species reveals that with the exception of Drosophila and mosquito CCS, all CCS molecules identified to date harbor these cysteines (Table 1). Interestingly, we observed that Drosophila CCS is very poor at activating yeast SOD1 compared with the homologous yeast CCS expressed from the identical yeast CCS1 gene promoter (Fig. 6C, lanes 2 and 3). By comparison, fly CCS was nearly as effective as yeast CCS in activating human SOD1 (Fig. 6C, lanes 5 and 6), and Drosophila SOD1 exhibited no apparent preference for CCS and showed strong activation by both CCS molecules (lanes 8 and 9). To address whether the yeast SOD1 preference for yeast CCS reflected loss of the conserved MXCXXC cysteines, we tested the effects of a C17S,C20S substitution in yeast CCS. As seen in Fig. 6D, this mutant retains the ability to fully activate yeast SOD1. Despite the well conserved nature of domain I CXXC in CCS, these cysteines are not essential for activating SOD1. These results also demonstrate that the apparent poor reactivity between fly CCS and yeast SOD1 is not simply explained by the absence of these CXXC cysteines. In any case, SOD1 molecules from phylogenetically distant organisms exhibit unique preferences in copper activation pathways.
FIGURE 5. Expression of Drosophila SOD1 (dSOD1) in yeast. A, an alignment of the C-terminal region of SOD1 molecules from *S. cerevisiae*, human, and *Drosophila*, highlighting the proline residues known to preclude CCS-independent activation of yeast SOD1 (5). B, coding sequences for *S. cerevisiae*, human, and *Drosophila SOD1* were placed under control of the *S. cerevisiae* SOD1 gene promoter and used to transform a yeast strain that was sod1Δ and either CCS1 (yCCS: +) or ccs1Δ (yCCS: −) as indicated. SOD1 lysates were analyzed for SOD1 activity (top) or SOD1 protein levels using an antibody directed against *C. elegans* Cu,Zn-SOD1 that cross-reacts well with diverse SOD1 molecules (6). Amounts of lysate protein analyzed are as follows: lanes 1–6, 5 μg; lane 7, 10 μg; lane 8, 25 μg; lane 9, 50 μg. Strains analyzed include KS107 (sod1Δ) and LS101 (sod1Δ ccs1Δ).

FIGURE 6. Role of copper and CCS in stabilizing the dSOD1 expressed in yeast. A sod1Δ ccs1ΔΔ yeast strain expressing the designated SOD1 and CCS molecules was analyzed for SOD1 activity and SOD1 polypeptide levels using 10 μg of cell lysate protein for each sample. A, strains expressing dSOD1 and either wild type (WT) or the indicated mutant versions of *S. cerevisiae* CCS1 or empty vector were analyzed for steady state levels of the dSOD1 polypeptide. Top, immunoblot analysis; bottom, quantitation of immunoblot by Odyssey quantitation software (version 1.2) where 100% = amount of dSOD1 polypeptide accumulated in cells expressing WT CCS1. B, cells expressing dSOD1 and either *S. cerevisiae* CCS1 or *Drosophila* CCS driven by the *S. cerevisiae* CCS1 promoter were grown in the presence of 100 μM bathocuproine sulfonate where indicated (+), and dSOD1 polypeptide levels measured as in A where 100% = amount of dSOD1 polypeptide with no addition of bathocuproine sulfonate. C, cells expressing either yeast, human, or *Drosophila* SOD1 in combination with either yeast or *Drosophila* CCS or with vector as indicated were analyzed for SOD1 activity and SOD1 protein levels as in Fig. 5B. D, cells expressing yeast SOD1 and either wild type or the indicated mutant versions of *S. cerevisiae* CCS1 were analyzed for SOD1 activity and protein levels as in Fig. 5B.
Drosophila CCS

availability could, in principle, underpin such cell type specificity in CCS-independent activation of SOD1.

Drosophila SOD1 can now be added to the growing list of SOD1 molecules that can obtain copper independently of CCS. Currently, the only Cu,Zn-SOD known to solely rely on CCS is the SOD1 of *S. cerevisiae*. It is curious that yeast does not need a CCS independent pathway, yet retains the ability to activate heterologous SODs independent of CCS. A likely explanation is that the factors for CCS independent activation serve another function in the cell. For example, CCS independent activation may involve metal binding or thiol-reactive molecules that participate in more general metal homeostasis and/or redox control.

*Drosophila* SOD1 appears unique in that the apo-form is very unstable in the absence of the copper chaperone. The apparent instability is not because of the *Drosophila* host, as human SOD1 expressed in CCS-null flies shows no polypeptide loss, and in the heterologous yeast expression system, ccs/Δ mutations affect loss of dSOD1 but not yeast or human SOD1 examined in parallel. Moreover, in C57BL/6J mouse models, the mammalian SOD1 polypeptide is stable (32). This loss of dSOD1 in the absence of CCS is in fact reminiscent of what has been observed with certain amyotrophic lateral sclerosis mutants of human SOD1 associated with amyotrophic lateral sclerosis (33). Because CCS both inserts copper and oxidizes the SOD1 disulfide, either one or both of these post-translational modifications must be important stabilizing factors for the dSOD1 molecule.

Prior to these studies, apo-inactive SOD1 was generally thought to stably accumulate in cells, allowing for rapid enzyme activation by CCS without the need for new SOD1 synthesis (31, 34). Yet *Drosophila* SOD1 does not stably accumulate in the inactive apo-form. Consequently, the high level of enzyme activity that is required to defend against oxidative challenge would require *de novo* SOD1 synthesis. The inability of CCS-null adults, which are constitutively deficient in SOD1 activity, to mount a successful defense against paraquat-induced oxidative stress is consistent with this scenario. It is also possible that available copper is limiting in *Drosophila*, and it would be disadvantageous to produce more copper-binding SOD1 than is needed.

With the exception of certain insects, CCS molecules from diverse eukaryotes harbor an MXCXXC near the N terminus that is a well characterized Cu(I) site (35). Although earlier studies with human CCS indicated that the MXCXXC cysteines may be necessary for SOD1 activation *in vivo* (36), studies with recombinant CCS *in vitro* indicate that only the C-terminal CXC cysteines are needed to bind and transfer the metal (37, 38). We observed that the MXCXXC motif is not required for activation of yeast SOD1 *in vivo*; copper binding and transfer must occur exclusively via the C-terminal CXC motif of CCS and the same must be true for dCCS lacking the N-terminal cysteines. It remains possible that in certain species, the N-terminal MXCXXC site becomes critical for maintaining SOD1 activity when oxygen and/or copper are limiting reagents for CCS. If true, the absence of such a motif in *Drosophila* CCS would minimize copper loading of SOD1 when pools of the metal are sparse. However, the biological consequences of transient fluctuations in copper availability may be minimized by the relative stability of metalated SOD1 coupled with the low cell turnover in the post-mitotic *Drosophila* adult.

Acknowledgments—We thank Kalpana White and Maryanne Fenerjian for sending us the 29E (Cex<sup>−/−</sup>) and H340 strains that provided the genetic foundation for the *Drosophila* work. We also thank Pete Anderson and Shawna Wicks for assistance in constructing and maintaining *Drosophila* stocks, for help with phenotypic assays, and for many helpful discussions.

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