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Instability of Superoxide Dismutase 1 of *Drosophila* in Mutants Deficient for Its Cognate Copper Chaperone*[S]

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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 (*Ccs*^{n29E}) 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 *Ccs*^{n29E} 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 (*ccs1*Δ) yeast. The residual dSOD1 polypeptide in *ccs1*Δ yeast was nevertheless active, consistent with CCS-independent activation. Stability of dSOD1 in *ccs1*Δ 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.

Dismutation of superoxide ($O_2^{\cdot-}$) by cytosolic superoxide dismutase (SOD1)[3] is dependent upon the cyclic reduction and oxidation of the prosthetic transition metal, copper. The cell must strictly limit concentrations of free copper, while simultaneously ensuring efficient delivery of copper to the SOD1 apoprotein. Copper is inserted into the SOD1 apoprotein by a specific chaperone, the copper chaperone for SOD1 (CCS). Initially identified in yeast as the protein product of the *LYS7* gene (1), CCS molecules have been identified from a wide range of organisms ranging from fungi to various metazoans (2).

CCS is composed of three separate protein domains that function in concert to activate SOD1 with copper (see Fig. 1*B*). At the N terminus of CCS, domain I resembles the ATX1 family of copper chaperones that harbor the well conserved C*XX*C Cu(I)-binding motif. The central domain II of CCS has significant homology to SOD1 and is important for forming a CCS-SOD1 heterodimer-docked complex as a prerequisite to copper transfer. Finally the C-terminal domain III contains a critical C*X*C copper-binding site that inserts copper and oxidizes the intramolecular disulfide in SOD1 (3, 4). In many models domain I and domain III cysteines together coordinate a copper ion (5, 6), although the role of domain I cysteines in SOD1 activation *in vivo* is uncertain.

In addition to CCS, SOD1 can be activated by a so-called CCS-independent pathway that is currently of unknown nature but requires reduced GSH (7). The mode of copper activation (CCS *versus* CCS-independent) can vary among different organisms. For example, the SOD1 of bakers' yeast *Saccharomyces cerevisiae* is totally dependent on CCS for activation, whereas that of the nematode *Caenorhabditis elegans* only acquires copper through the CCS-independent pathway (8). In fact, *C. elegans* lacks an obvious CCS-encoding gene. Mammals express a CCS that is homologous to that of yeast, but mammalian SOD1 can acquire copper by either pathway (7, 9). CCS-encoding genes have been identified in a wide array of metazoans, yet the biology of CCS from nonmammalian metazoans has not been investigated.

The invertebrate, *Drosophila melanogaster*, has provided an ideal organism in which to explore the role of SOD1 in devel-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[3] 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.

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 $Sod1^{n108}$ 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 SmaI site of pBluescriptIISK (Stratagene) to generate the vector pBCC. The transformation vector pUCC was constructed by subcloning the EcoRI-NotI fragment of pBCC into pUAST (15).

Yeast Strains, Growth Conditions, Plasmids—Yeast strains used in this study were derived from EG103 (*MATα, leu2-3,112, his3Δ1, trp1-289, ura3-52*) (16) and include KS107 (*sod1*Δ) (17) and LS101 (*sod1*Δ *ccs1*Δ) (18). Cells were propagated at 30 °C either in enriched yeast extract, peptone-based medium supplemented with 2% glucose (YPD), or in synthetic complete (SC) medium (19). Solid medium was supplemented with 15 mg/liter ergosterol and 0.5% Tween 80 to enhance growth in anaerobic conditions.

Yeast expression plasmids for dSOD1 and dCCS were produced using cDNA clone RE52090 (Open Biosystems) for dSOD1 and plasmid pUCC for dCCS. The coding sequences for dSOD1 and dCCS were PCR0-amplified introducing 5′ BglII and 3′ SnaBI sites in dSOD1 and 5′ MluI and 3′ RsrII sites in dCCS. The dSOD1 PCR product was digested with the appropriate enzymes and ligated into pLS108 (18) cut with BglII/SnaBI, replacing the yeast SOD1 coding sequence with that of dSOD1 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 yCCS1 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 (C17S, C20S) 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 K_2HPO_4, 0.035 mM riboflavin, 0.3% TEMED for 7 min and then washed several times in H_2O 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 nondenaturing 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 autooxidation 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 $MnCl_2$, 2 mM citric acid, 50 mM Tris-HCl, pH 8.0) and centrifuged at 13,000 × *g*. Samples were electrophoresed on Sepraphore 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-diphenyltetrazolium bromide, 0.3 mM phenazine methosulfate, 25 mM $MgCl_2$, 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

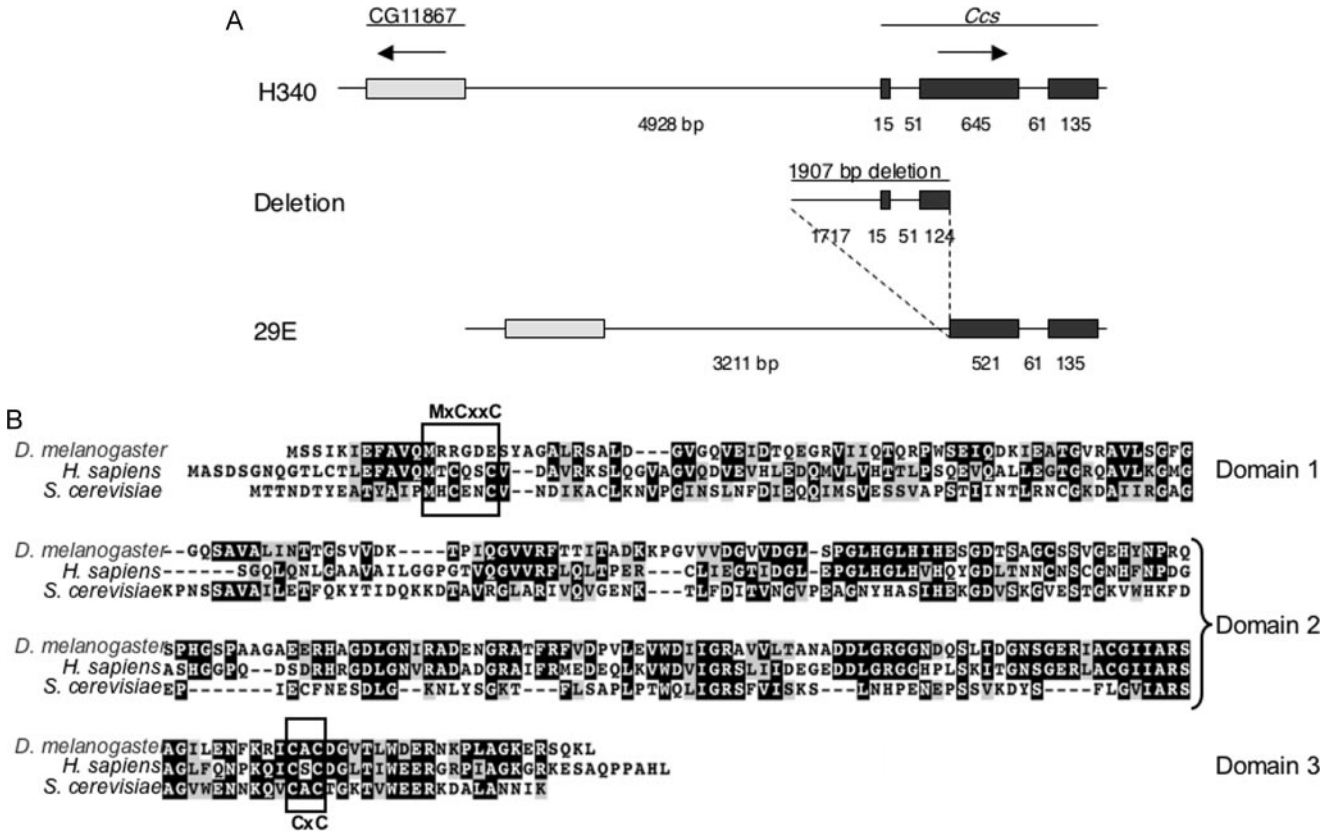

FIGURE 1. ***Drosophila* CCS.** *A,* genomic architecture of *Ccs*, the *Drosophila* copper chaperone for superoxide dismutase 1 (Fly Base Annotation CG17753, FBgn0010531). The gene is located in cytogenetic region 46F1 of *D. melanogaster* chromosome 2. H340 is the Ccs^{+} parent chromosome. 29E is the mutant chromosome containing a 1907-bp deletion extending upstream from bp 124 of the 2nd exon through the first intron, the first exon and 1717 bp of the 5′-noncoding region. The precise details of the origin of 29E from H340 are unknown. *B,* domain structure of *Drosophila* CCS.

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 (Licor 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. 1*A*). 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 C*X*C is conserved. Unexpectedly, the domain I C*XX*C copper-binding motif is absent (Fig. 1*B*). A comparison to all known CCS-like molecules in the data base revealed that the absence of a C*XX*C motif in *Drosophila* CCS is unique. Of 20 CCS sequences analyzed from fungi to humans, only *D. melanogaster* and *Anopheles gambiae* lack the M*X*C*XX*C copper-binding site (Table 1). However, all 20 CCS molecules retain the C*X*C copper site in domain III.

Ccs Deletion Mutation—A loss of function mutation of *Ccs* (Ccs^{n29E}), 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 Ccs^{n29E} 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. 1*A*). *Ccs* transcripts are relatively rare throughout normal develop-

TABLE 1

Drosophila* CCS lacks domain 1 MTC*XXC

The presence of the domain I M*XCXXC* and domain III C*XC* sequences was examined in CCS proteins from fungi, plants, and animals. Sequences were obtained from the GenBank™ database. Alignments were performed with Geneious (version 3.0.6).

Organism	Domain 1 MxCxxC	Domain 3 CxC
Saccharomyces cerevisiae	MHCENC	VCACT
Candida glabrata	MHCTDC	VCACT
Aspergillus fumigatus	MTCDGC	VCSCS
Neurospora crassa	MHCESC	VCSCT
Oryza sativa	MKCDGC	LCTCD
Solanum lycopersicum	MSCQGC	LCTCD
Glycine max	MKCEGC	LCTCD
Solanum tuberosum	MSCQGC	ICACD
Arabidopsis thaliana	MTCEGC	LCSCD
Anopheles gambiae	FRGAGC	ICACD
Nasonia vitripennis	MTCQKC	ICACD
Tribolium castaneum	MTCNSC	ICACD
Drosophila melanogaster	MRRGDE	ICACD
Strongylocentrotus purpuratus	MTCNSC	ICACD
Xenopus tropicalis	ITCESC	LCTCD
Rattus norvegicus	MSCQSC	ICSCD
Mus musculus	MSCQSC	ICSCD
Canis lupus familiaris	MTCQSC	ICSCD
Sus scrofa	MTCQSC	ICSCD
Homo sapiens	MTCQSC	ICSCD

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. 4*A*). 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. 2*C*). 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]

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 affect on the activity of mACON (Fig. 2*D*). Taken together, these data indicate that at the biochemical level, the absence of dCCS 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. 3*A* 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. 3*B*). 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. 4*A*). Moreover, hSOD1 functions biologically to rescue the early mortality and reduced median life span of CCS-null flies (Fig. 4*B*). 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-

[4] The possibility remained that the lack of SOD1 activity in the *Ccs* 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 $Sod1^s$, 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 $SOD1^F/SOD1^S$ dimeric protein in the 29E/$Sod1^S$ hybrid. The data in supplemental Fig. S1, showing the presence of an enzymatically active $SOD1^F/SOD1^S$ 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.

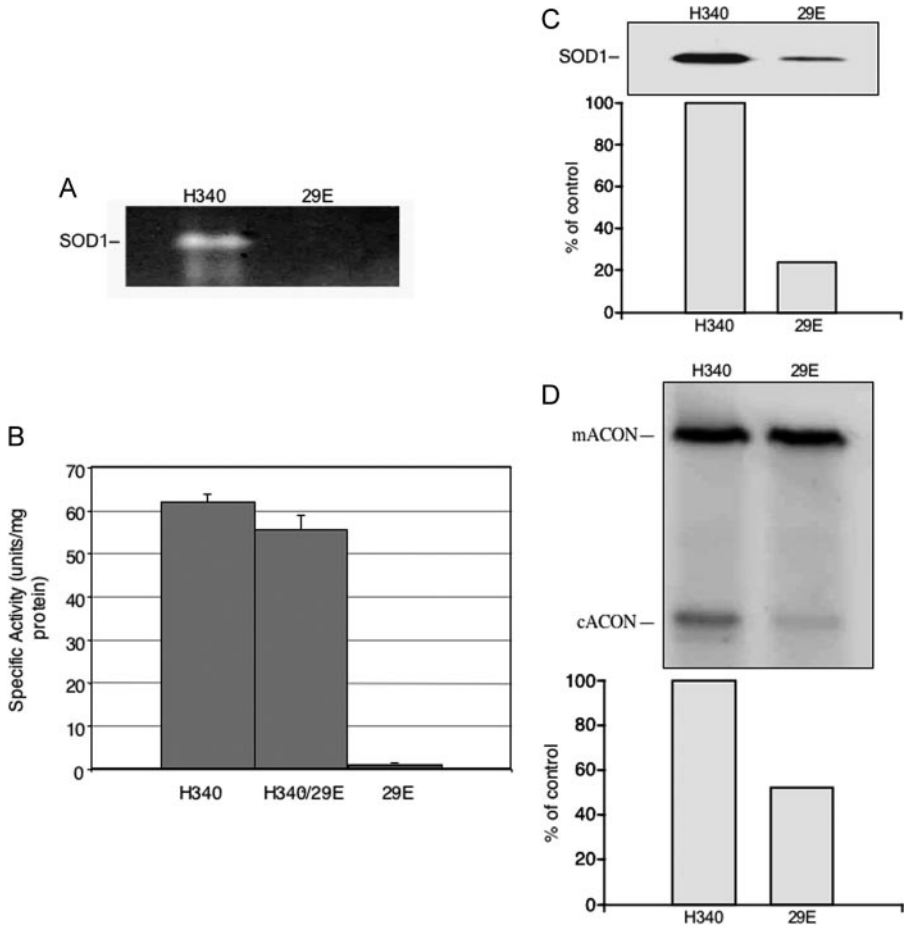

FIGURE 2. **Loss of CCS affects SOD1 and mAconitase.** *A,* in-gel activity assay of SOD1. H340 is the $dCcs^{+}$ parent stock from which the CCS-null mutant, Ccs^{n29E}, was derived. 29E is Ccs^{n29E}/Ccs^{n29E}. H340/29E is Ccs^{+}/Ccs^{n29E}. Both strains are homozygous for $Sod1^{+}$. *B,* spectrophotometric assay of SOD1 activity based on 6-HD reduction. Extracts were treated with Triton X-100 to eliminate interference from mitochondrial superoxide dismutase (SOD2). See "Experimental Procedures" for details. Note, the "background activity" found in 29E extracts is also present in SOD1-null mutant extracts. *C,* dSOD1 is reduced in the absence of CCS. Extracts were prepared from H340 and 29E adult males, and SOD1 protein was detected in Western immunoblots with anti-SOD1 antibody. *Top,* immunoblot analysis; *bottom*, densitometric quantitation of immunoblot where 100 = amount of dSOD1 polypeptide in H340. 29E is Ccs^{n29E}/Ccs^{n29E}. H340 is Ccs^{+}/Ccs^{+}. Both strains are homozygous for $Sod1^{+}$. *D,* selective loss of cytosolic aconitase (*cACON*) activity in the absence of CCS. Aconitase activities in extracts of 2–3-day-old adult males were assayed after electrophoretic separation. mACON and cACON indicate mitochondrial and cytosolic aconitase activities, respectively. *Top,* zymogram analysis; *bottom,* densitometric quantitation of zymogram where 100 = level of cACON activity in H340. mACON activity remained unaffected in the absence of CCS. 29E is Ccs^{n29E}/Ccs^{n29E}. H340 is Ccs^{+}/Ccs^{+}. H340/29E is Ccs^{+}/Ccs^{n29E}. All strains are homozygous for $Sod1^{+}$.

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. 5*A*) 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* *ccs1*Δ yeast. As in CCS-null flies (Fig. 2*C*), dSOD1 appears highly unstable in *ccs1*Δ yeast (Fig. 5*B*), and dSOD1 activity was initially difficult to discern (Fig. 5*B, 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. 5*B, 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 *ccs1*Δ null yeast (Fig. 5*B*) and in CCS null flies (Fig. 2*C*) is atypical, and it is not observed with yeast and human SOD1 analyzed in parallel (Fig. 5*B, lanes 2* and *4*). This loss in dSOD1 can be rescued by expressing either yeast CCS or *Drosophila* CCS in the *ccs1*Δ yeast (Fig. 6*C*, *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. 6*A, 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. 6*A, 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. 6*B*). 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 M*X*C*XX*C copper-binding motif that is well conserved in CCS molecules from phyloge-

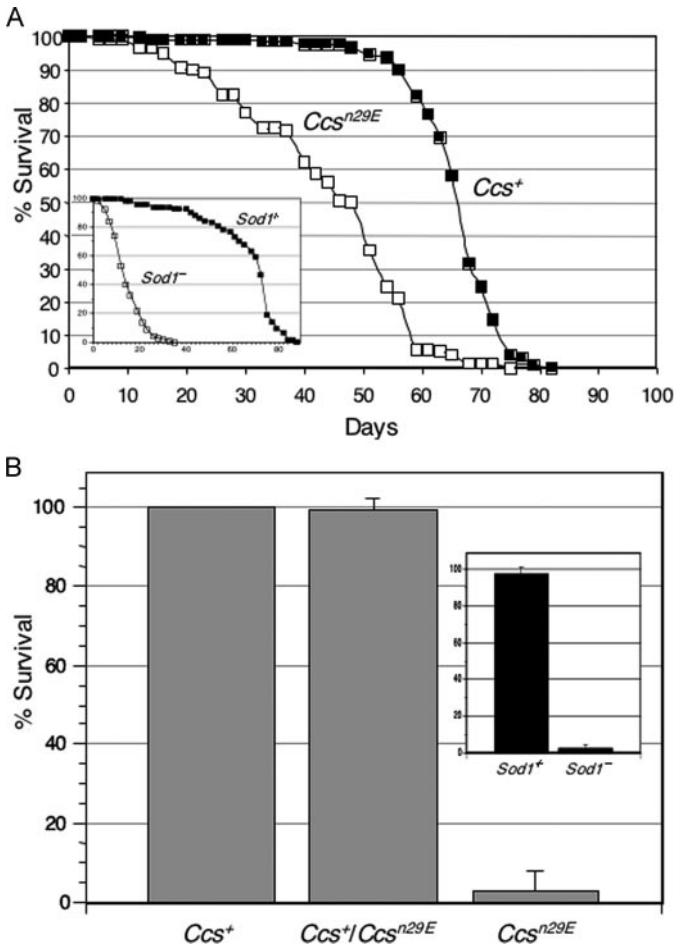

FIGURE 3. **CCS deficiency confers early adult mortality and enhanced sensitivity to the redox cycling agent, paraquat.** *A,* early mortality of CCS-null adults. Survival of at least 125 males of each genotype on standard cornmeal food was followed at 25 °C with enumeration and transfer of survivors to fresh bottles every 2–3 days. Ccs^{+} is H340, the Ccs^{+} parent stock from which Ccs^{n29E} was derived. Ccs^{n29E} is Ccs^{n29E}/Ccs^{n29E}. Both stocks are homozygous for $Sod1^{+}$. *Inset,* early mortality of SOD1-null adults. $Sod1^{-}$ is $Sod1^{n108}/Sod1^{n108}$, $Sod1^{+}$ is the wild type stock, ry^{+5}. Both stocks are homozygous for Ccs^{+}. *B,* paraquat sensitivity of CCS-null adults. Ccs^{+} is H340, the Ccs^{+} parent stock from which Ccs^{n29E} was derived. Ccs^{n29E} is Ccs^{n29E}/Ccs^{n29E}. Both stocks are homozygous for $Sod1^{+}$. *Inset,* paraquat sensitivity of SOD1-null adults. $Sod1^{-}$ is $Sod1^{n108}/Sod1^{n108}$. $Sod1^{+}$ is the wild type stock, ry^{+5}. 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.

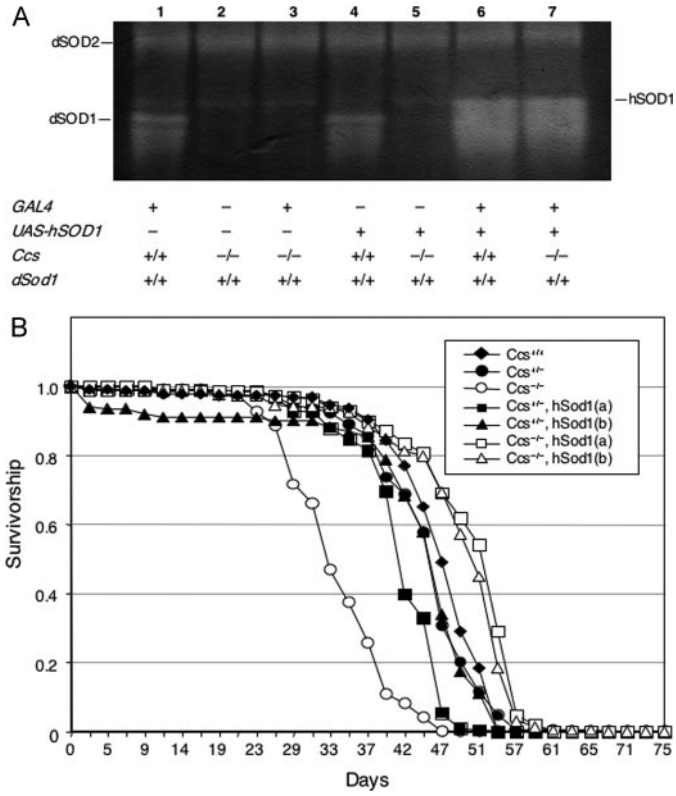

FIGURE 4. **Expression of hSOD1 in CCS-null *Drosophila*.** *A,* hSOD1 expressed in *Drosophila* is enzymatically active in the absence of CCS. In-gel activity assay of SOD used extracts of young adult males. hSOD1 was expressed in $Ccs^{+/+}$ *and* $Ccs^{-/-}$ flies using the GAL4/UAS system. Genotypes (by lane) are as follows: *lane 1,* Ccs^{+}/Ccs^{+}; $dSod1^{+}/dSod1^{+}$, $daGAL4^{G32}$; *lane 2,* Ccs^{-}/Ccs^{-}; $dSod1^{+}/dSod1^{+}$; *lane 3,* Ccs^{-}/Ccs^{-}; $dSod1^{+}/dSod1^{+}$; $daGAL4^{G32}$; *lane 4,* Ccs^{+}/Ccs^{+}, *UAS-hSod1*; $dSod1^{+}/dSod1^{+}$; *lane 5,* Ccs^{-}/Ccs^{-}, *UAS-hSod1*; $dSod1^{+}/dSod1^{+}$; *lane 6,* Ccs^{+}/Ccs^{+}, *UAS-hSod1*; $dSod1^{+}/dSod1^{+}$, $daGAL4^{G32}$; *lane 7,* Ccs^{-}/Ccs^{-}, *UAS-hSod1*; $dSod1^{+}/dSod1^{+}$, $daGAL4^{G32}$. The *UAS-hSod1* transgene is on recombinant 2nd chromosomes carrying Ccs^{+} or Ccs^{-}. $Ccs^{-} = Ccs^{n29E}$. *B,* human SOD1 rescues flies deficient in CCS. Genotypes are as follows: $Ccs^{+/+}$,Ccs^{+}/Ccs^{+}; $dSod1^{+}/dSod1^{+}$, $daGAL4^{G32}$. $Ccs^{+/-}$, Ccs^{+}/Ccs^{n29E}; $dSod1^{+}/dSod^{+}$, $daGAL4^{G32}$. $Ccs^{-/-}$, Ccs^{n29E}/Ccs^{n29E}; $dSod1^{+}/dSod1^{+}$, $daGAL4^{G32}$. $Ccs^{+/-}$, hSod1(a), Ccs^{+}/Ccs^{n29E}, *UAS-hSod1*(*a*); $dSod1^{+}/dSod^{+}$, $daGAL4^{G32}$. $Ccs^{-/-}$, hSod1(a), Ccs^{n29E}/Ccs^{n29E}, *UAS-hSod1*(*a*); $dSod1^{+}/dSod1^{+}$, $daGAL4^{G32}$. $Ccs^{+/-}$, hSod1(b): Ccs^{+}/Ccs^{n29E}, *UAS-hSod1*(*b*); $dSod1^{+}/dSod1^{+}$, $daGAL4^{G32}$. $Ccs^{-/-}$, hSod1(b): Ccs^{n29E}/Ccs^{n29E}, *UAS-hSod1*(*b*); $dSod1^{+}/dSod1^{+}$, $daGAL4^{G32}$. Note that all strains are homozygous for $dSod1^{+}$ and hemizygous for $daGAL4^{G32}$, the latter of which is known to impose a mild dominant reduction of adult life span (compare with Fig. 3*A*). hSod1(a), hSod1(b) are independently selected recombinant 2nd chromosomes carrying both Ccs^{n29E} and *UAS-hSod1*. Note that hSOD1 appears to super-rescue in the absence of CCS.

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. 6*C, lanes 2* and *3*). By comparison, fly CCS was nearly as effective as yeast CCS in activating human SOD1 (Fig. 6*C, 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 M*XCXX*C cysteines, we tested the effects of a C17S,C20S substitution in yeast CCS. As seen in Fig. 6*D*, this mutant retains the ability to fully activate yeast SOD1. Despite the well conserved nature of domain I C*XX*C 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 C*XX*C cysteines. 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

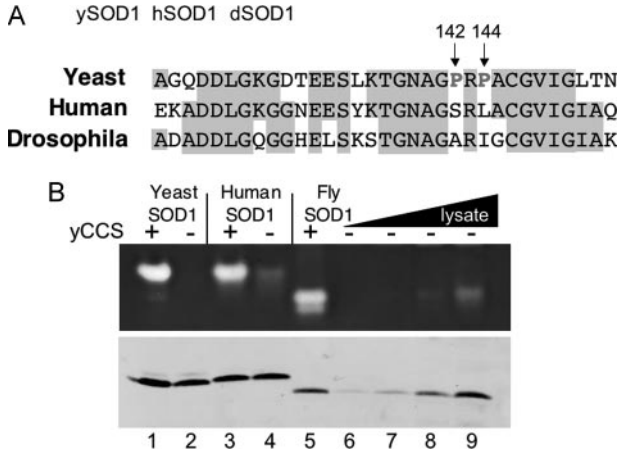

FIGURE 5. **Expression of *Drosophila* SOD1 (dSOD1) in yeast.** *A,* an alignment of the C-terminal region of SOD1 molecules from *S. cerevisiae*, human, and *Drosophila,* highlighting the proline residues known to preclude CCS-independent activation of yeast SOD1 (5). *B,* coding sequences for *S. cerevisiae*, human, and *Drosophila* SOD1 were placed under control of the *S. cerevisiae SOD1* gene promoter and used to transform a yeast strain that was *sod1*Δ and either *CCS1* (yCCS:+) or *ccs1*Δ (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 (*sod1*Δ) and LS101 (*sod1*Δ *ccs1*Δ).

mediate the activation of apo-SOD1 by copper. Compared with the severity of phenotypes caused by SOD1 deficiency (either genomic null mutation or RNA interference knockdown), the CCS-null phenotypes are unexpectedly mild. This likely arises from a vanishingly small amount of CCS-independent SOD1 activity that is at or just below the threshold of detectability in fly extracts and could only be discerned in concentrated lysates from yeast cells expressing dSOD1. Considering the rather severe phenotype of SOD1-null flies with respect to adult life span and oxidative stress resistance (10), these findings suggest that only a very small fraction of wild type SOD1 is actually required for adult viability in the absence of oxidative stress, whereas the bulk of SOD1 is utilized in defense against oxidative stress. This view is supported by the contrasting modest and severe effects of CCS deficiency on life span and paraquat resistance, respectively. Thus, the age of onset and severity of early adult mortality in CCS-nulls is markedly less than in SOD1-nulls, whereas the hypersensitivity of CCS-nulls to paraquat is essentially equivalent to that of SOD1-nulls.

It is also possible that the residual SOD1 activity in CCS-nulls reflects the presence of enzymatically active SOD1 in just one or a small number of cell types in which the CCS-independent activation pathway is particularly active. Although we know of no direct evidence in support of this possibility, the ectopic expression of the CCS-independent hSOD1 in motor neurons, which confers robust rescue of the SOD1-null adult life span (14), suggests that motor neurons do possess an active CCS-independent activation pathway; consequently, motor neurons could potentially be one such cell type that could activate dSOD1 in the absence of CCS. Because *Drosophila* life span is sensitive to SOD1 activity in motor neurons, the functioning of the CCS-independent activation pathway in motor neurons could also help explain why CCS deficiency has such a modest effect on adult life span relative to the severe life span reduction conferred by SOD1 deficiency. Alternatively, and as discussed later, our results are consistent with the possibility that although most cells can survive under normal conditions with little SOD1, some cells may require more than the residual level of SOD1 activity present in CCS-nulls under conditions of applied oxidative stress (paraquat) or increased metabolic activity (motor neurons). Finally, it is also plausible that cell type variation in copper

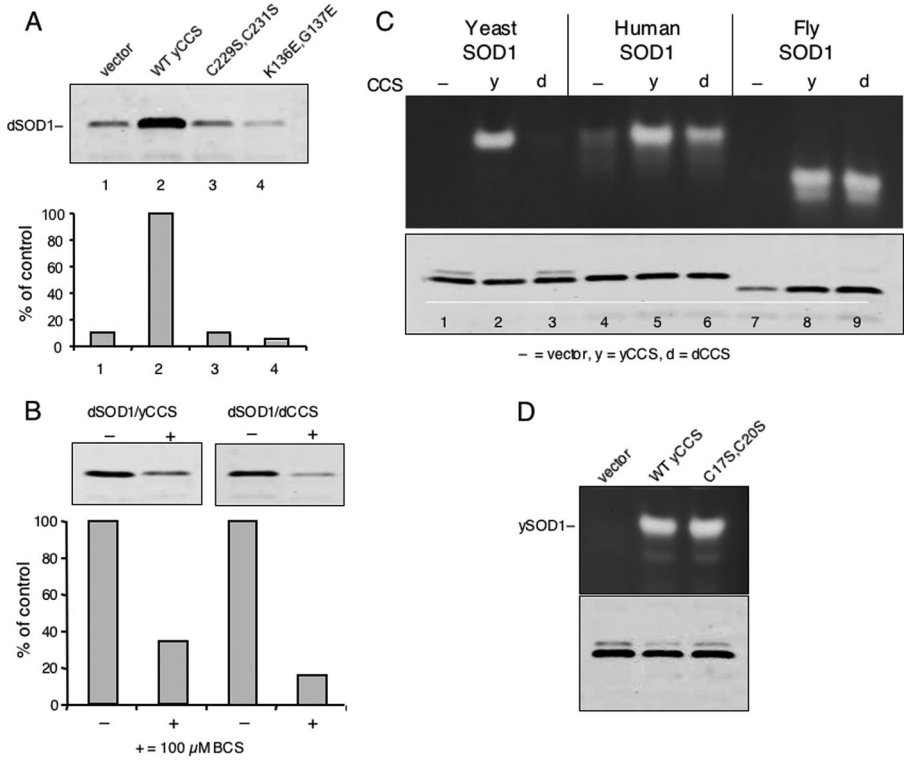

FIGURE 6. **Role of copper and CCS in stabilizing the dSOD1 expressed in yeast.** A *sod1*Δ *ccs1*Δ yeast strain expressing the designated SOD1 and CCS molecules was analyzed for SOD1 activity and SOD1 polypeptide levels using 10 μg of cell lysate protein for each sample. *A,* strains expressing dSOD1 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 Fig. 5*B*. *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. 5*B*.

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, *ccs1*Δ mutations affect loss of dSOD1 but not yeast or human SOD1 examined in parallel. Moreover, in $CCS^{-/-}$ 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 M*XCXX*C near the N terminus that is a well characterized Cu(I) site (35). Although earlier studies with human CCS indicated that the M*XCXX*C cysteines may be necessary for SOD1 activation *in vivo* (36), studies with recombinant CCS *in vitro* indicate that only the C-terminal C*X*C cysteines are needed to bind and transfer the metal (37, 38). We observed that the M*XCXX*C motif is not required for activation of yeast SOD1 *in vivo*; copper binding and transfer must occur exclusively via the C-terminal C*X*C 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 M*XCXX*C 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.

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