Activation of Cu,Zn-Superoxide Dismutase in the Absence of Oxygen and the Copper Chaperone CCS*

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Eukaryotic Cu,Zn-superoxide dismutases (SOD1s) are generally thought to acquire the essential copper cofactor and intramolecular disulfide bond through the action of the CCS copper chaperone. However, several metazoan SOD1s have been shown to acquire activity in vivo in the absence of CCS, and the Cu,Zn-SOD from Caenorhabditis elegans has evolved complete independence from CCS. To investigate SOD1 activation in the absence of CCS, we compared and contrasted the CCS-independent activation of C. elegans and human SOD1 to the strict CCS-dependent activation of Saccharomyces cerevisiae SOD1. Using a yeast expression system, both pathways were seen to acquire copper derived from cell surface transporters and compete for the same intracellular pool of copper. Like CCS, CCS-independent activation occurs rapidly with a preexisting pool of apo-SOD1 without the need for new protein synthesis. The two pathways, however, strongly diverge when assayed for the SOD1 disulfide. SOD1 molecules that are activated without CCS exhibit disulfide oxidation in vivo without oxygen and under copper-depleted conditions. The strict requirement for copper, oxygen, and CCS in disulfide bond oxidation appears exclusive to yeast SOD1, and we find that a unique proline at position 144 in yeast SOD1 is responsible for this disulfide effect. CCS-dependent and -independent pathways also exhibit differential requirements for molecular oxygen. CCS activation of SOD1 requires oxygen, whereas the CCS-independent pathway is able to activate SOD1s even under anaerobic conditions. In this manner, Cu,Zn-SOD from metazoans may retain activity over a wide range of physiological oxygen tensions.

Oxygen is essential for aerobic respiration, but reactive byproducts of oxygen metabolism, such as the superoxide anion, can damage cellular molecules, including proteins, DNA, and lipids (1–3). SOD1s (copper- and zinc-containing superoxide dismutases) provide the primary defense against superoxide damage by catalytically removing it through a disproportionation reaction (4). This reaction involves redox cycling at the copper active site (5). SOD1s require several post-translational modifications to form an active molecule. Copper and zinc are bound by the enzyme, and an intramolecular disulfide bond is formed between two conserved cysteine residues. Although the zinc ion and disulfide bond are not directly involved in the disproportionation reaction, these modifications are required for proper stability and formation of the active site (6–10). The presence of an intramolecular disulfide bond is intriguing, given the fact that the cytosol favors reduced thiols.

The activity of SOD1s in vivo is largely controlled through the aforementioned post-translational modifications. Most of what is currently known about activation of SOD1 in vivo has emerged through studies of the bakers’ yeast Saccharomyces cerevisiae SOD1. Here insertion of the catalytic copper requires the action of the copper chaperone for SOD3 (CCS) (11). CCS physically interacts with SOD1 to deliver the copper ion and catalyze the disulfide bond formation in an oxygen-dependent manner (12–15). In fact, S. cerevisiae SOD1 (γSOD1) is completely dependent on CCS for insertion of the catalytic copper and oxidation of the disulfide bond (11, 15, 16).

Although γSOD1 is dependent on CCS for activity, other eukaryotic SOD1s are not. Mouse and human SOD1 (hSOD1), when expressed in CCS−/− mouse fibroblasts and in ccs1Δ yeast, still retain some SOD1 activity (17–19). Moreover, the genome for the nematode Caenorhabditis elegans does not contain a CCS-like gene, yet harbors several Cu,Zn-SODs. Previous studies with C. elegans SOD-1 (wSOD-1) have shown that this SOD is activated completely independently of CCS (20). Together, these studies present a strong case for a second SOD1 activation mechanism independent of CCS.

There must be inherent differences in SOD1 sequences that dictate whether the enzyme uses CCS or the CCS-independent pathway or both. Through targeted mutagenesis, sequences near the C terminus have been previously identified as being important (19). Yeast SOD1 contains dual prolines at positions 142 and 144, which when mutated in combination allow for CCS-independent activation. Conversely, hSOD1 and wSOD-1

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3 The abbreviations used are: SOD, superoxide dismutase; IAM, iodoacetamide; γSOD1, S. cerevisiae SOD1; wSOD-1, C. elegans SOD-1; hSOD1, human SOD1; BCS, bathocuproinedisulfonic acid; WT, wild type.
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contain non-proline residues at these positions, and if dual prolines are introduced, then CSS-independent activation is blocked (19, 20). How this pair of prolines influences SOD1 activation is not understood.

It is interesting that nature has developed two activation mechanisms for such a key enzyme in oxidative stress protection, and these are not likely to be redundant. It was previously predicted that the two pathways draw upon distinct sources of copper (19), since the addition of the catalytic copper ion is limiting for enzyme activation. However, since disulfide oxidation is also limiting for enzyme activity, it is possible that the two pathways diverge at this level. In the current study, we investigate the requirements and regulation of the CCS-dependent and -independent SOD1 activation pathways. Our results strongly indicate that the two pathways do not diverge at the level of upstream copper transporter sources or the kinetics of copper incorporation into SOD1 but rather at the level of disulfide bond formation. Copper is required for CCS-mediated disulfide bond oxidation in yeast SOD1, whereas SOD1s that can be activated without CCS show no such requirement for copper in disulfide oxidation. Moreover, oxygen is required for enzyme activation through CCS, but the CCS-independent pathway is able to bypass the need for molecular oxygen. This allows for significant SOD1 activity to be found at a variety of oxygen concentrations by utilizing two activation pathways.

MATERIALS AND METHODS

Strains and Culture Conditions—Yeast strains used in this study were derived from EG103 (Mat, leu2-3,112, his3Δ1, trp1–289, ure3–52) (21) and include KS107 (sod1Δ:TRP1) (22), LS101 (sod1Δ:TRP1 ccslΔ:URA3) (23), LS102 (sod1Δ:TRP1 ccslΔ:URA3 ura3– from 5-FOA), RR101 (ctr1Δ::URA3), RR103 (ctr2Δ::HIS3), and RR201 (ctr1Δ::URA3 ctr2Δ::HIS3) (24). Cells were propagated at 30 °C either in enriched yeast extract, peptone-based medium supplemented with 2% glucose, or minimal synthetic complete medium (25). For anaerobic growth, medium also contained 15 mg/l ergosterol and 0.5% Tween 80. Yeast transformations were performed using the lithium acetate procedure (26).

To achieve anaerobic conditions for growth on solid medium, BBL GasPak and anaerobic culture jars were used, as described previously (27). For liquid cultures, anaerobic conditions were maintained inside an Invivo2 400 hypoxic work station using 10% H2 balanced with N2 in the presence of a palladium catalyst. For growth at 3% oxygen, a work station maintained at 3% oxygen with a nitrogen balance was used. To achieve hyperoxygen conditions of liquid cultures, cells were grown in a Billups-Rothenberg modular incubator chamber flushed with 100% O2 for 30 min at 2 p.s.i.

Plasmids—Plasmids pLS108, pLS121, and pLJ256 (CEN LEU2) express yeast, human, and worm Cu,Zn-SOD under the control of the S. cerevisiae SOD1 promoter (20, 23). Plasmid pLC1 (2μ URA3) expresses WT human SOD1 from the S. cerevisiae PGK1 promoter (17). The S142P/L144P variant of human SOD1 was derived from pLC1 (19). Plasmids pLJ305 and pLJ304 expressing P142A and P142S mutants of ySOD1 were generated using the QuikChange mutagenesis kit (Stratagene) and derived from pLS108. pLJ101 (CEN HIS3) expressing ySOD1 under control of the S. cerevisiae SOD1 promoter was created by digestion of pLS108 with Sall and BamHI and ligated into pRS413 (28) cut with these same enzymes. The sequence integrity of plasmids was ensured by double-stranded DNA sequencing (DNA Analysis Facility, Johns Hopkins University).

Screen for CCS-independent Yeast SOD1 Mutations—Random mutagenesis of the S. cerevisiae SOD1-expressing plasmid pLS108 was performed using the GeneMorph II random mutagenesis kit (Stratagene) and SOD1 primers such that only the yeast SOD1 open reading frame would be mutated at a rate of ~2 residues/molecule. The PCR product was transformed into bacteria to construct a library of ~10,000 clones, which were then transformed into the sod1Δ ccs1Δ strain LS101. 10,000 yeast colonies were screened for aerobic growth on medium lacking lysine. From the positive colonies, plasmids were rescued and retested for CCS independence by a second transformation into naive LS101. The SOD1 mutations conferring CCS independence were identified by DNA sequencing (DNA Analysis Facility, Johns Hopkins University).

Biochemical Assays—SOD enzymatic activity was assayed from yeast cells grown overnight with shaking at 30 °C to an A600 of 2. Yeast cells were washed and lysed with glass beads in 600 mM sorbitol, 10 mM HEPES, pH 7.5, containing protease inhibitors, as described previously (29). SOD activity analysis was carried out by non-denaturing gel electrophoresis and staining with nitro blue tetrazolium (30). SOD activity was quantified through densitometric tracings using ImageJ (NIH) software. Integrated density of pixel intensity was recorded. Immunoblot of SOD1 levels were carried out using an antibody generated against C. elegans SOD-1, as described previously, to detect S. cerevisiae, human, and C. elegans Cu,Zn-SOD (20), and an immunoblot of CCS was performed using an antibody generated against S. cerevisiae Ccs1p (31), followed by incubation with a goat anti-rabbit Alexafluor 680 secondary antibody (Invitrogen) and imaging and quantification with a LI-COR Odyssey imager (32).

Disulfide oxidation was monitored using a method adapted from tissue culture cells (32). Here, yeast cells are lysed as above but in the presence of 100 mM iodoacetamide (IAM) to alkylate free thiols and prevent artificial disulfide oxidation. Separation of disulfide-oxidized and disulfide-reduced Cu,Zn-SOD was then accomplished by non-reducing SDS-PAGE using precast 16% Tris-glycine gels. Prior to transfer to polyvinylidene difluoride membranes, gels were incubated for 30 min with SDS-electrophoresis buffer containing 5% dithiothreitol. Immunoblotting and quantification were as above.

For analysis of the SOD1 disulfide and enzymatic activity from anaerobic cultures, cultures maintained in nitrogen work stations described above were harvested and lysed in the same work station. Buffers and growth medium were all deoxygenated for 24 h prior to use. Yeast cell lysis was conducted in the presence of 100 mM IAM, and lysates were allowed to react with IAM anaerobically for 30 min at 30 °C prior to air exposure, thereby preventing any postlysis oxidation of the SOD1 disulfide- and oxygen-mediated enzyme activation.

C. elegans SOD1 and yeast SOD1 were expressed and purified from Escherichia coli, as described previously (20, 33). Purified...
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**FIGURE 1.** CCS and the CCS-independent pathway share common upstream copper transporters and compete for intracellular copper. A, the indicated yeast strains expressing wSOD-1 from the pJL256 plasmid and endogenous yeast SOD1 were grown aerobically to mid-log phase in enriched medium supplemented with 50 μM CuSO₄ where indicated (+). Cell lysates were assayed for SOD activity by native gel electrophoresis and nitro blue tetrazolium staining (30) and SOD1 protein levels using an anti-C. elegans SOD-1 antibody that cross-reacts with yeast SOD1 (bottom) (20). Strains utilized include the following: ctr3Δ, EG103; ctr3Δ ctr1Δ, RR101; ctr3Δ ctr2Δ, RR103; and ctr3Δ ctr1Δ ctr2Δ, RR201. B, the sod1Δ strain K5170 expressing either ySOD1 and/or wSOD-1 from plasmids pLS108 and pJL256, respectively, were assayed for SOD1 activity and protein levels as in A.

fied human SOD1 from *E. coli* was purchased from RayBiotech, Inc. For analysis of the SOD1 disulfide reduction potential, apo-disulfide-oxidized SOD1 was prepared as described elsewhere (33). Apo-disulfide-oxidized SOD1s were then incubated with buffer containing various ratios of reduced and oxidized glutathione (Sigma) or reduced and oxidized dithiothreitol (Sigma) (34, 35) under anaerobic conditions maintained by an InviVo₂ 400 hypoxic work station for 16 h. The disulfide reduction/oxidation reaction was stopped by the addition of 10% final trichloroacetic acid, and the protein was precipitated on ice for 30 min under anaerobic conditions. The protein samples were then pelleted and resuspended in 2X SDS sample buffer containing 100 mM IAM and incubated at room temperature for 1 h under aerobic atmosphere. Disulfide bond formation was then monitored by non-reducing SDS-PAGE as described above. Disulfide-oxidized and -reduced SOD1 were quantified, and the fraction reduced was plotted as a function of the

**FIGURE 2.** Kinetics of copper activation with CCS-dependent versus CCS-independent SOD1 molecules. The sod1Δ strain K5170 expressing either ySOD1 from plasmid pLS108 (top) or wSOD-1 from pJL256 (bottom) were grown as in Fig. 1 in medium treated with 50 μM BCS to deplete intracellular copper and inactivate SOD1. Cells were treated with 100 μM cycloheximide for 30 min to block protein synthesis, followed by treatment with 20 μM CuSO₄ for the indicated times prior to cell lysis. SOD1 activity and protein levels were determined as in Fig. 1.

buffer reduction potential and SOD1 reduction potentials fit Equation 1,

\[
f_{\text{reduced}} = \frac{2F}{e^RT} \left( \frac{f_{\text{buffer}} - f_{\text{SOD1}}}{f_{\text{buffer}} - f_{\text{buffer}}} \right)
\]

(Eq. 1)

where \(F\), \(R\), and \(T\) represent the Faraday constant, gas constant, and temperature, respectively.

**RESULTS**

Copper Delivery to Cu,Zn-SOD through CCS Versus the CCS-independent Pathway—We sought to determine if the CCS-mediated and CCS-independent pathways for activating SOD1 use similar pools of intracellular copper. These studies used *S. cerevisiae* SOD1 (ySOD1) as a read-out for CCS activation and *C. elegans* SOD-1 (wSOD-1) to monitor CCS-independent activation. As seen in Fig. 1A, these two Cu,Zn-SODs exhibit differential mobility on native gels for SOD activity, allowing us to simultaneously monitor CCS-dependent and -independent Cu,Zn-SOD in the same cell. Note that the two SOD1s do not form active heterodimers (data not shown).

High affinity copper uptake in yeast is accomplished through the cell surface Ctr1p and Ctr3p copper transporters (36–38). In most laboratory strains, Ctr3p is inactivated by a Ty2 inserion, as is the case for the EG103 strain used in these studies (24, 38). As seen in Fig. 1A, both ySOD1 and wSOD-1 display some activity in this CTR1Δ, ctr3Δ strain that can be augmented by supplementation of 50 μM (non-toxic) copper to the growth medium (Fig. 1A, lanes 1 and 2). When CTR1 is deleted, both the CCS-dependent and -independent Cu,Zn-SODs are inactive (lane 3), demonstrating the requirement for cell surface high affinity copper uptake. However, ySOD1 and wSOD-1 activities can be fully restored by copper supplementation of the growth medium, where the metal can enter the cell through
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FIGURE 3. CCS-independent SOD1s display higher disulfide oxidation propensity. Lysates were prepared from the sod1Δ (CCS) and sod1Δ ccs1Δ (ccsΔ) strains KS107 and LS101 expressing plasmid-borne ySOD1 (pLS108) (A), wSOD-1 (pLJ256) (B), or hSOD1 (pLS121) (C). Cells were grown to mid-log phase in enriched medium containing either 50 μM CuSO₄ (lanes 1 and 4), no treatment (lanes 2 and 5), or 50 μM BCS to deplete copper (lanes 3 and 6). The wedge illustrates the drop in copper availability. Lysates prepared in the presence of IAM to block free thiols were assayed for (top) the SOD1 disulfide bond by non-reducing SDS-PAGE and immunoblotting. The disulfide-oxidized form of SOD1 (small arrow) has a faster mobility than the disulfide-reduced form (large arrow). Quantification of the immunoblots is represented in the graphs (middle), showing the fraction of SOD1 molecules with an oxidized disulfide. Bottom, lysates were analyzed for SOD1 activity as in Fig. 1.

low affinity transporters (24, 38) (lane 4). A second source of cytosolic copper is through Ctr2p, the copper transporter in the vacuole (24, 39). Previous results showed that Ctr2p does not normally contribute much copper to CCS (24), but the role of vacuolar stores of copper in CCS-independent activation was unknown. We observed that, as is the case with CCS-activation of ySOD1, CCS-independent activation of wSOD-1 was unaffected by single ctr2 mutations (compare lanes 5 and 6 with lanes 1 and 2). In the background of a ctr1Δ mutation, the additional loss of Ctr2p resulted in a slight inhibition in activity of both SODs during copper supplementation (compare lanes 8 and 4), although the effects were mild (~30–50% reduction; see supplemental Fig. S1). Overall, the CCS-dependent and -independent pathways show precisely the same pattern of requirement for upstream copper transporters.

We tested whether the two pathways can compete for the same pool of intracellular copper. In the experiment of Fig. 1B, we compared the level of ySOD1 (CCS-dependent) and wSOD-1 (CCS-independent) activity when the two SODs are co-expressed versus their expression in separate yeast cells. When ySOD1 and wSOD-1 are co-expressed, there is diminish-ished activity for both pathways compared with when each Cu,Zn-SOD is expressed alone (lanes 1–3). The addition of 50 μM copper to the growth medium attenuates this competition and allows for nearly full activity of both pathways (lanes 4–6). Together, these data of Fig. 1 are consistent with the notion that CCS and the CCS-independent pathways compete for a common pool of intracellular copper that originates from cell surface transporters rather than vacuolar stores of the metal.

Previous studies have shown that the substrate for CCS activation is a preexisting pool of apo-SOD1 (13). However, it was not known whether the same was true for CCS-independent activation. A pool of inactive ySOD1 or wSOD-1 was created by growing cells in the presence of the Cu(I) chelator bathocuproinedisulfonic acid (BCS). Copper was then supplemented to the growth medium, and the time course for activating the Cu,Zn-SODs in vivo was monitored in the presence of cycloheximide to block new SOD1 synthesis. As seen in Fig. 2, the apo pool of the CCS-independent wSOD-1 was rapidly activated in the absence of new protein synthesis. Moreover, the CCS-dependent ySOD1 and CCS-independent wSOD-1 exhibited virtu-
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Fig. 3A, the disulfide of ySOD1 is nearly fully oxidized in yeast cells that are wild type for CCS1 and are supplemented with high (non-toxic) copper (lane 1), and this correlates with maximal activity of the enzyme (bottom panel). As available copper concentrations diminish inside the cell, the disulfide becomes fully reduced, and this is observed even in CCS1 wild type cells (lanes 2 and 3). In ccs1Δ null cells (lanes 4 and 6), the disulfide of ySOD1 remains reduced irrespective of copper. Oxidation of the yeast SOD1 disulfide in vivo requires both copper and CCS, as predicted.

The effect of copper and CCS on the disulfide of C. elegans Cu,Zn-SOD is shown in Fig. 3B. As expected, the disulfide is equally oxidized in CCS1+ and ccs1Δ cells (lanes 1 and 4). However, unlike the case with yeast SOD1, the disulfide of wSOD-1 remains largely oxidized irrespective of copper. Even under copper starvation conditions, where there is insufficient copper for SOD1 enzyme activity (lanes 3 and 6, bottom panel), the disulfide is greater than 70% oxidized (middle panels). The same result is obtained in copper-replete cells expressing a H48F mutant of wSOD-1 that cannot bind copper (data not shown). Based on studies with wSOD-1 alone, it would appear that CCS independence relies on a disulfide that has a high propensity to oxidize in vivo even in the absence of copper.

The effects of CCS and copper on the disulfide of human SOD1 (both CCS-dependent and -independent) are shown in Fig. 3C. The effects are intermediate to that observed with the CCS-dependent (ySOD1) and CCS-independent (wSOD-1) SODs. With copper starvation, the enzyme is inactive, yet the disulfide remains ~40–50% oxidized (lanes 3 and 6). Even with copper starvation and in cells lacking CCS1, the hSOD1 disulfide is partially oxidized (lane 6).

Overall, the level of CCS-independent activation seen for the three tested SOD1s correlates with the propensity to oxidize the disulfide. The CCS-dependent yeast SOD1 is totally reliant on copper and the copper chaperone for disulfide oxidation, whereas the partially CCS-independent (hSOD1) and fully CCS-independent (wSOD-1) molecules show correspondingly lower requirements for copper in disulfide oxidation. The ability of Cu,Zn-SOD1 to use the CCS-independent pathway seems to require a disulfide that can be oxidized without copper.

We tested whether the differential propensities for disulfide oxidation in vivo reflected differences in the inherent reduction potentials for three Cu,Zn-SOD1 disulfides. Purified recombinant Cu,Zn-SOD proteins from human, S. cerevisiae, and C. elegans were subject to redox potential measurements under anaerobic conditions, as described (15, 35, 40). As seen in Fig. 4, incubation in redox buffers poised at decreasing redox potentials (left to right) leads to an increase in the amount of disulfide-reduced SOD1. Quantification of the results and calculation of the reduction potentials demonstrate that yeast SOD1 has the highest redox potential (~234 mV) in close agreement with previous measurements (15), whereas the reduction potentials of human and worm Cu,Zn-SOD are more negative at ~248 and ~270 mV, respectively. Overall, the relative disulfide redox potentials from E. coli-expressed SOD1s show the anticipated pattern of worm < human < yeast, precisely as we observe in the yeast expression system. However, even so, the absolute numbers are all such that the disulfide cysteines are expected to be predominantly reduced in the eukaryotic cytosol if they are influenced by the intracellular GSH:GSSG redox state (~290 mV) (41).
Perhaps there are post-translational modifications in the euakaryotic cell that contribute to the greater disulfide oxidation of these SOD1s in vivo (see “Discussion”).

**The CCS-independent Pathway Allows for SOD1 Activation in the Absence of Molecular Oxygen**—Elegant experiments by O’Halloran and co-workers (14) have shown that CCS requires molecular oxygen for activation of SOD1. However, these experiments were conducted with yeast, which is incapable of CCS-independent activation, or with hSOD1 under in vitro conditions, where CCS-independent factors are unlikely to be present. We therefore addressed the oxygen requirement for CCS-dependent versus -independent activation of human SOD1 in vivo.

Previous studies on the O2 dependence of CCS utilized the endogenous SOD1 of yeast that is down-regulated under anaerobic conditions (14). Consistent with this, we observed that either yeast or human SOD1 driven by the S. cerevisiae SOD1 promoter is strongly down-regulated under anaerobic conditions (Fig. 5A, lanes 3 and 6, top) compared with normoxia (lanes 1 and 4) and 3% oxygen (lanes 2 and 5, top). By comparison, yeast Ccs1p (bottom panel) levels remain constant throughout all tested oxygen concentrations. To circumvent the transcriptional down-regulation of SOD1 under anaerobic conditions, we used a human SOD1 construct expressed from the S. cerevisiae PGK1 promoter that allows for constant expression of hSOD1 in yeast under all oxygen tensions (Fig. 5B, bottom panel).

In CCS1 wild type cells, hSOD1 activity is maximal at 20% oxygen (air) and 3% oxygen (Fig. 5B, lanes 3 and 5) and virtually absent under anaerobic conditions (lane 7), consistent with the oxygen dependence of CCS. Compared with activity in the presence of CCS, activity of hSOD1 in the ccs1Δ null cells does not fluctuate much over the range in oxygen tensions. In fact, this CCS-independent activity is not greatly impaired under anaerobic conditions (Fig. 5B, lane 8). The CCS-independent activation of hSOD1 does not exhibit the same dependence on oxygen as CCS.

In cells expressing CCS1, a low level of hSOD1 activity is seen with anaerobic cultures (Fig. 5B, lane 7). This is likely to represent CCS-independent activation of hSOD1 as opposed to adventitious CCS activation in cell lysates. To confirm this, we employed a S142P/L144P derivative of hSOD1 that was previously shown to block CCS-independent but not CCS-mediated activation of hSOD1 (19). As seen in Fig. 5C, the residual activity of WT hSOD1 under anaerobic conditions (lanes 3 and 6) is completely obliterated in the CCS-dependent S142P/L144P hSOD1 (lane 9). This confirms that there is no adventitious CCS-dependent activation during analysis of our anaerobic cultures. Moreover, the activity of human SOD1 that remains under anaerobic conditions can be ascribed to the CCS-independent pathway, even in cells expressing CCS.

As shown above in Fig. 3C, oxidation of the human SOD1 disulfide in air can occur in the absence of CCS and under copper starvation conditions. We tested whether the same was true under anaerobic conditions. As seen in Fig. 5C, the disulfide of hSOD1 is partially oxidized under anaerobic conditions (lane 3), and this reflects CCS-independent disulfide oxidation as it is observed in ccs1Δ cells (lane 6) but not in the S142P/L144P hSOD1 that is incapable of CCS-independent activation (lane 9). Interestingly, the CCS-independent and oxygen-independent oxidation of the disulfide also appears copper-independent. As seen in Fig. 5D, the disulfide of hSOD1 expressed in anaerobic cultures remains oxidized even when copper is limiting such that there is no SOD1 activity (lanes 3 and 6). Expression of CCS1 has no effect on the degree of SOD1 disulfide (lanes 1–3) or activity (bottom panel) under anaerobic conditions. The disulfide of human SOD1 indeed has a high propen-
ySOD1 is active only in yeast cells expressing activation of individual Pro-144 mutants of yeast SOD1 activity (11). CCS protects the lysine biosynthetic pathway from superoxide damage (44); therefore, yeast cells lacking SOD1 activity are unable to grow aerobically on medium lacking lysine. A library of ySOD1 mutants created through error-prone PCR were transformed into sod1Δ ccs1Δ yeast and screened for lysine-independent aerobic growth.

The spectrum of ySOD1 mutations that permitted CCS-independent activation is shown in supplemental Table S1. The CCS-independent activity of a number of these was too low to monitor SOD1 activity directly on activity gels, yet they were sufficiently active to sustain aerobic growth in the absence of lysine. Previous studies have shown that less than 1% of the normal yeast SOD1 activity is sufficient to protect the lysine biosynthetic pathway (17). However, a number of ySOD1 mutants did show strong CCS-independent activity that could be monitored by enzyme assays, similar to levels obtained with human SOD1. Interestingly, all contained mutations at Pro-144.

Fig. 6A shows the CCS-mediated and CCS-independent activation of individual Pro-144 mutants of yeast SOD1. WT ySOD1 is active only in yeast cells expressing CCS1 (lanes 1 and 2), whereas P144Q (lanes 3 and 4), P144L (lanes 5 and 6), and P144S (lanes 7 and 8) derivatives are also active in ccs1Δ cells. Of these, P144S displays the highest level of CCS-independent activity (lane 8). Previously, we had shown through targeted mutagenesis that a P142S/P144L double mutant of ySOD1 was CCS-independent and postulated that both prolines were involved in blocking CCS-independent activation (19). The results of Fig. 6A indicate that Pro-144 by itself is sufficient to block enzyme activity in the absence of CCS. To confirm this, single P142S and P142A derivatives of ySOD1 were created to match the analogous residues in the CCS-independent human and C. elegans Cu,Zn-SODs, respectively. Fig. 6B shows that these Pro-142 mutants retain strong dependence on yeast CCS1 for activity (lanes 3–6). Hence, it is Pro-144 and not Pro-142 that is the key to blocking activity in the absence of CCS.

As shown above in Fig. 3, CCS-independence closely correlates with the propensity for disulfide bond formation. Therefore, the effect of Pro-144 mutations on the yeast SOD1 disulfide were examined. In Fig. 7A, WT ySOD1 requires yeast CCS1 and copper for disulfide oxidation (top) and enzyme activity (bottom). However, with all three variants of Pro-144, there is a higher tendency to oxidize the disulfide, even in ccs1Δ cells starved for copper (Fig. 7, B–D). This is particularly obvious with P144S, which shows the strongest level of CCS-independent activity (Fig. 7D). These findings underscore the notion that activation of SOD1 in the absence of CCS requires a disulfide bond that is more prone to oxidize, even in the absence of copper. These studies also demonstrate that a single Pro-144 in SOD1 is sufficient to block disulfide oxidation under low copper and in the absence of CCS.

**DISCUSSION**

Depending on the organism, Cu,Zn-SOD molecules can be activated in vivo through either the CCS copper chaperone or through a CCS-independent pathway (11, 20, 45). Here we have compared and contrasted SOD1 molecules that have evolved to favor one pathway over the other. We observe that as with CCS, CCS-independent activation occurs rapidly in vivo with preexisting apo-SOD1 molecules and employs the same pool of available copper as CCS, largely derived from cell surface copper transporters. However, two major differences were noted in the two pathways for activating SOD1. First, CCS exhibited a strong dependence on copper for oxidizing the SOD1 disulfide in vivo, whereas CCS-independent disulfide oxidation occurred even under severe copper deplete conditions. Second, the CCS-independent activation of SOD1 does not exhibit the same requirement for molecular oxygen as does CCS. In this manner, SOD1 molecules that are activated without CCS can retain activity over a wide range of oxygen tensions.

Previous studies reporting the requirement for copper in disulfide oxidation and the requirement for oxygen in SOD1 activation were all conducted with S. cerevisiae SOD1 (14, 15).
We now know that yeast Cu,Zn-SOD may be an exception rather than a rule, based on the strict CCS-dependence of this molecule. To date, the only Cu,Zn-SODs known to be fully dependent on CCS are those from Ascomycota fungi, such as S. cerevisiae, and we show here this is due in large part to a single proline at position 144. In our survey of Cu,Zn-SOD molecules from various metazoans, including mammals, insects, birds, and nematodes that all lack Pro-144, CCS-independent activation is evident (19, 20, 45). Unlike the unicellular yeasts that are directly exposed to atmospheric O2, tissues and cells from multicellular metazoans experience a wide range of oxygen tensions from aerobic to hypoxic. By obviating dependence on CCS, SOD1 activity can be maintained in very low oxygen concentrations at Pro-144 help to obviate the requirement for copper in disulfide oxidation. Pro-144 is situated less than 5.5 Å away from the disulfide involving Cys-146 and may therefore restrict conformational dynamics and/or affect the pKa of the cysteines such that the disulfide is less prone to oxidation. In fact, previous results with thioredoxin have shown that substitution of the proline in the CGPC active site causes a large shift in the disulfide reduction potential (40, 47–49). Prolines may play a more widespread role in thiol oxidation than previously appreciated.

Pro-144 is not the only residue to influence CCS-independent SOD1 activation and disulfide bond formation. Like human SOD1, C. elegans contains a non-proline residue at position 144, yet worm and human SOD1 display vast differences in both disulfide oxidation and degree of CCS-independent activation (20). Very recently, the three-dimensional structure of C. elegans SOD1 has been solved, revealing an unprecedented conformation of the Cu,Zn-SOD that could explain the complete lack of CCS activation in this molecule.5

Overall, these findings obtained through yeast expression systems can have important implications in human cellular physiology. Having dual mechanisms for activating human SOD1 under a wide range of oxygen tensions provides versatility in control of SOD1 activity. Although classically known as an oxidative stress enzyme, SOD1 is gaining recognition in mammalian signaling pathways under non-oxidatively stressed or

4 J. M. Leitch, L. T. Jensen, S. D. Bouldin, C. E. Outten, P. J. Hart, and V. C. Culotta, unpublished results.
5 O. Pakhamova, A. B. Taylor, L. T. Jensen, J. M. Leitch, V. C. Culotta, and P. J. Hart, manuscript in preparation.
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even hypoxic conditions (50–52). The disparate activation pathways for SOD1 are likely to come into play over a range of physiological oxygen tensions.

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