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Post-Translational Modification of Cu/Zn Superoxide Dismutase under Anaerobic Conditions

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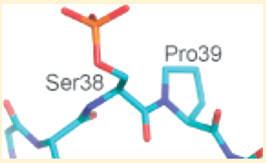
Supporting Information

ABSTRACT: In eukaryotic organisms, the largely cytosolic copper- and zinc-containing superoxide dismutase (Cu/Zn SOD) enzyme represents a key defense against reactive oxygen toxicity. Although much is known about the biology of this enzyme under aerobic conditions, less is understood regarding the effects of low oxygen levels on Cu/Zn SOD enzymes from diverse organisms. We show here that like bakers' yeast (*Saccharomyces cerevisiae*), adaptation of the multicellular *Caenorhabditis elegans* to growth at low oxygen levels involves strong downregulation of its Cu/Zn SOD. Much of this regulation occurs at the post-translational level where CCS-independent activation of Cu/Zn SOD is inhibited. Hypoxia inactivates the endogenous Cu/Zn SOD of *C. elegans* Cu/Zn SOD as well as a P144 mutant of *S. cerevisiae* Cu/Zn SOD (herein denoted Sod1p) that is independent of CCS. In our studies of *S. cerevisiae* Sod1p, we noted a post-translational modification to the inactive enzyme during hypoxia. Analysis of this modification by mass spectrometry revealed phosphorylation at serine 38. Serine 38 represents a putative proline-directed kinase target site located on a solvent-exposed loop that is positioned at one end of the Sod1p β-barrel, a region immediately adjacent to residues previously shown to influence CCS-dependent activation. Although phosphorylation of serine 38 is minimal when the Sod1p is abundantly active (e.g., high oxygen level), up to 50% of Sod1p can be phosphorylated when CCS activation of the enzyme is blocked, e.g., by hypoxia or low-copper conditions. Serine 38 phosphorylation can be a marker for inactive pools of Sod1p.



The large family of superoxide dismutase (SOD) enzymes represents a primary defense against reactive oxygen toxicity. Using copper, iron, manganese, or nickel as a catalytic cofactor, these enzymes disproportionate reactive superoxide anion radicals into hydrogen peroxide and oxygen. Most eukaryotes express two distinct SOD molecules that differ in cellular location and metal ion cofactor. A manganese-containing form of the enzyme (often denoted SOD2) localizes to the mitochondrial matrix, whereas an unrelated copper- and zinc-containing SOD (often denoted SOD1) localizes diffusely throughout the cell, including the cytosol, nucleus, and intermembrane space of mitochondria.[1,2]

SOD enzyme activity can be regulated at the transcriptional and post-translational levels, where post-translation control involves the rapid conversion of an apo-inactive polypeptide to an enzymatically active SOD enzyme through insertion of the metal ion cofactor. The best-studied example of such post-translation control involves the Cu/Zn SODs of eukaryotes. Each subunit of the Cu/Zn SOD homodimer harbors three key post-translational modifications: the catalytic copper ion, a noncatalytic but structurally important zinc ion that promotes the proper geometry of the copper site, and an intramolecular disulfide that also serves an essential structural role.[1,3,4] While virtually nothing is known about insertion of zinc, copper acquisition and disulfide oxidation have been studied in great detail. In 1997, the CCS copper chaperone that serves to insert copper and oxidize the disulfide in eukaryotic Cu/Zn SOD molecules was identified.[5] Much of the work on CCS has been completed in the bakers' yeast *Saccharomyces cerevisiae* where the Cu/Zn SOD (herein denoted Sod1p) is completely dependent on the Ccs1p copper chaperone for activation.[6−8] However, in non-yeast organisms, Cu/Zn SOD molecules can also be activated through a secondary pathway that is independent of CCS but is reliant on abundant intracellular glutathione.[9,10] Most eukaryotic Cu/Zn SODs can be activated through both pathways, with the exception of the Cu/Zn SOD of *Caenorhabditis elegans* (denoted Sod-1). The nematode genome does not encode CCS, and accordingly, worm Sod-1 is only activated in a manner independent of CCS.[11]

Key structural determinants in the Cu/Zn SOD polypeptide dictate whether the SOD is activated solely by CCS (e.g., yeast Sod1p), only by the CCS-independent pathway (e.g., *C. elegans* Sod-1), or by both pathways (e.g., human Cu/Zn SOD, known as SOD1).[9,10,12] For example, prolines at position 142 and 144 in *S. cerevisiae* Sod1p preclude this Cu/Zn SOD from being

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activated in a manner independent of CCS. These proline residues are positioned at the end of loop VII at one end of the Greek key β-barrel of the Cu/Zn SOD structure.[13,14] Human SOD1 harbors serine and leucine at the equivalent positions, and an S142P/L144P variant of human SOD1 was shown to gain complete dependence on CCS.[10] In our more recent studies, we observed that of the two prolines, P144 of yeast Sod1p was most critical and that a single P144S substitution was sufficient to confer CCS independence to yeast Sod1p.[9] The P144S variant of yeast Sod1p provides a unique tool for exploring the distinct mechanisms for SOD enzyme activation.

Oxygen is also a key factor for activation of Cu/Zn SOD molecules. Rotilio and co-workers were the first to observe an oxygen dependence on yeast SOD1 activity in 1991.[15] O'Halloran and colleagues then demonstrated that the inactivity of yeast SOD1 under anaerobic conditions reflected a strict requirement for CCS in its activation of the SOD.[16] This was not likely due to a limitation of the Cu(I) cofactor for SOD1, as the total level of copper if anything is increased under anaerobic conditions and is predominantly Cu(I).[15,17] If anything, oxidation of the SOD1 disulfide would be limiting under these conditions, not copper. We previously reported that human SOD1 that is a recipient for the both the CCS-dependent and CCS-independent pathways is not so rigorously dependent on oxygen for activity as is the case with yeast SOD1. When expressed in yeast, residual human SOD1 activity was retained without oxygen via the CCS-independent pathway.[9] Nevertheless, these previous studies did not exclude a role for oxygen in regulating the efficacy of CCS-independent activation. Aside from post-translation effects, oxygen can also control SOD enzymes at the transcriptional level as seen in yeast where the level of expression of both *SOD1* and *SOD2* is decreased as cells become hypoxic.[15,18] The adaptation of yeast to low oxygen levels clearly involves a loss of SOD enzymes, although it is not clear if a similar scenario exists in multicellular organisms that use CCS-independent pathways.

In this study, we revisit the oxygen control of SOD enzymes. We find that the decrease in SOD enzyme levels with low oxygen levels is not unique to CCS-dependent Sod1p from bakers' yeast. The CCS-independent Sod-1 of *C. elegans* is likewise subject to downregulation when the nematode grows at low oxygen levels. Oxygen control of CCS-independent activation was also observed in a yeast expression system where the activity of the CCS-independent P144S yeast Sod1p decreased with a low oxygen level. Finally, these studies revealed a new post-translational modification to yeast Sod1p that was favored with low oxygen levels, namely phosphorylation at serine 38. This modification correlated specifically with loss of CCS-dependent activation of yeast Sod1p and appears to mark cellular pools of inactive enzyme.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions. Yeast strains used in this study include BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), the *sod1Δ kanMX4* derivative (Research Genetics), and BY4741 harboring a C-terminally TAP-tagged version of Tir1p (Open Biosystems).[19] A *sod1Δ::kanMX4* mutation was introduced into this Tir1-TAP strain using the pJAB002 plasmid (described below) creating strain JL116. Additional strains include *sod1Δ::TRP* strain KS107 (*Mat α, leu2-3, 112, his3Δ1, GAL+, trp1-289a, ura3-52, sod1Δ::TRP1*)[20] and isogenic *ccs1Δ sod1Δ* strain LS102 (*Mat α, leu2-3, 112, his3Δ1, GAL+, trp1-289a, ura3-52, sod1Δ::TRP1, lys7Δ::URA3 ura3-*).[9]

Cells were propagated at 30 °C in enriched yeast extract, peptone-based medium supplemented with 2% glucose (YPD), a minimal synthetic medium (SC), or the same medium containing 15 mg/L ergosterol and 0.5% Tween 80 for support of anaerobic growth (YPDE or SCE).[21] Anaerobic conditions ("0 oxygen") were achieved either by growth in anaerobic culture jars (BBL GasPak) as previously described[22] or by growth in a Coy Laboratory anaerobic workstation equilibrated to 30 °C. For growth at 1 or 3% oxygen, cultures were placed in an Almore Vacu-Quik jar and pre-equilibrated in a COY chamber under a 100% nitrogen atmosphere. The jars were sealed and then alternately placed under vacuum and flushed with either a 1% oxygen/99% nitrogen gas mixture or a 3% oxygen/97% nitrogen gas mixture (Airgas) five times.

Yeast Plasmids. The pLJ486 2 μm *URA3* plasmid for expressing *S. cerevisiae SOD1* from the strong *ADH1* promoter was a gift from L. Jensen. pJL111 is a derivative of pLJ486 that expresses P144S Sod1p created by QuikChange mutagenesis (Stratagene). The S38D, P39A, S38E, S38T, and S38A derivatives of *S. cerevisiae* Sod1p were created similarly by mutagenesis using as a template *CEN LEU2* plasmid pLS108 where *SOD1* falls under control of the endogenous *S. cerevisiae SOD1* promoter.[9] *sod1::kanMX4* plasmid pJAB002 was created by amplifying via polymerase chain reaction (PCR) *S. cerevisae SOD1* sequences from position −744 to −204 with primers that introduced a BamHI site at position −732 and an XbaI site at position −223, and amplification of residues 500−920 with primers including 5′ and 3′ end XhoI and BamHI sequences, respectively. The PCR products were digested with the respective enzymes and ligated in a trimolecular reaction into *kanMX4* plasmid pRS400[23] digested with XbaI and XhoI. The resulting pJAB002 plasmid digested with BamHI was used to delete chromosomal *SOD1* residues −223 to 500.

***C. elegans* Strains and Growth.** *C. elegans* strain N2 was provided by the *Caenorhabditis* Genetics Center funded by the National Institutes of Health National Center for Research Resources (NCRR). The sod-1 (tm776) and sod-2 (gk257) strains were provided by S. Mitani (Tokyo Women's Medical University School of Medicine, Tokyo, Japan). Nematodes were maintained at 25 °C on enriched NGM plates supplemented with *Escherichia coli* OP50 as a food source. Worms were synchronized by harvesting animals from four 10 cm plates by rinsing in M9 buffer,[24] yielding a pellet of approximately 0.5 mL; worms were then synchronized by bleaching of gravid hermaphrodites.[25] Isolated eggs were hatched in M9 at 20 °C for 24 h. The resulting starved L1 animals were then transferred to fresh plates and allowed to grow for 2 days at 25 °C to young adult stages. For hypoxia experiments, the young adults were placed in an Almore Vacu-Quik jar that was flushed with nitrogen five times and incubated for an additional 24 h at 25 °C. Upon inspection of worms following this hypoxic treatment, full recovery of movement was observed within minutes of the worms being transferred to air.

SOD Activity and Protein Analysis in Crude Lysates. For SOD activity and immunoblot analysis of yeast cells, cells were lysed by glass bead homogenization in a Hepes or phosphate buffer (pH 7.5) also containing 1.2 mM sorbitol and protease inhibitors as described previously.[9] In the case of *C. elegans*, we harvested one plate of worms by rinsing the plate in M9 buffer, yielding approximately 100 μL of animals that were

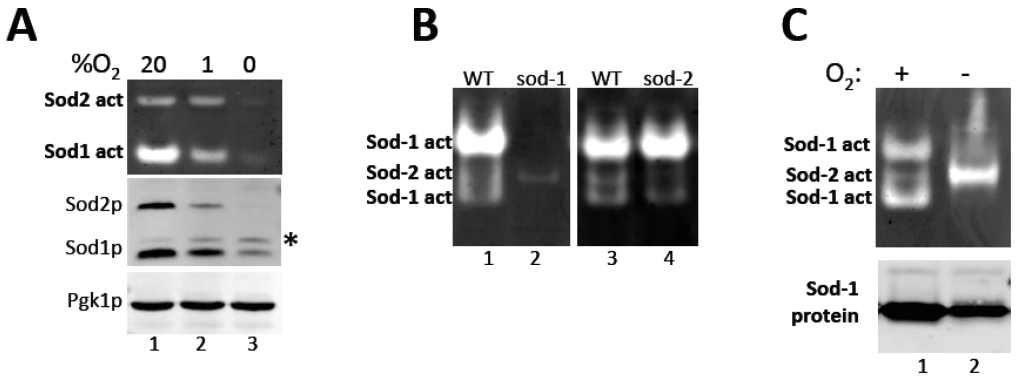

Figure 1. Effects of low oxygen levels on SOD enzymes in *S. cerevisiae* and *C. elegans*. (A) The BY4741 yeast strain was grown for 18 h to confluency in YPDE medium under the indicated oxygen tensions. Cells were lysed and assayed for (top) SOD activity by native gel electrophoresis and NBT staining and (middle and bottom) Sod1, Sod2, and Pgk1 protein levels by SDS−PAGE and immunoblot analyses. Pgk1p serves as a loading control. Sod1 act and Sod2 act mark the positions of the respective active enzymes on the native gel. An asterisk marks the position of post-translationally modified Sod1p (see the text). (B and C) Synchronized *C. elegans* worms were grown for 2 days to young adult stage in air (B) and where indicated (C) grown for an additional 24 h at 25 °C either in air (O_2: +) or under nitrogen in an anaerobic culture jar (O_2: −) Worms were lysed and analyzed for SOD activity (B and top gel of panel C) or Sod-1 protein levels (bottom gel of panel C) as in panel A. Worms were either the wild-type N2 strain (B and C) or the *sod-1* or *sod-2* null derivatives (B). Enzymatically active Sod-1 typically migrates as two bands on a native gel and Sod-2 as a single band marked by Sod-1 act and Sod-2 act.

suspended in lysis buffer containing 10 mM $NaPO_4$ (pH 7.8), 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, 0.1% Triton X-100, 10% glycerol, 500 μM phenylmethanesulfonyl fluoride, and 1:100 protease inhibitor (Sigma catalog no. P8340) and lysed in a TissueLyser using 0.7 mm zirconium oxide beads and agitation for two 1.0 min cycles. Lysates were clarified by centrifugation at 20000*g* and 4 °C for 10 min. Supernatants were filtered through a 0.22 μm membrane (Costar, Spin-X Centrifuge Tube Filter) by centrifugation at 20000*g* and 4 °C for 2 min. Whole cell lysates from either yeast or worms were analyzed for SOD activity by native gel electrophoresis using 12% precast gels (Invitrogen) and staining with nitroblue tetrazolium (NBT).[26] Immunoblot analysis was conducted by denaturing gel electrophoresis on 14% SDS−PAGE gels, followed by gel transfer to membranes and hybridization to antibodies directed against *C. elegans* Sod-1 (cross reacts well with Cu/Zn SODs from various species[11]), to *S. cerevisiae* Sod2p,[27] to *S. cerevisiae* Pgk1p (Invitrogen), and to a TAP tag (Open Biosystems). Immunoblots were visualized by the Odyssey infrared imaging system (Licor Biosciences). Quantitation was performed with LI-COR Odyssey and ImageJ (W. S. Rasband, National Institutes of Health, Bethesda, MD)

Protein Purification. Purified, recombinant yeast Sod1p was isolated from an *S. cerevisiae* expression system as previously described.[28] To obtain recombinant yeast Sod1p from a bacterial expression system, the gene encoding the full-length wild-type yeast Sod1p protein was subcloned into a pET-3d plasmid (Stratagene) and expressed at 37 °C in *E. coli* strain BL21(DE3) under the control of the IPTG-inducible lac UV5 promoter. Cells were grown to an A_{600} of 0.7 before being induced using 1 mM IPTG, and the cells were permitted to grow for an additional 3 h before being harvested by centrifugation. The pelleted cells were resuspended in 50 mM phosphate buffer (pH 7.4) and lysed by sonication on ice. After the cell debris had been removed by centrifugation, the cleared lysate was dialyzed against distilled deionized water to bring the phosphate concentration to 5 mM at pH 7.4. The yeast Sod1p protein was subsequently purified via anion-exchange chromatography on DEAE-cellulose (Whatman) followed by gel filtration on Sephadex G-75 (Pharmacia). The purity of wild-type yeast Sod1p was confirmed through the observation of a single band using 15% SDS−PAGE. To remove adventitiously bound metals, we subjected purified yeast Sod1p to dialysis against 100 mM acetate buffer (pH 3.8) and 100 mM EDTA, followed by dialysis with the same buffer but with 100 mM NaCl replacing the EDTA, followed by dialysis against 100 mM acetate (pH 5.5). The yeast Sod1p apoprotein concentration was calculated using a molar extinction coefficient of 3000 M^{-1} cm^{-1} at 278 nm (derived from the single tyrosine residue per protein monomer). The yeast Sod1p protein was reconstituted with zinc first, followed by copper as described previously.[29]

Mass Spectrometry Analysis. For analysis of recombinant yeast Sod1 proteins by mass spectrometry, 1.0 μg of the aforementioned recombinant proteins was resolved by SDS−PAGE and identified by colloidal blue staining (Invitrogen). Bands were excised and submitted for in-gel tryptic digest and mass spectral analysis and sequencing to the Johns Hopkins University School of Medicine Mass Spectrometry and Proteomics Facility. The data were then analyzed with Scaffold 2 (Proteome Software) for differences in sequence coverage and mass.

RESULTS

Downregulation of SOD Enzymes by Low Oxygen Levels. Superoxide dismutase enzymes in the bakers' yeast *S. cerevisiae* are downregulated by low oxygen levels. In the experiment depicted in Figure 1A, cells were cultured for 24 h under varying oxygen tensions. Steady state SOD activity was monitored by a native gel assay, and SOD protein levels were monitored by an immunoblot. Both Sod1p (largely cytosolic Cu/Zn) and Sod2p (mitochondrial manganese) in yeast were strongly downregulated by low oxygen levels at the levels of enzymatic activity (top panel) and protein (middle panel). Activity was maximal at atmospheric 20% oxygen and virtually absent with 0 oxygen/anaerobic conditions (Figure 1A).

We tested whether the downregulation of SOD enzymes by low oxygen levels was unique to yeast or was also a property of multicellular organisms, e.g., the metazoan *C. elegans*. In a SOD activity analysis of whole worms, the Cu/Zn largely cytosolic Sod-1 is often seen as dual bands and mitochondrial manganese

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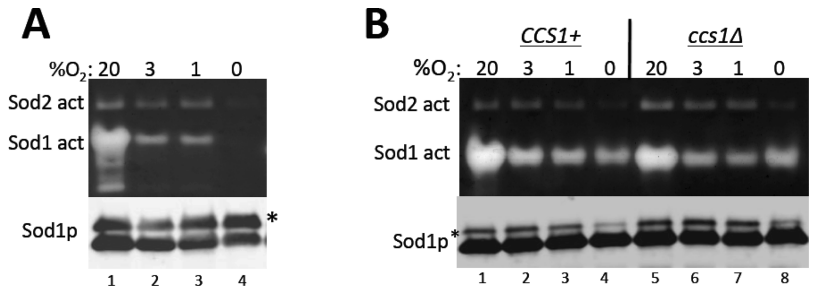

Figure 2. Effect of oxygen on CCS-dependent vs CCS-independent activation of *S. cerevisiae* Sod1p. Yeast strains overexpressing the indicated Sod1p molecules under the strong *ADH1* promoter were grown in selecting SCE medium for 18 h either aerobically (20% oxygen) or under 3, 1, or 0% oxygen/anaerobic conditions as indicated. Cells were lysed and assayed for (top) SOD activity and (bottom) Sod1 protein levels as in Figure 1A: (A) *sod1*Δ KS107 strain expressing WT Sod1p and (B) *sod1*Δ strain KS107 (lanes 1−4) or *sod1*Δ *ccs1*Δ strain LS102 (lanes 5−8) expressing P144S Sod1p. Sod1 act and Sod2 act mark the positions of the respective active enzymes on the native gel. An asterisk marks the position of post-translationally modified Sod1p (see the text).

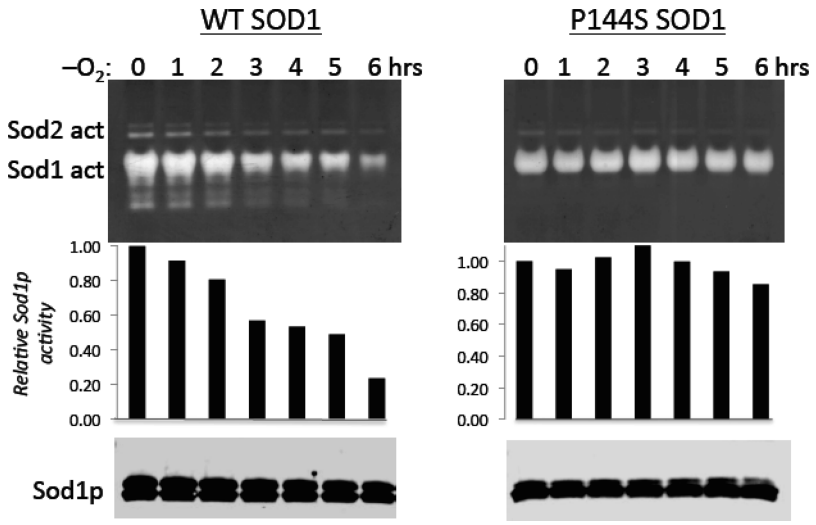

Figure 3. Time course for loss of CCS activation upon a switch to anaerobic conditions. *sod1*Δ strain JL116 expressing either WT Sod1p (left) or P144S Sod1p (right) under the strong *ADH1* promoter was grown aerobically in SC medium to an OD_{600} of 0.8 and then placed under anaerobic conditions for the indicated time points prior to analysis of Sod1p activity (top) and protein levels (bottom) as in Figure 1A. The bar graphs represent quantitation of the Sod1p activity bands from the native gel.

Sod-2 as a single band; these SODs can be discerned by comparative analysis of *sod-1* null and *sod-2* null animals (Figure 1B, lanes 2 and 4). In the experiment depicted in Figure 1C, synchronized young adult worms were incubated at 25 °C for 24 h either in air or under anaerobic conditions that do not affect worm survival. Sod-1 activity was virtually absent under anaerobic conditions (Figure 1C, lane 2). By comparison, Sod-2 activity in the mitochondria was not downregulated and, if anything, was seen to increase with hypoxia (Figure 1C, lane 2). Although the effects of hypoxia on mitochondrial Mn SODs are not conserved in yeast and worms, both organisms show a dramatic decrease in the level of the major Cu/Zn SOD enzyme with low oxygen levels.

It is noteworthy that Sod1p of *S. cerevisiae* is a CCS-dependent enzyme whereas worm Sod-1 is CCS-independent,[9,11] yet both are downregulated by hypoxia. Oxygen control of the CCS-independent worm Sod-1 could lie at either the pre- or post-translational levels because both Sod-1 protein and enzymatic activity decreased in hypoxic worms (Figure 1C). To specifically test whether post-translational activation by the CCS-independent pathway is subject to oxygen control, we used a *S. cerevisiae* expression system. In the experiment depicted in Figure 2, a CCS-dependent Cu/Zn SOD and CCS-independent Cu/Zn SOD were expressed under the *S. cerevisiae* *ADH1* promoter to eliminate any transcriptional effects of oxygen. As such, SOD protein levels were basically unchanged with varying oxygen levels (bottom panel, Figure 2A,B). The CCS-dependent and -independent Cu/Zn SODs chosen for these studies varied by only a single amino acid, namely, wild-type (WT) yeast Sod1p (CCS-dependent) and its P144S variant that is CCS-independent.[9] Consistent with previous results,[16] CCS activation of WT Sod1p is strongly downregulated by low oxygen levels (Figure 2A). The extent of CCS-independent activation of P144S Sod1p also decreased with lower oxygen levels in both *CCS1*+ (Figure 2B, lanes 1−4) and *ccs1*Δ null yeast (lanes 5−8). Activity was maximal at 20% oxygen and then decreased at ≤3% O_2 (Figure 2B). The major difference was seen under 0 oxygen/anaerobic conditions where the CCS-dependent WT Sod1p was completely inactive (Figure 2A, lane 4), whereas the P144S variant retained residual activity (Figure 2B, lanes 4 and 8), consistent with previous findings on human SOD1 expressed in yeast.[9] The studies depicted in Figure 2 were conducted under steady state conditions, i.e., growth for 18 h at various oxygen tensions. To more closely examine oxygen effects, we conducted a time course experiment in which cells were pregrown in atmospheric

oxygen prior to switching to anaerobic conditions for various time points. As seen in the left panel of Figure 3, the extent of CCS-mediated activation of WT Sod1p declined ≈5-fold within 6 h of growth under anaerobic conditions. By comparison, there was relatively little change in CCS-independent activation of P144S Sod1p during this shorter time course (Figure 3, right). Together, the studies depicted in Figures 1−3 demonstrate that atmospheric oxygen is needed for maximal Sod1p activation by both CCS-dependent and -independent pathways; however, the oxygen requirement for CCS at least in the yeast expression system appears to be more stringent.

Post-Translational Modification of Sod1p by Phosphorylation. During the course of our studies on oxygen control of yeast Sod1p, we noted an aberrant migration of the Sod1 polypeptide on SDS gels (see the asterisks in Figures 1 and 2). In addition to the major Sod1 polypeptide, a secondary species with a slightly slower mobility is evident. Because it is SDS-resistant, this secondary band may represent a covalent modification of the polypeptide. With endogenous Sod1p of aerobic cultures, this modification is of low abundance (Figure 1A, lane 1); however, it can represent up to 50% of the total Sod1p protein in anaerobically grown cells (Figures 1A, lane 3, Figure 2A, lane 4, and Figure S1 of the Supporting Information). The appearance of the modification can be visualized over time when cells are switched from aerobic to anaerobic conditions (Figure 4). The frequency of the

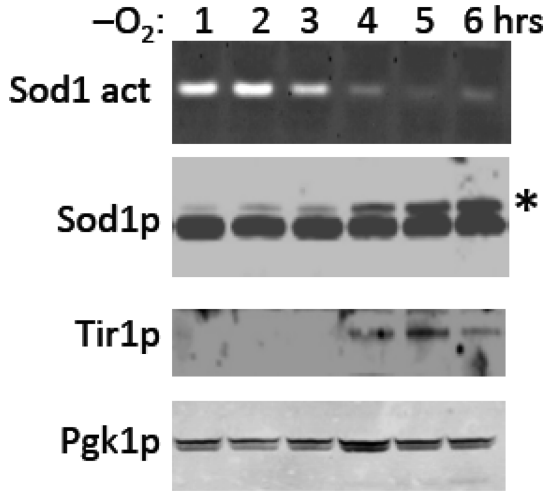

Figure 4. Molecular events associated with a switch to anaerobic conditions. The yeast strain expressing the TAP-tagged version of Tir1p was grown under anaerobic conditions for the indicated times precisely as described in the legend of Figure 3. Endogenous Sod1p activity (top) and Sod1 protein levels (second panel) were assayed as described in the legend of Figure 1A. Immunoblots were also probed for Tir1-TAP using an anti-TAP antibody (third panel) and for Pgk1p (bottom) as a loading control. An asterisk marks the position of modified Sod1p on denaturing gels.

modification increases in parallel with the decline in Sod1p activity and also follows the induction of Tir1p, a marker of anaerobic gene expression[30] (Figure 4).

The Sod1p modification appears to be specific to eukaryotic expression systems. Recombinant yeast Sod1p purified from *E. coli* migrates as a single band on denaturing gels, whereas recombinant Sod1p purified from a *S. cerevisae* expression system exhibits the characteristic doublet (Figure 5A). To investigate the nature of this modification, we subjected both

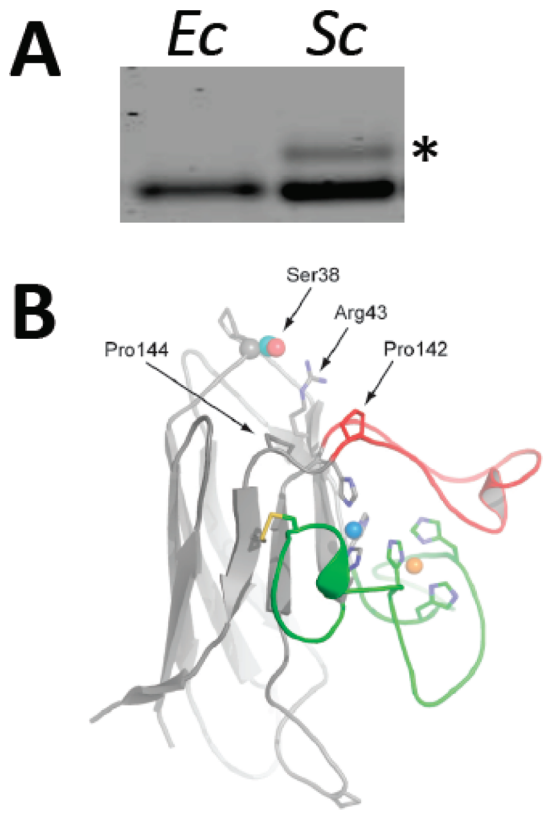

Figure 5. Yeast Sod1p is phosphorylated at serine 38. (A) Recombinant yeast Sod1p (1.0 μg) that was purified from either an *E. coli* (Ec) or *S. cerevisiae* (Sc) expression system was analyzed by denaturing gel electrophoresis and brilliant blue staining. An asterisk marks the position of modified Sod1p unique to the yeast expression system. (B) Crystal structure of yeast Sod1p (Protein Data Bank entry 2jcw[14]) highlighting the region where S38 is positioned. The zinc loop (loop IV) is colored green and the electrostatic loop (loop VII) red. Copper and zinc ions are shown as blue and orange spheres, respectively. The disulfide bond between C57 and C146 is shown as yellow sticks. This image was created with PyMol.

forms of yeast-expressed recombinant Sod1p and the single recombinant Sod1p from *E. coli* (Figure 5A) to mass spectrometry analysis. The results summarized in Figure S2 of the Supporting Information revealed a single post-translational modification. Specifically, the slower-migrating form of yeast-expressed Sod1p showed an 80 Da modification to serine 38 consistent with phosphorylation, whereas this modification was absent in the faster-migrating form from yeast and from *E. coli* expression systems (Figure S2 of the Supporting Information). Consistent with phosphorylation at S38, the slower-migrating Sod1p band on SDS gels is susceptible to phosphatase treatment (Figure S3 of the Supporting Information) and is abolished in an S38A mutant of Sod1p (Figure 6A, lane 4).

Figure 5B reveals that S38 in *S. cerevisiae* Sod1p is located on a solvent-exposed loop (loop III) at one end of the β-barrel, a region immediately adjacent to proline residues 142 and 144 that have been previously implicated in dictating the CCS dependence of the fungal Sod1p.[9,10] Figure 5C shows how this region might appear when S38 is phosphorylated.

Serine 38 is followed by a proline, suggesting it may be recognized by proline-directed kinases.[31,32] To address this possibility, we analyzed substitution mutations at S38 and P39. As with an S38A mutation, a P39A substitution blocked

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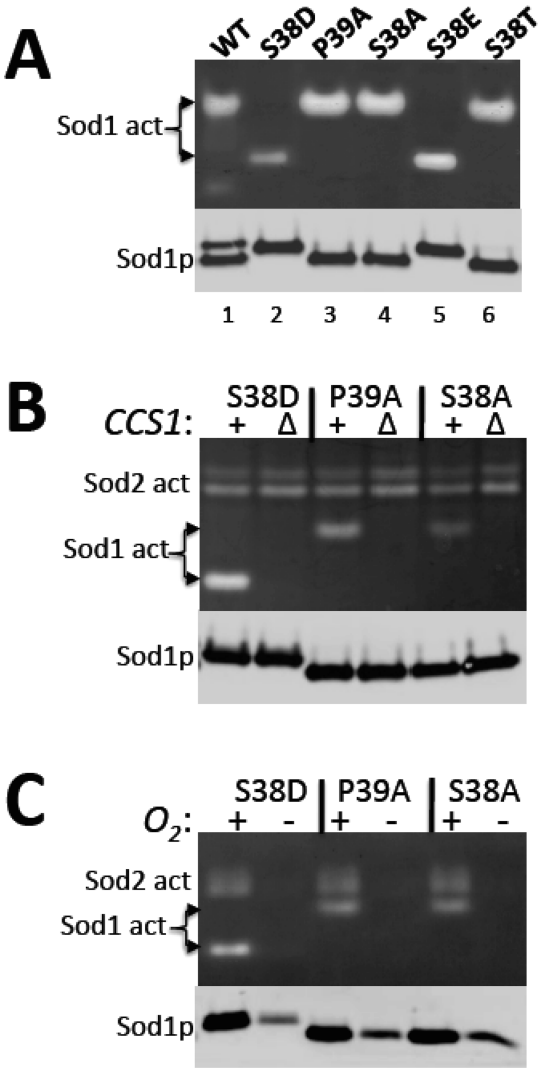

Figure 6. Requirement for S38 and P39 in post-translational modification of Sod1p. The *sod1*Δ strain expressing either WT Sod1p on plasmid pLS108 or the indicated mutant derivatives of Sod1p was grown for 18 h in selecting SC (A and B) or SCE (C) medium prior to analysis of Sod1p activity by the native gel assay (top) and Sod1 polypeptide levels (bottom) by an immunoblot as in Figure 1A. For Sod1 act, the double arrow indicates the two positions for Sod1p migration on native gels where S38D and S38E Sod1p migrate faster than WT Sod1p and other mutant variants. Cells were either the *sod1*Δ*::kanMX4* derivative of BY4741 (A and C), *sod1*Δ strain KS107 (CCS1: +, panel B), or *sod1*Δ *ccs1*Δ strain LS102 (CCS1: Δ, panel B). Cell growth occurred under either aerobic conditions (A, B, and O_2: +, panel C) or anaerobic conditions (O_2: −, panel C).

modification of Sod1p (Figure 6A, lane 3), consistent with the notion that a proline-directed kinase is involved in S38 phosphorylation. The phospho mimic S38D and S38E mutations resulted in a single Sod1p species that exhibited slower migration on SDS gels, similar to effects of phosphorylation at S38 (Figure 6A, lanes 2 and 5). Surprisingly, an S38T substitution also inhibited modification of Sod1p (lane 6), indicating that SP, not TP, is a preferred site for phosphorylation of yeast Sod1p. It is noteworthy that all the aforementioned substitutions produced enzymatically active Sod1p (Figure 6A). Like WT Sod1p, variants with substitutions at S38 and P39 were dependent on the CCS copper chaperone (Figure 6B) and also dependent on oxygen for enzymatic activity (Figure 6C).

Inactive Pools of Sod1p Favor Phosphorylation at Serine 38. As mentioned above, phospho-S38 can become more prominent under anaerobic conditions. This was observed with endogenous Sod1p (Figures 1A and 4) as well as WT Sod1p overexpressed from the *ADH1* promoter (Figure 2A and Figure S1 of the Supporting Information). However, P144S Sod1p that lacks CCS dependence is not phosphorylated to the same degree as WT Sod1p examined in parallel, and there is no increased level of phosphorylation under anaerobic conditions (Figure 2 and Figure S1 of the Supporting Information). These results suggest that a loss of CCS activation of Sod1p triggers an increased level of phosphorylation at Ser38. If true, loss of CCS in air should mimic the anaerobic effects on Sod1p. CCS activation of WT Sod1p in air can be blocked by lowering the copper availability or through null mutations in *ccs1*. As one can see in Figure 7, Sod1p activity in air is highest with copper-supplemented cells and lowest in cells treated with the BCS copper chelator (Figure 7A) or in *ccs1*Δ cells (Figure 7B). We observed that in all cases, the degree of Sod1p phosphorylation as determined by immunoblot increases with a loss of Sod1p activity (Figure 7). Hence, it appears that the loss of CCS activation of Sod1p both in air and under anaerobic conditions favors phosphorylation of Sod1p at Ser38.

■ DISCUSSION

In organisms as diverse as bakers' yeast and *C. elegans*, the major SOD of the cell, Cu/Zn SOD, is strongly downregulated by hypoxia. Previous studies have shown that with bakers' yeast Sod1p, this downregulation occurs at the transcriptional as well as post-translational level via an oxygen-dependent step in CCS activation of the SOD.[9,16,18] Although *C. elegans* does not express CCS, we observe that Sod-1 is still downregulated by hypoxia to a point at which activity is virtually undetectable without oxygen. Mammalian SOD1 may be subject to similar oxygen control, as SOD1 activity and/or the level of mRNA was seen to be diminished during hypoxia in isolated macrophages and in hypoxic kidneys.[33,34] The loss of activity of the largely cytosolic Cu/Zn SOD with low oxygen tensions appears quite conserved. At first glance, it might seem illogical to downregulate a major antioxidant defense with low oxygen levels, given the potential risks of severe oxidative stress with a sudden rise in oxygen level. However, reactive oxygen species have been implicated in various signaling pathways induced by hypoxia,[35−39] and it is possible that the decrease in Cu/Zn SOD activity helps to amplify such adaptive signals.

A major finding of these studies was identification of phosphorylated serine 38 as a post-translational modification to *S. cerevisiae* Sod1p during the switch to anaerobic conditions. This modification is not unique to low oxygen levels but can be observed under aerobic conditions when pools of apo-inactive Sod1p accumulate because of the loss of CCS-dependent activation. Phosphorylation of Ser38 has also been noted in a global screen for yeast proteins phosphorylated in response to mating pheromone.[40] We note that cells treated with mating pheromone have roughly 40% lower Sod1p activity (Figure S2 of the Supporting Information). These findings corroborate the notion that inactive Sod1p is a more likely target for phosphorylation at Ser38. Serine 38 in yeast Sod1p is a candidate for a proline-directed kinase, as substitution at P39 abolished such modification of Sod1p. Using the available

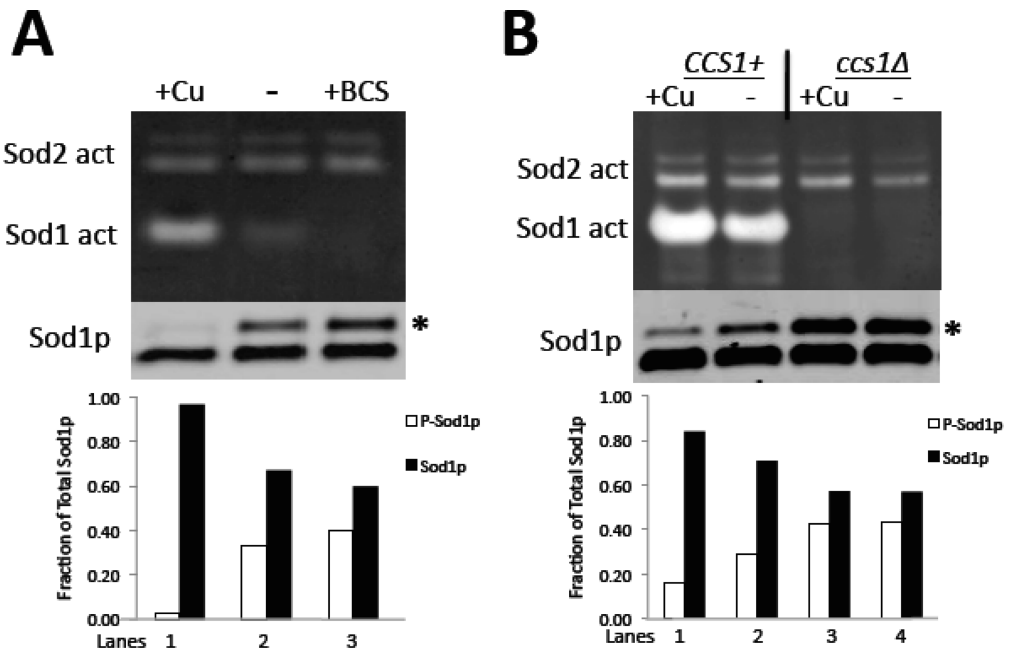

Figure 7. Extent of modification of Sod1p increases with low enzyme activity. Yeast strains were grown aerobically for 18 h to confluency in YPD medium prior to analysis of Sod1p activity (top) and Sod1 protein levels (bottom) as in Figure 1A. Bar graphs represent quantitation of modified (P-Sod1p) and unmodified (Sod1p) forms represented as a fraction of total Sod1p. Strains were transformed with WT Sod1p expression plasmid pLS108 and were either *sod1*Δ strain KS107 (A or CCS1: +, panel B) or *sod1*Δ *ccs1*Δ strain LS102 (CCS1: Δ, panel B). (A) Cells were grown in YPD medium supplemented with either 100 μM $CuSO_4$ (+Cu) or 100 μM Cu(I) chelator bathocuproine sulfonate (+BCS) or were not treated (−).

collection of *S. cerevisiae* deletion mutants, we were unable to identify a single kinase gene mutation that abolished this modification (not shown), suggesting more than one proline-directed kinase can phosphorylate Ser38.

We were surprised to find that Ser38 is relatively non-conserved among Cu/Zn SOD molecules. In human SOD1 and *C. elegans* Sod-1, there is a threonine at this site (Figure S4 of the Supporting Information). Phospho-mapping of human SOD1 has revealed phosphorylation at Thr2 and possibly Thr58 and/or Ser59,[41] but not Thr38, yet on the basis of our studies with yeast Sod1p, phoshorylation at Thr38 should become obvious only when inactive pools of human SOD1 accumulate in cells. Most surprising is the lack of S38 conservation among closely related fungi. In 82 of 90 related fungi examined, Ser38 is replaced with Asp, the phospho-mimic; only four species were seen to harbor S38 (not shown). Thus, while the vast majority of fungal Sod1p molecules are constitutively acidic at position 38, *S. cerevisae* has evolved to phoshorylate and, hence, modulate the charge at this position. Recently, an evolutionary analysis of phosphorylation sites was conducted using as examples the phospho-proteomes of the yeasts *S. cerevisae*, *Schizosaccharomyces pombe*, and *Candida albicans*. These studies revealed a remarkably rapid evolution of kinase sites on proteins that facilitate enhanced diversity in protein function.[42] The appearance of the kinase site S38 on *S. cerevisiae* Sod1p is an excellent example of such an evolution of diversity.

The rationale for the appearance of the S38 kinase site on *S. cerevisiae* Sod1p is still not known. As of yet, we have not identified a phenotype associated with the phospho-mimic S38D versus S38A alleles of yeast Sod1p in a wide array of tests for known Sod1p function in yeast. Nevertheless, this phosphorylation is indeed biologically relevant in that it closely correlates with a loss of CCS-dependent activation of Sod1p. Intriguingly, as shown in Figure 5C, S38 is positioned close in three-dimensional space to P142 and P144, the critical region that helps dictate the CCS dependence of Cu/Zn SOD molecules.[9,10] Thus, phosphorylation at S38 could conceivably modulate activation by CCS. Although we found no effects of S38 substitutions on steady state Sod1p activation by CCS, we cannot exclude all-important kinetic effects. As a plausible hypothesis, phosphorylation of S38 may tag apo-Sod1p for rapid activation by CCS and/or facilitate interactions with other partner molecules.

■ ASSOCIATED CONTENT

Supporting Information

Quantitation of phosphorylated Sod1p (Figure S1), mass spectrometric analysis of purified *S. cerevisiae* Sod1p (Figure S2), reduction in Sod1p activity with α-factor (Figure S3), and alignment of Cu/Zn SOD molecules (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

SOD, superoxide dismutase; CCS, copper chaperone for SOD; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NBT, nitroblue tetrazolium; YPD and YPDE, yeast extract peptone and yeast extract peptone with ergosterol and Tween, respectively; SC and SCE, synthetic complete and synthetic complete with ergosterol and Tween, respectively; BCS, bathocuproine sulfonate.

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