Published in final edited form as:

Biochemistry. 2012 January 17; 51(2): 677–685. doi:10.1021/bi201353y.

Post-translational modification of Cu/Zn superoxide dismutase under anaerobic conditions†

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Abstract

In eukaryotic organisms, the largely cytosolic copper and zinc containing superoxide dismutase enzyme (Cu/Zn SOD) 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 on Cu/Zn SOD from diverse organisms. We show here that like bakers' yeast (*Saccharomyces cerevisiae*), adaptation of the multicellular *Caenorhabditis elegans* to growth in low oxygen involves strong down-regulation 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 as 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 on 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 beta-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), 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 catalytic co-factor, these enzymes disproportionate reactive superoxide anion radicals into hydrogen

[†]This work was supported by the JHU NIEHS Center, NIH grants GM 50016 (VCC) and NS 39112 (PJH), NIH postdoctoral fellowship F32 GM 087904 (JML) and by NIH training grants ES 07141 (CXL) and T32 CA009110 (JAB). XC was supported in part by a grant by the Judith and Jean Pape Adams Charitable Foundation.

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SUPPORTING INFORMATION

This information is available free of charge via the internet at <http://pubs.acs.org>. Four figures are provided as follows:

Figure S1: Quantitation of phosphorylated Sod1p

Figure S2: Mass spec analysis of purified *S. cerevisiae* Sod1p

Figure S3: Reduction in Sod1p activity with alpha factor

Figure S4: Alignment of Cu/Zn SOD molecules

peroxide and oxygen. Most eukaryotes express two distinct SOD molecules that differ in cellular location and metal ion co-factor. 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 co-factor. 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 non-catalytic but structurally important zinc ion that promotes 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 was identified that serves to insert copper and oxidize the disulfide in eukaryotic Cu/Zn SOD molecules ⁵. Much of the work on CCS has been completed in the bakers yeast *S. cerevisiae* where the Cu/Zn SOD (denoted herein as 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 *C. elegans* (denoted as Sod-1). The nematode genome does not encode CCS and accordingly, worm Sod-1 is only activated 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 activated 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 a 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 1991¹⁵. 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) co-factor for SOD1, as total 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 pathway 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 in seen in yeast where expression of both *SOD1* and *SOD2* is decreased as cells become hypoxic ^{15, 18}. The adaptation of yeast to low oxygen

clearly involves a loss in SOD enzymes, although it is not clear if a similar scenario exists in multicellular organisms that use CCS-independent pathways.

In this study we re-visited the oxygen control of SOD enzymes. We find that the drop in SOD enzyme levels with low oxygen is not unique to CCS-dependent Sod1p from bakers' yeast. The CCS-independent Sod-1 of *C. elegans* is likewise subject to down regulation when the nematode is grown in low oxygen. Oxygen control of CCS independent activation was also observed in a yeast expression system where activity of the CCS independent P144S yeast Sod1p decreased with low oxygen. Finally, these studies revealed a new post-translational modification to yeast Sod1p that was favored with low oxygen, 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 *sod1ΔkanMX4* 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 the *sod1Δ::TRP* strain KS107 (*Mat a, leu2-3, 112, his3Δ1, GAL+, trp1-289a, ura3-52, sod1Δ::TRP1*)²⁰ and the isogenic *ccs1Δsod1Δ* strain LS102 (*Mat a, 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 media 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 described²² 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 or a 3% Oxygen/97% Nitrogen gas mixture (Airgas) 5 times.

Yeast plasmids—The pLJ486 2 micron *URA3* plasmid for expressing *S. cerevisiae SOD1* from the strong *ADHI* 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 template the *CEN LEU2* plasmid pLS108 where *SOD1* falls under control of the endogenous *S. cerevisiae SOD1* promoter⁹. The *sod1::kanMX4* plasmid pJAB002 was created by PCR amplifying *S. cerevisiae SOD1* sequences from -744 to -204 with primers that introduced a BamHI site at -732 and an XbaI site at -223, and amplification of sequences +500 to +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 the *kanMX4* plasmid pRS400²³ digested with XbaI and XhoI. The resulting pJAB002 plasmid digested with BamHI was used to delete chromosomal *SOD1* sequences -223 to +500.

C. elegans strains and growth

The *C. elegans* strain N2 was provided by the *Caenorhabditis* Genetics Center funded by the NIH National Center for Research Resources (NCRR). The *sod-1* (tm776) and *sod-2* (gk257) strains were provided by Shohei Mitani, Tokyo Women's Medical University School of Medicine, Japan. Nematodes were maintained at 25°C on enriched NGM plates supplemented with *E. coli* OP50 as food source. Worms were synchronized by harvesting animals from four 10 cm plates by rinsing in M9 buffer²⁴, yielding a pellet of approximately 0.5 mls; worms were then synchronized by bleaching of gravid hermaphrodites²⁵. Isolated eggs were hatched in M9 at 20°C for 24 hours. The resulting starved L1 animals were then transferred to fresh plates and allowed to grow 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 hours at 25°C. Upon inspection of worms following this hypoxic treatment, full recovery of movement was observed within minutes of transferring 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 described⁹. In the case of *C. elegans*, 1 plate of worms were harvested by rinsing in M9 buffer yielding approximately 100 µl of animals that were suspended in lysis buffer containing 10mM NaPO₄ pH 7.8, 5mM EDTA, 5mM EGTA, 50mM NaCl, 0.1% Triton X-100, 10% glycerol, 500µM phenylmethanesulfonyl fluoride and 1:100 protease inhibitor (Sigma #P8340) and lysed in a TissueLyser using 0.7 mm zirconium oxide beads and agitation for two 1.0 minute cycles. Lysates were clarified by centrifugation at 20,000xg, 4°C for 10 min. Supernatants were filtered through a 0.22 µm membrane (Costar, Spin-X Centrifuge Tube Filter) by centrifugation at 20,000xg, 4°C for 2 min. Whole cell lysates from either yeast or worms were analyzed for SOD activity by native gel electrophoresis using 12% pre-cast gels (Invitrogen) and staining with nitroblue tetrazolium (NBT)²⁶. Immunoblot analysis was carried out by denaturing gel electrophoresis on 14% SDS polyacrylamide 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* Sod2p²⁷, to *S. cerevisiae* Pgl1p (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 system software and ImageJ (Rasband, W. S. NIH, USA)

Protein Purification

Purified, recombinant yeast Sod1p was isolated from an *S. cerevisiae* expression system as previously described²⁸. 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 *Escherichia coli* strain BL21(DE3) under the control of the IPTG-inducible lac UV5 promoter. Cells were grown to an A₆₀₀ of 0.7 before induction using 1mM IPTG and the cells were permitted to grow an additional 3 h before being harvested by centrifugation. The pelleted cells were resuspended in 50mM phosphate buffer pH 7.4 and lysed by sonication on ice. After removing the cell debris 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, purified yeast Sod1p was subjected 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 $3000 \text{ M}^{-1} \text{ cm}^{-1}$ 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 were 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

Down regulation of SOD enzymes by low oxygen

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

We tested whether the down-regulation of SOD enzymes by low oxygen was unique to yeast or was also a property of multicellular organisms, e.g., the metazoan *C. elegans*. In SOD activity analysis of whole worms, the Cu/Zn largely cytosolic Sod-1 is often seen as dual bands and mitochondrial manganese Sod-2 as a single band; these SODs can be discerned by comparative analysis of *sod-1* null and *sod-2* null animals (Fig. 1B, lanes 2 and 4). In the experiment of Fig. 1C, synchronized young adult worms were incubated at 25°C for 24 hours either in air or under anaerobic conditions that do not affect worm survival. Sod-1 activity was virtually absent under anaerobic conditions (Fig. 1C, lane 2). By comparison, Sod-2 activity in the mitochondria was not down-regulated and if anything, was seen to increase with hypoxia (Fig. 1C, lane 2). Although the effects of hypoxia on mitochondrial Mn SODs are not conserved in yeast and worms, both organisms show a dramatic drop in the major Cu/Zn SOD enzyme with low oxygen.

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 down regulated 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 (Fig. 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 of Fig. 2, a CCS-dependent and CCS-independent Cu/Zn SOD were expressed under the *S. cerevisiae* *ADHI* promoter to eliminate any transcriptional effects of oxygen. As such, SOD proteins levels were basically unchanged with varying oxygen (bottom panel, Fig 2A, 2B). The CCS-dependent and independent Cu/Zn SODs chosen for these studies varied by only a single amino acid: namely 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 down-regulated by low oxygen (Fig. 2A). CCS-independent activation of P144S Sod1p also decreased with lower oxygen in both *CCS1+* (Fig. 2B lanes 1–4) and *ccs1Δ* null

yeast (lanes 5–8). Activity was maximal at 20% oxygen and then decreased with O₂ levels $\leq$ 3% (Fig. 2B). The major difference was seen at 0 oxygen/anaerobic conditions where the CCS-dependent WT Sod1p was completely inactive (Fig. 2A lane 4) whereas the P144S variant retained residual activity (Fig. 2B lanes 4,8), consistent with previous findings on human SOD1 expressed in yeast⁹. The studies of Fig. 2 were conducted at steady state conditions, i.e., 18 hours of growth in various oxygen tensions. To more closely examine oxygen effects we conducted a time course experiment in which cells were pre-grown in atmospheric oxygen prior to switching to anaerobic conditions for various time points. As seen in Fig. 3 left, CCS-mediated activation of WT Sod1p declined $\approx$ 5 fold within 6 hours of growth under anaerobic conditions. By comparison, there was relatively little change in CCS-independent activation of P144S Sod1p during this shorter time course (Fig. 3 right). Together the studies of Figs. 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 more stringent.

Post-translation 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 asterisks Fig. 1 and 2). In addition to the major Sod1 polypeptide, a secondary specie of slightly slower mobility is evident. Since 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 (Fig. 1A, lane 1); however it can represent up to 50% of the total Sod1p protein in anaerobically grown cells (Figs 1A lane 3, 2A lane 4 and Fig. S1). The appearance of the modification can be visualized over time when cells are switched from aerobic to anaerobic conditions (Fig. 4). The modification increases in parallel with the decline in Sod1p activity and also follows the induction of Tir1p, a marker of anaerobic gene expression³⁰ (Fig. 4).

The Sod1p modification appears 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. cerevisiae* expression system exhibits the characteristic doublet (Fig. 5A). To investigate the nature of this modification, we subjected both forms of yeast-expressed recombinant Sod1p and the single recombinant Sod1p from *E. coli* (Fig. 5A) to mass spectrometry analysis. The results summarized in Figure S2 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 (Fig. S2). Consistent with phosphorylation at S38, the slower migrating Sod1p band on SDS gels is susceptible to phosphatase treatment (Fig. S3) and is abolished in a S38A mutant of Sod1p (Fig. 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 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 a S38A mutation, a P39A substitution blocked modification of Sod1p (Fig. 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 (Fig. 6A lanes 2 and 5). Surprisingly, a S38T substitution also inhibited modification of Sod1p (lane 6), indicating that SP and not TP, are preferred sites for phosphorylation of

yeast Sod1p. It is noteworthy that all the aforementioned substitutions produced enzymatically active Sod1p (Fig. 6A). Like WT Sod1p, variants with substitutions at S38 and P39 were dependent on the CCS copper chaperone (Fig. 6B) and also dependent on oxygen for enzymatic activity (Fig. 6C).

Inactive pools of Sod1p favor phosphorylation at serine 38

As mentioned above, phospho-S38 can become more prominent during anaerobic conditions. This was observed with endogenous Sod1p (Figs. 1A and 4) as well as WT Sod1p over-expressed from the *ADHI* promoter (Fig. 2A and Fig. S1). 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 phosphorylation under anaerobic conditions (Fig. 2 and Fig. S1). These results suggest that a *loss of CCS activation* of Sod1p triggers increased phosphorylation at ser38. If true, loss of CCS in air should mimic anaerobic effects on Sod1p. CCS activation of WT Sod1p in air can be blocked by lowering copper availability or through null mutations in *ccs1*. As seen in Fig. 7, Sod1p activity in air is highest with copper supplemented cells and lowest in cells treated with the BCS copper chelator (Fig. 7A) or in *ccs1Δ* cells (Fig. 7B). We observed that in all cases, the degree of Sod1p phosphorylation as determined by immunoblot increases with a loss of Sod1p activity (Fig. 7). Hence it appears that the loss of CCS activation of Sod1p in both air and under anaerobic conditions favors phosphorylation of Sod1p at serine 38.

DISCUSSION

In organisms as diverse as bakers yeast and *C. elegans*, the major SOD of the cell, namely Cu/Zn SOD, is strongly down-regulated by hypoxia. Previous studies have shown that with bakers yeast Sod1p, this down-regulation 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 the Sod-1 is still down-regulated by hypoxia to the point of where activity is virtually undetectable without oxygen. Mammalian SOD1 may be subject to similar oxygen control, as SOD1 activity and/or mRNA was seen to diminished during hypoxia in isolated macrophages and in hypoxic kidneys^{33, 34}. The loss in activity of the largely cytosolic Cu/Zn SOD with low oxygen tensions appears quite conserved. At first glance, it might seem illogical to down-regulate a major antioxidant defense with low oxygen, given the potential risks of severe oxidative stress upon sudden rise in oxygen. However, reactive oxygen species have been implicated in various signaling pathways induced by hypoxia^{35–39} and it is possible that the drop 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, but can be observed under aerobic conditions when pools of apo inactive Sod1p accumulate due to loss of CCS-dependent activation. Phosphorylation of ser38 has also been noted in a global screen for yeast proteins phosphorylated in response to mating pheromone⁴⁰. We note that mating pheromone treated cells have roughly 40% lower Sod1p activity (Fig. S2). 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 the Sod1p. Using the available 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 (Fig. S4). Phospho-mapping of human SOD1 has revealed phosphorylation at Thr2 and possibly Thr58 and/or Ser59⁴¹, but not Thr38. Yet based on our studies with yeast Sod1p, phosphorylation at Thr38 should only become obvious when cells accumulate inactive pools of human SOD1. Most surprising is the lack of S38 conservation among closely related fungi. In 82 out of 90 related fungi examined, ser38 is replaced by asp, the phospho-mimic; only 4 species were seen to harbor S38 (not shown). Thus, while the vast majority of fungal Sod1p molecules are constitutively acidic at position 38, *S. cerevisiae* has evolved to phosphorylate and hence modulate, the charge at this position. Recently, an evolutionary analysis of phosphorylation sites was carried out using as examples the phospho proteomes of the yeasts *S. cerevisiae*, *S. pombe* and *C. albicans*. These studies revealed a remarkably rapid evolution of kinase sites on proteins that facilitate enhanced diversity in protein function⁴². The appearance of the kinase site S38 on *S. cerevisiae* Sod1p is an excellent example of such 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 loss of CCS-dependent activation of the Sod1p. Intriguingly, as shown in Figure 5C, S38 is positioned close in 3-D space to P142 and P144, the critical region that helps dictate 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Nathan Kim for analysis of fungal SOD1 sequences, Julie Gleason for expert advice on *C. elegans* and help with manuscript preparation, Laran Jensen for the pLJ486 plasmid and the NIH NCRR and Shohei Mitani for *C. elegans* strains.

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 the same with ergosterol/tween
SC and SCE	synthetic complete and the same with ergosterol/tween
BCS	bathocuproine sulfonate

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Fig. 1. Effects of low oxygen on SOD enzymes in *S. cerevisiae* and *C. elegans*

(A) The BY4741 yeast strain was grown for 18 hours 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, for (middle and bottom) Sod1, Sod2 and Pgk1 protein levels by SDS-PAGE and immunoblot analyses. Pgk1p serves as loading control. "Sod1 act, Sod2 act" mark positions of the respective active enzymes on the native gel. Asterisk marks position of post-translationally modified Sod1p (see text). (B, 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 hours 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 C top) or Sod-1 protein levels (C bottom) as in part A. Worms were either the WT N2 strain (B,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."

Fig. 2. The effect of oxygen on CCS-dependent versus CCS-independent activation of *S. cerevisiae* Sod1p

Yeast strains over-expressing the indicated Sod1p molecules under the strong *ADHI* promoter were grown in selecting SCE medium for 18 hours either aerobically (20% oxygen), or under 3%, 1% or no oxygen/anaerobic conditions as indicated. Cells were lysed and assayed for (top) SOD activity and (bottom) Sod1 protein levels as in Fig. 1A. (A) The *sod1Δ*KS107 strain expressing WT Sod1p. (B) The *sod1Δ*strain KS107 (lanes 1–4) or the *sod1Δccs1Δ*strain LS102 (lanes 5–8) expressing P144S Sod1p. “Sod1 act, Sod2 act” mark positions of the respective active enzymes on the native gel. Asterisk marks position of post-translationally modified Sod1p (see text).

Fig. 3. Time course for loss of CCS activation upon a switch to anaerobic conditions

The *sod1Δ* strain JL116 expressing either WT Sod1p (left) or P144S Sod1p (right) under the strong *ADH1* promoter was grown aerobically in SC medium to an $OD_{600} = 0.8$, and then placed under anaerobic conditions for the indicated time points prior to analysis of Sod1p activity (top) and protein levels (bottom) as in Fig. 1A. Graph in middle represents quantitation of the Sod1p activity bands from the native gel.

Fig. 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 Fig. 3. Endogenous Sod1p activity (top) and Sod1 protein levels (second panel) were assayed as described in Fig. 1A. Immunoblots were also probed for Tir1-TAP using an anti-TAP antibody (third panel) and for Pgk1p (bottom) as loading control. Asterisk marks position of modified Sod1p on denaturing gels.

Fig. 5. Yeast Sod1p is phosphorylated at serine 38

(A) 1.0 μ g of recombinant yeast Sod1p 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. Asterisk marks position of modified Sod1p unique to the yeast expression system. (B) Crystal structure of yeast Sod1p (pdb code 2jcw; ¹⁴) highlighting the region where S38 is positioned. The zinc-loop (loop IV) is shown in green and the electrostatic loop (loop VII) is shown in 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 the program PyMol {DeLano, 2002 #2180.

Fig. 6. The requirement for S38 and P39 in post-translational modification of Sod1p

The *sod1Δ* strain expressing either WT Sod1p on plasmid pLS108 or the indicated mutant derivatives of Sod1p were grown for 18 hours in selecting SC (A,B) or SCE (C) medium prior to analysis of Sod1p activity by the native gel assay (top) and Sod1 polypeptide levels (bottom) by immunoblot as in Fig. 1A. For “Sod1 act”, 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 *sod1Δ::kanMX4* derivative of BY4741 (A,C), the *sod1Δ* strain KS107 (“CCS1: +” part B) or the *sod1Δccs1Δ* strain (“CCS1: Δ” part B) LS102. Cell growth was either under aerobic conditions (A, B, and “O₂: +” part C) or anaerobic conditions (“O₂: -” part C)

Fig. 7. Modification of Sod1p increases with low enzyme activity

Yeast strains were grown aerobically for 18 hours to confluency in YPD medium prior to analysis of Sod1p activity (top) and Sod1 protein levels (bottom) as in Fig. 1A. Bottom graph represents quantitation of modified (P-Sod1p) and unmodified (Sod1p) represented as a fraction of total Sod1p. Strains were transformed with the WT Sod1p expression plasmid pLS108 and were either the *sod1Δ* strain KS107 (part A or “CCS1: +” part B) or the *sod1Δccs1Δ* strain (“CCS1: Δ” part B) LS102. (A) Cells were grown in YPD medium supplemented with either 100 μ M CuSO₄ “+Cu”, or 100 μ M of the Cu(I) chelator bathocuproine sulfonate “+BCS” or no treatment “-”.