Inactivation of Homocitrate Synthase Causes Lysine Auxotrophy in Copper/Zinc-containing Superoxide Dismutase-deficient Yeast Schizosaccharomyces pombe*

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The fission yeast Schizosaccharomyces pombe lacking copper/zinc-containing superoxide dismutase (CuZn-SOD) is auxotrophic for lysine and sulfuric amino acids under aerobic growth conditions. A multicopy suppressor gene (phx1) that restored the growth of CuZn-SOD-deficient cells on minimal medium was isolated. It encodes a putative DNA-binding protein with a conserved homeobox domain. Overproduction of Phx1 increased the amount of several proteins, and one of those turned out to be a putative homocitrate synthase (HCS) encoded by the lys4 gene in S. pombe as judged by mass spectrometric analysis. Consistent with this observation, overexpression of the lys4 gene increased HCS enzyme activity and was sufficient to suppress the lysine requirement of the CuZn-SOD-deficient cells. Enzyme activity and Western blot analyses revealed that the activity and protein level of HCS were dramatically reduced upon depletion of CuZn-SOD. Treatment of exponentially growing S. pombe cells with paraquat, a superoxide generator, caused a decrease in the amount of Lys4 protein as expected. These results led us to conclude that HCS, the first enzyme in the α-aminoadipate-mediated pathway for lysine synthesis common in fungi and some bacteria, is a labile target of oxidative stress caused by CuZn-SOD depletion and that its synthesis is positively regulated by the putative transcriptional regulator Phx1.

Superoxide dismutases (SODs) scavenge superoxide (O₂⁻) anions that are formed as by-products of aerobic metabolism and protect cell components from oxidative damages (1). Depending on the metals in their active sites, four types of SODs have been characterized: manganese-, iron-, copper/zinc-, and nickel-containing SODs (1, 2). Although all four types are found in prokaryotes, eukaryotes have manganese- and copper/zinc-containing SODs, primarily in mitochondria and the cytosol, respectively.

Defects in copper/zinc-containing SOD (CuZn-SOD) cause several disease conditions in humans, such as amyotrophic lateral sclerosis (3), and hypersusceptibility to neuronal injury and decreased fertility in mice (4, 5). The role of CuZn-SOD in eukaryotes has been investigated in most detail in yeast SOD-deficient mutants. In the budding yeast Saccharomyces cerevisiae, CuZn-SOD deficiency causes a high mutation rate and an auxotrophy for lysine and methionine or cysteine under aerobic growth conditions (6). In the fission yeast Schizosaccharomyces pombe, CuZn-SOD deficiency causes severe growth defects even in rich media (7) and auxotrophy for lysine and methionine or cysteine (8).

In S. cerevisiae, several multicopy suppressor genes that overcome the biosynthetic defects and oxidant-sensitive phenotype of a CuZn-SOD-deficient mutant (sod1Δ) have been isolated. They include the ATX1 and ATX2 genes, which cause accumulation of copper and manganese, respectively (9, 10), and which could serve as metal-based mimics for SOD. Mutations in genes such as PMR1 and BSD2 also suppress oxidative damage in sod1Δ by accumulation of manganese and copper plus manganese, respectively (11, 12). Other mutational suppressors of biosynthetic defects in sod1Δ, called spo (suppressors of endogenous oxidation) mutations, contain defects in the assembly or repair of iron-sulfur clusters (13), and a recent study suggests that the suppression might be due to increased mitochondrial iron in spo mutants (14).

Even though lysine auxotrophy is generally observed in yeast lacking CuZn-SOD, the labile target(s) in the lysine biosynthetic pathway has remained elusive. In yeast, filamentous fungi, and some bacteria, lysine is synthesized by the α-aminoadipate pathway (15–17). In this pathway, the first step is catalyzed by homocitrate synthase (HCS), which converts α-ketoglutarate to homocitrate. Homocitrate is then converted to homoaconitate and further to homoisocitrate by homocitrate dehydrase and homoaconitate dehydratase (homocitrate), respectively (18). The reactions from α-ketoglutarate to α-aminoadipate are similar to those in leucine biosynthesis and a portion of the tricarboxylic acid cycle. A close similarity in amino acid sequence between the corresponding enzymes has been revealed (available at www.ncbi.nlm.nih.gov/COG) (18, 19). One recent study hypothesized that homoaconitate (encoded by the lys4 gene in S. cerevisiae) containing a 4Fe-4S cluster could be the damaged target in the sod1Δ mutant of S. cerevisiae (20). In this study, we screened for multicopy suppressors of growth defects caused by deletion of CuZn-SOD in the fission yeast S. pombe and found that overproduction of HCS, encoded by the lys4 gene in S. pombe (21), is sufficient to suppress lysine auxotrophy caused by CuZn-SOD depletion. We present evidence that HCS is the labile target that is compromised and thus causes lysine auxotrophy under pro-oxidative (superoxide-rich) conditions.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—S. pombe strains 972, ED665, ED668, and JLA6 were used as described by Lee et al. (7). Cells were routinely grown in yeast extract/supplement (YES) medium and syn-
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thetic minimal medium (Edinburgh minimal medium (EMM)) as described by Alfa et al. (22). For conjugation and sporulation analyses, cells were grown in malt extract medium according to standard procedures (23).

Disruption of the sod1+ Gene and Tetrad Analysis—A HindIII fragment containing the ura4+ gene of S. pombe (24) was inserted into the BstEII site of the sod1+ gene cloned into pSOD1 (7), generating pSUD1. The BstYI/SpeI fragment (2 kb) from pSUD1, which contains the ura4+ cassette (1.26 kb) flanked by the sod1+ gene sequence, was used to transform a diploid cell (ED665/ED668). Following selection by the ura4+ marker, the expected heterozygous (sod1+/sod1:ura4+) gene structure of the diploid transformant was confirmed by PCR and Southern hybridization. For tetrad analysis, the spores from each ascus were dissected on YES plates and allowed to grow at 30 °C for 3 days.

Auxotrophy Test—Strain JL46, an ED665 derivative that contains the nmt1 promoter-driven sod1+ gene in the chromosome (7), was grown to the exponential phase (A600 = 0.5) in EMM. For survival tests on plates, aliquots from serial dilutions were spotted onto EMM plates with or without thiamine (10 μM) and amino acid supplements (250 mg/liter) and incubated at 30 °C. To monitor growth in liquid medium, freshly grown cells were inoculated in liquid EMM with or without thiamine (10 μM) and amino acid supplements (250 mg/liter), and the absorbance at 600 nm was measured.

Screening of Multicopy Suppressors of Growth Defects Caused by Depletion of CuZn-SOD—An S. pombe genomic library was made by cloning partial HindIII-cut DNA fragments from strain 972 into the pWH5 shuttle plasmid with a 2μ circle origin (25). Following transformation into strain JL46 (nmt1-sod1+), transformants were selected on EMM plates containing 10 μM thiamine, on which parental JL46 cells are unable to grow. Transformants that restored the normal level of CuZn-SOD (Sod1+) due to introduction of the sod1+ gene or constitutive nmt1 promoter mutations were excluded by Western blot analysis using antibody against Sod1. Plasmids that confirmed JL46 growth on thiamine plates in the absence of sod1+ gene expression were then selected, and the multicopy suppressor genes were analyzed by nucleotide sequencing. The pWH5-34 clone containing a 8.07-kb genomic DNA fragment harboring the phs1+ gene was selected and named pWH5-pxs1+

Two-dimensional PAGE Analysis—Cell extracts were prepared from ED665 cells harboring the pWH5 or pWH5-pxs1+ plasmid grown to the stationary phase in EMM. Extracts containing 200 μg of proteins were loaded onto ImmobilineTM DryStrip gels (pH 4–7, 13-cm length; Amersham Biosciences) for isoelectric focusing as recommended by the manufacturer. Gel strips were then equilibrated in SDS equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 m urea, 30% glycerol, and 2% SDS) and subjected to second dimensional electrophoresis on a 12.5% polyacrylamide gel containing SDS. Following electrophoresis, gels were stained with silver nitrate as recommended by the manufacturer and compared for changed spots.

Matrix-assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry—The gel spots visualized by silver staining were excised, followed by destaining, reduction, and alkylation with 10 mM dithiothreitol and 50 mM iodoacetamide (26). Following dehydration of gel slices with 50% acetonitrile, 2 volumes of freshly prepared trypsin (20 ng/μl in 25 mM NH4HCO3) were added and incubated overnight at 37 °C. Peptides were extracted with 10 μl of trifluoroacetic acid (5%)/acetonitrile (50%) solution in two consecutive steps, dried by vacuum centrifugation, redisolved in 10 μl of 0.1% trifluoroacetic acid, and loaded onto ZipTip®C18 Pipette tips (Millipore Corp.). The washed peptides were eluted with saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 60% acetonitrile and 0.1% trifluoroacetic acid). Mass analysis was carried out on a Voyager-DE™ STR spectrometer (Applied Biosystems) at the Center for Natural Science Research Facilities of Seoul National University. External and internal calibrations were done with a Sequazyme peptide mass standard kit (Perspective Biosystems) and autolytic trypsin peptides, respectively. For data analysis, the Mascot peptide mass fingerprint program (available at www.matrixscience.com) was used.

Cloning of the lys4+ Gene—We generated a PCR fragment containing the entire coding region of the lys4+ gene using the mutagenic forward primer CATCCATATGTCGTGTCGAAGC (with the Ndel site underlined) and reverse primer TAAAGGATCCAGCATTCGCGAACC (with the BamHI site underlined). The 1.26-kb Ndel/BamHI-cut lys4+ gene was cloned into the Ndel/BamHI site of the pAEPI expression vector, a prePI-based vector with the ade1+ promoter preceding the cloning site.

Homocitrate Synthase Activity Assay—A classical method for measuring HCS activity was employed with slight modifications (27, 28). Cell extracts were obtained by vortexing cells with glass beads (0.5 mm). The supernatant was transferred to a new tube and centrifuged at 12,000 rpm for 30 min at 4 °C. It was then dialyzed twice for 3 h each against 50 mM Tris-HCl (pH 7.8) containing 10% (v/v) glycerol to remove small thiol compounds that could interfere with the HCS activity assay utilizing 5,5′-dithiobis(2-nitrobenzoic acid). Cell extracts containing 200 μg of proteins were incubated in 0.5 ml of reaction buffer containing 0.5 mM acetyl-CoA and 50 mM α-ketoglutaric acid in 50 mM Tris-HCl (pH 7.8) at 30 °C. The reaction was terminated by adding 0.5 ml of absolute ethanol, followed by 0.5 ml of 5 mM 5,5′-dithiobis(2-nitrobenzoic acid). The amount of CoA generated by HCS reaction was indirectly measured by its reduction of 5,5′-dithiobis(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid, which was then quantified by its absorbance at 412 nm (ε = 13,600/mol/cm). One unit of enzyme is defined as the activity that produces 1 nmol of 5-thio-2-nitrobenzoic acid/min. The same cell extract sample containing 20 μg of proteins was analyzed for the amount of Lys4 (HCS) protein by Western blot analysis using anti-Lys4 polyclonal antibody raised in mice.

Preparation of Total RNA and Northern Hybridization—Total cellular RNA was prepared as described by Schmitt et al. (29). Each RNA sample (7 μg) was electrophoresed on a 1% agarose gel containing 18% formaldehyde, transferred to a Hybond-N membrane (Amersham Biosciences), and fixed with a UV cross-linker. Hybridization was performed with the PCR-generated lys4+ probe in Rapid-Hyb buffer (Amersham Biosciences) as recommended by the manufacturer.

RESULTS

Lack of CuZn-SOD Causes Auxotrophy for Lysine and Cysteine/Methionine—It has been suggested previously that the sod1+ gene encoding CuZn-SOD is absolutely required for the proliferation of S. pombe cells (7). In contrast, Mutoh et al. (8) reported that its absence causes auxotrophy for lysine and cysteine or methionine as judged by random spore analysis. To resolve this controversy, we created a sod1 null mutation in a diploid cell (ED665/ED668) using the ura4+ cassette. Ura4+ heterozygous cells (sod1+/sod1::ura4+) were selected and induced to sporulate for tetrad analysis. Of 15 asci tested, 12 exhibited similar spore growth patterns, generating two large (normal) and two small (slow growing) colonies on YES plates, suggesting that the sod1 disruption causes poor growth even in rich medium (data not shown). When each colony grown on a YES plate was streaked onto an EMM plate without uracil, no growth was observed, suggesting that the sod1 null mutant (with the ura4+ marker) requires some growth supple-
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When cysteine and lysine were added to the EMM plate, two colony streaks from each ascus grew (Fig. 1), consistent with the observations. When cysteine alone did not suggest that lysine is more limiting than cysteine in the thiamine-deficient S. pombe strain. In liquid EMM, cell growth was also retarded by adding thiamine (Fig. 2B). Growth retardation was completely relieved by adding lysine, but not cysteine. Considering that O2 partial pressure is lower in liquid than on plate cultures, these results support the hypothesis that the biosynthetic pathway for lysine is more labile and damaged than that for sulfurous amino acids under pro-oxidative conditions, as occur in the wild-type cells.

Multicopy Suppression of Lysine Auxotrophy by phe1 and phe2 Mutations

We employed a repressible sod1 strain (JL46, nmt1-sod1) that expresses the sod1 gene under the control of the thiamine-repressible nmt1 promoter (7) for further analyses. As shown in Fig. 2A, JL46 cells required cysteine and lysine to grow efficiently on thiamine-containing plates. Addition of lysine alone slightly increased growth, whereas cysteine alone did not suggest that lysine is more limiting than cysteine in the thiamine-deficient S. pombe strain. In liquid EMM, cell growth was also retarded by adding thiamine (Fig. 2B). Growth retardation was completely relieved by adding lysine, but not cysteine. Considering that O2 partial pressure is lower in liquid than on plate cultures, these results support the hypothesis that the biosynthetic pathway for lysine is more labile and damaged than that for sulfurous amino acids under pro-oxidative conditions, as occur in the wild-type cells.

Multicopy Suppression of Lysine Auxotrophy by phx1 and phx2 Mutations

To identify genes related to superoxide-mediated damage and protection in the thiamine-deficient S. pombe strain, we screened for multicopy suppressors of the poor growth phenotype of strain JL46 on thiamine-containing plates by introducing the S. pombe genomic library into strain JL46. The transformants that acquired either the sod1 or thi1 gene, which allowed sod1 expression in the presence of thiamine and thus restored normal levels of CuZn-SOD activity, were excluded. Of several suppressor clones, one clone showed a pronounced effect. This clone contained a gene encoding a putative homeodomain protein, thus named phx1 for pho homeobox gene. It encodes a large protein of 942 amino acids (103,986 Da), with the conserved homeobox domain near the N termini.
nus. When the \textit{phx1}\textsuperscript{+} gene cloned into the pWH5 vector was reintroduced into strain JL46, it enhanced growth of JL46 cells on EMM plates containing thiamine (Fig. 3A). In liquid EMM culture with thiamine, the multicopy \textit{phx1}\textsuperscript{+} gene restored growth of strain JL46 significantly, but to a lesser extent than that achieved by adding lysine (Fig. 3B).

Enhanced Production of Homocitrate Synthase in Phx1-overproducing Cells—As an initial attempt to determine the mechanism by which Phx1 suppresses growth defects in CuZn-SOD-deficient cells, we compared the proteomes of the control and Phx1-overproducing cells by two-dimensional PAGE. Multiple protein spots were affected by Phx1 overproduction, and they were subjected to MALDI-TOF mass spectrometric analysis. One of the spots that greatly increased in Phx1-overproducing cells was identified as the \textit{lys4}\textsuperscript{+} gene product, a putative HCS (Fig. 4A) (21). HCS catalyzes the first step in the \textit{α}-aminoadipate pathway for lysine biosynthesis common in fungi and some bacteria (Fig. 4B). Western blot analysis using polyclonal antibody against recombinant Lys4 protein produced in \textit{Escherichia coli} revealed that the amount of Lys4 protein was enhanced by >5-fold in Phx1-overproducing cells compared with control cells harboring the parental vector only (data not shown). These results suggest that the mechanism by which Phx1 suppresses lysine auxotrophy in Sod1-deficient cells involves provision of lysine by overproducing the Lys4 protein, a putative HCS.

Suppression of Lysine Auxotrophy by Overproducing Lys4—\textit{S. pombe} harbors only one gene (\textit{lys4}\textsuperscript{+}) predicted to encode HCS. We tested whether overproduction of Lys4 suppresses the lysine auxotrophy caused by CuZn-SOD deficiency as predicted. The \textit{lys4}\textsuperscript{+} gene was cloned downstream of the strong \textit{adh1} promoter in the expression vector pAEP1 (derived from pREP1), and the resulting plasmid (pAEP1-\textit{lys4}\textsuperscript{+}) was introduced into strain JL46 (\textit{nmt1-sod1}\textsuperscript{+}). HCS enzyme activity in Lys4-overproducing cells was ∼20 times higher than that in control cells (with the parental vector only), confirming that the \textit{lys4}\textsuperscript{+} gene indeed encodes HCS in \textit{S. pombe} (data not shown). We then monitored the effect of Lys4 overproduction on the growth of JL46 cells in liquid EMM with thiamine. Fig. 5A demonstrates that, whereas growth of JL46 cells with the parental vector was inhibited by thiamine in liquid EMM, growth of cells with pAEP1-\textit{lys4}\textsuperscript{+} was not, indicating that overproduction of HCS reverses growth defects caused by CuZn-SOD depletion.

On EMM plates in the presence of thiamine, the multicopy \textit{lys4}\textsuperscript{+} gene enhanced proliferation of JL46 cells as expected (Fig. 5B). Addition of lysine did not further enhance growth, indicating that the growth
we conclude that the decrease in HCS enzyme activity in CuZn-SOD-depleted growth-retarded cells is due to the specific loss of HCS (Lys4) protein, which might reflect the vulnerability of HCS to reactive oxygen species (ROS), superoxide, and/or its derivatives.

**Decrease in Homocitrate Synthase in Paraquat-treated Cells**—To test whether HCS is indeed vulnerable under superoxide-rich conditions, we treated cells with paraquat, a superoxide generator, and monitored changes in HCS (Fig. 7). Cells were grown in liquid EMM to early exponential phase and treated with 2 mM paraquat. Slight growth retardation was observed in paraquat-treated cells (Fig. 7A). Cell-free extracts were prepared at 2, 4, 6, and 9 h after paraquat treatment and from non-treated cells for comparison. Western blot analysis revealed that the amount of HCS dramatically decreased upon paraquat treatment (Fig. 7B). Neither the lys4+ transcript level (Fig. 7B) nor the total protein profile (data not shown) was affected by paraquat. These results confirm the proposal that HCS is the labile target damaged under superoxide-rich conditions, as in CuZn-SOD-depleted or paraquat-treated cells.

**DISCUSSION**

We have demonstrated in this study that overproduction of the Lys4 protein, a HCS, overcomes lysine auxotrophy caused by CuZn-SOD deficiency. We also found that the amount of this enzyme is greatly reduced under superoxide-rich (Sod1-depleted or paraquat-treated) conditions in fission yeast. Unlike *S. cerevisiae*, which contains two genes (LYS20 and LYS21) that encode two isoenzymes of HCS, *S. pombe* contains only one gene (lys4+) for HCS.

HCS contains two conserved CXXXC motifs predicted to be involved in metal binding. Similar motifs are found in *S. cerevisiae* Sco1p, in which they are involved in Cu(I) ligation and have been implicated in the delivery of copper to cytochrome c oxidase (30, 31). Consistent with the idea that HCS may have bound metal, HCS activity is strongly inhibited by metal-chelating agents such as o-phenanthroline and α,α′-dipyridyl (27). HCS activity is also inhibited by a sulfhydryl-binding agent, p-hydroxymercuribenzoate (27), implying that metal coordination by sulfhydryl residues is critical for its activity. Our work demonstrates that HCS is a labile target that is damaged under superoxide-rich conditions. Exposure of some proteins to ROS has been reported to cause structural modification, resulting in proteolytic degradation (32–34). In the case of the cell cycle-regulating phosphatase Cdc25C, H2O2 destabilizes the protein, most likely by modification of its critical cysteine residue (34). Alternatively, proteins can be fragmented by hydroxyl radicals produced from H2O2 in the presence of transition metals such as Cu(I) and Fe(II) by the Fenton reaction (32). Hydroxyl radicals generated within the metal-binding protein may react with the protein, resulting in fragmentation of nonrandom lengths. Further study is necessary to reveal the mechanism of HCS sensitivity to ROS. Considering various modulators of HCS activity such as activators (acetyl-CoA and α-ketoglutarate) and inhibitors (lysine and CoA) (35, 36), an indirect effect of ROS on changing the level and/or ratio of these modulators needs to also be assessed. The recent observation by microarray analysis that the lys4+ gene in *S. pombe* is induced by H2O2 is intriguing (available at www.sanger.ac.uk/perl/SPGE/geexview). This suggests that more Lys4 is needed under oxidative stress condition and supports our hypothesis that it is a labile target of oxidative stress that needs to be replenished for survival under such conditions.

In *S. cerevisiae*, it has been proposed that homoaconitase, the second enzyme in the lysine biosynthetic pathway in fungi (Fig. 4B), is the superoxide-labile target (20). It is widely accepted that enzymes that contain solvent-exposed 4Fe-4S clusters are labile to superoxide-specific damages (37), and homoaconitase, a sequence homolog of aconi-
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**FIGURE 7.** Effect of paraquat on HCS. A wild-type JH43 cells were grown in liquid EMM to an A<sub>600</sub> of 0.45 and equally distributed into new flasks. They were further incubated with (△) or without (●) 2 mM paraquat, and their growth was monitored. Cells were harvested at 2, 4, 6, and 9 h (arrows) after paraquat (PQ) treatment. B, changes in the amounts of Lys4 protein and lys4<sup>+</sup> transcript following paraquat treatment. Cells harvested at the indicated time points were used for Western or Northern analysis to detect HCS or lys4<sup>+</sup> transcripts as described in the legend to Fig. 6B and under “Experimental Procedures.” Samples from non-treated cells were prepared at 2 (lanes 1) and 6 (lanes 2) h after parallel culture.

tase containing superoxide-labile 4Fe-4S clusters, could be a vulnerable target. However, there still remains a possibility that another enzyme(s) in the lysine biosynthetic pathway could be inactivated by superoxide in *S. cerevisiae* because lysine is synthesized normally in manganese-containing SOD-deficient mutants, in which the level of homoaconitase is greatly reduced as in CuZn-SOD-deficient mutants (20). Therefore, it is possible that homoaconitase may not be the rate-limiting enzyme in lysine biosynthesis in *S. cerevisiae*. In *S. pombe*, we were not able to suppress lysine auxotrophy by overproducing the Lys2 protein, a putative homoaconitase (data not shown). If homoaconitase is also critically inactivated in CuZn-SOD-depleted *S. pombe* cells, provision of its substrates by HCS would not rescue lysine auxotrophy, as we observed in this study. Therefore, whether homoaconitase is damaged or not by superoxide or its derivatives, it is not likely that it is the critically damaged target causing lysine auxotrophy in the sod1 mutant of *S. pombe*.

The subcellular localization of HCS in *S. cerevisiae* has been in dispute for some time. Both HCS isoenzymes of *S. cerevisiae* have been shown to localize mainly in the nucleus using monoclonal antibodies against nuclear proteins (38) and HCS-green fluorescent protein (GFP) fusion proteins (39). In contrast, in *Penicillium chrysogenum*, HCS enzyme activity has been detected in both mitochondrial and cytosolic cell extracts (28) and has been reported to reside primarily in the cytoplasm when detected by GFP fusion proteins (40). By sequence analysis (available at psort.ims.u-tokyo.ac.jp), the HCS of *S. pombe* is predicted to be a cytoplasmic protein with marginal 57% probability. According to our preliminary observations, different results are obtained depending on the detection methods. When fluorescence from the HCS-GFP fusion gene is monitored by microscopy, HCS is distributed evenly throughout the cell. Detection by antibody against GFP also provides similar results. On the other hand, when HCS itself is detected by Western analysis, it resides primarily in the non-cytoplasmic fraction. This discrepancy suggests that GFP fusion to the C terminus of HCS could have perturbed subcellular localization in *S. pombe*, and we need to take precautions when interpreting GFP fusion data. Further systematic investigation to elucidate the exact distribution of HCS in *S. pombe* is necessary. If we accept the Western results detecting HCS directly, it appears that most of the HCS resides in a different location compared with cytosolic Sod1. However, the difference between the localizations of Sod1 and HCS does not contradict the model that HCS is the target of pro-oxidative damage caused by Sod1 deficiency. Because protein synthesis, folding, and targeting occur all in the cytosol, accumulation of ROS in the cytosol of Sod1-deficient cells can affect nearly all labile target proteins, including organellar proteins, before they reach their destination.

In budding yeast, it had been proposed that the cysteine/methionine auxotrophy of the *sod1* mutant is due to its hypersensitivity to sulfite, producing a sulfur tetroxide radical (SO<sub>4</sub><sup>•−</sup>). But more recently, a metabolic connection between Sod1p (CuZn-SOD) and the pentose phosphate pathway has been suggested (42) based on the observation that the aerobic methionine auxotrophy of the *sod1* mutant was fully rescued by overexpression of transketolase (TkI1p) and glucose-6-phosphate dehydrogenase (Zw1p), which can provide NADPH to PAPS reductase and/or sulfite reductase in the sulfurous amino acid biosynthetic pathway. Overproduction of Phx1 could also partially suppress the cysteine auxotrophy of the *sod1* mutant in fission yeast in aerobic plate cultures.

It can be proposed that Phx1 serves as a global regulator to respond against pro-oxidative conditions. In addition to its homology to Hox proteins with DNA-binding homeodomains, its overproduction causes changes in multiple proteins as observed by two-dimensional PAGE and confirms resistance to various oxidants. The investigation of genes that are regulated by Phx1 is expected to provide further clues toward understanding the mechanism of ROS toxicity in yeast and other eukaryotes.

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