JBC Papers in Press. Published on May 27, 2004 as Manuscript M403590200

Superoxide inhibits 4Fe-4S cluster enzymes involved in amino acid biosynthesis: Cross-compartment protection by CuZnSOD.

Matthew Alan Wallace, Lee-Loung Liou, Jacob Martins, Matthew H. S. Clement, Sasaneh Bailey, Valter D. Longo*, Joan Selverstone Valentine, and Edith Butler Gralla†

Department of Chemistry and Biochemistry, University of California at Los Angeles (UCLA), 607 Charles E. Young Dr. East, Los Angeles, CA 90095-1569.

*Present address: Division of Biogerontology Andrus Gerontology Center, University of Southern California, 3715 McClintock Avenue, Los Angeles, CA 90089-0191

† To whom correspondence should be addressed at:

Department of Chemistry and Biochemistry

UCLA

607 Charles E. Young Dr. East

Los Angeles, CA 90095-1569.

Phone: 310 825-1946

FAX: 310 206-7197

email: egralla@chem.ucla.edu

Running title: Superoxide-sensitive targets in amino acid biosynthesis

Summary

Among the phenotypes of *Saccharomyces cerevisiae* mutants lacking copper-zinc superoxide dismutase (Sod1p) is an aerobic lysine auxotrophy; in the current work we show an additional leaky auxotrophy for leucine. The lysine and leucine biosynthetic pathways each contain a four iron-four sulfur cluster enzyme homologous to aconitase and likely to be superoxide sensitive—homoaconitase (Lys4p) and isopropylmalate dehydratase (Leu1p), respectively. We present evidence that direct aerobic inactivation of these enzymes in *sod1*Δ yeast results in the auxotrophies. Located in the cytosol and intermembrane space (IMS) of the mitochondria, Sod1p likely provides direct protection of the cytosolic enzyme Leu1p. However, surprisingly, Lys4p does not share a compartment with Sod1p, but is located in the mitochondrial matrix. The activity of a second matrix protein, the tricarboxylic acid cycle enzyme aconitase, was similarly lowered in *sod1*Δ mutants. We measured only slight changes in total mitochondrial iron and found no detectable difference in mitochondrial "free" (EPR-detectable) iron making it unlikely that a gross defect in mitochondrial iron metabolism is the cause of the decreased enzyme activities. Thus, we conclude that, when Sod1p is absent, a lysine auxotrophy is induced because Lys4p is inactivated in the matrix by superoxide that originates in the IMS and diffuses across the inner membrane.

Introduction

The antioxidant enzyme copper-zinc superoxide dismutase (CuZnSOD or Sod1p)[1] plays an integral role in the protection of many organisms from the oxidative aerobic environment. CuZnSOD is localized to the cytosol, nucleus, and the intermembrane space (IMS) of the mitochondria suggesting that it exerts its protective effect in multiple compartments (1). Another SOD that contains manganese (MnSOD, or Sod2p) is located in the matrix of mitochondria. *Saccharomyces cerevisiae* lacking CuZnSOD (*sod1*Δ) have distinct, well-established aerobic phenotypes including diminished growth, auxotrophies for lysine and either methionine or cysteine, decreased ability to grow on non-fermentable carbon sources (2,3), increased "free" (EPR-detectable) iron (4), hypersensitivity to millimolar concentrations of zinc (5), and exquisite sensitivity to redox-cycling drugs such as the herbicide paraquat (6,7). All of these phenotypes (except zinc sensitivity) are also observed in mutants lacking the copper chaperone for CuZnSOD[2] (*lys7*Δ or *ccs1*Δ) and thus contain a form of the Sod1p polypeptide that is inactive due to a lack of copper in the active site (5,8). Mutants lacking Sod2p have a less dramatic phenotype—they are sensitive to redox cycling drugs and grow poorly on non-fermentable carbon sources, but show no aerobic auxotrophies (2).

Many cellular components are susceptible to oxidative damage, including proteins, lipids, and DNA (9,10). However, targets of superoxide-specific damage are much more limited. A particular type of protein prosthetic group, solvent-exposed four iron-four sulfur (4Fe-4S) clusters occurring in non-electron transfer proteins, have been shown to be specifically damaged by superoxide at low concentrations (11). These clusters differ from the iron-sulfur clusters found in electron transfer proteins in that one iron atom, rather than being covalently bound to the enzyme, is labile and able to interact with its substrate to promote dehydration. The most extensively studied enzyme of this class is aconitase, a dehydratase of the tricarboxylic acid

(TCA) cycle located in the mitochondrial matrix. Aconitase is subject to reversible inactivation by superoxide resulting in loss of the labile iron atom, leaving a 3Fe-4S cluster (12,13). We have previously observed reversible inactivation of aconitase in yeast lacking MnSOD (14) supporting a model of superoxide mediated inactivation of 4Fe-4S cluster enzymes in the mitochondrial matrix. Studies in bacterial systems have shown that two 4Fe-4S cluster containing dehydratases, dihydroxyacid dehydratase, and 6-phosphogluconate dehydratase (15), are specifically sensitive to superoxide (16,17). Inactivation of these enzymes leads to a deficiency in branched chain amino acid biosynthesis and a decreased capacity to utilize gluconate as a carbon source, respectively.

In the eukaryotic organism *S. cerevisiae*, two enzymes share distinct homology with aconitase—homoaconitase (Lys4p) and isopropylmalate dehydratase (Leu1p). These enzymes are involved in the biosynthesis of lysine and leucine, respectively. Lys4p catalyzes the conversion of cis-homoaconitate to homoisocitrate as part of the α-aminoadipate pathway of lysine biosynthesis (18). Although it has not been directly proven to contain a 4Fe-4S cluster, Lys4p is homologous to aconitase, particularly at active site residues involved in the 4Fe-4S cluster coordination (18,19), and its activity can be inhibited by iron chelators (20). Lys4p is therefore a potential target for superoxide-mediated inactivation. Leu1p catalyzes a two step reaction that converts α-isopropylmalate to β-isopropylmalate during leucine biosynthesis and is located in the cytosol (18). Yeast defective in formation of cytosolic Fe-S clusters exhibit a leucine auxotrophy (21) indicating the necessity of the Leu1p Fe-S cluster.

Fe-S cluster metabolism is an important process as indicated by (a) the abundance of enzymes requiring some form of cluster and (b) the fact that mutations affecting cluster synthesis are often lethal. Fe-S synthesis occurs in the mitochondria, and defects in the biosynthetic pathway generally result in dramatic accumulation of iron within the mitochondria (22-24). Iron

entry into the mitochondria for Fe-S cluster production, as well as heme synthesis, requires a mitochondrial membrane potential, a reducing environment, and the availability of ATP and NADH (25,26).

A tight lysine auxotrophy is a classic phenotype of *sod1*Δ yeast. We hypothesized that Lys4p is susceptible to superoxide inactivation, and that this sensitivity leads to the lysine auxotrophy. A leucine auxotrophy has not been reported for *sod1*Δ yeast, but based on the above information we decided to look for and found a heretofore undescribed leaky leucine auxotrophy in *sod1*Δ yeast. In this study we demonstrate inactivation of the iron-sulfur cluster enzymes Lys4p and Leu1p in the *sod1*Δ mutant under aerobic conditions. In addition, we localize Lys4p in the mitochondrial matrix and show that levels of mitochondrial total and "free" iron are similar in wild type and *sod1*Δ strains. Overall, we conclude that both auxotrophies are caused by direct inactivation of the respective protein by superoxide and that Sod1p protects them by lowering superoxide levels. In the case of Lys4p, the protection by Sod1p is exerted from one compartment (the IMS) across a membrane barrier to the matrix, and is required despite of the presence of MnSOD in the matrix.

Experimental Procedures

Yeast Strains, media, and growth conditions. Strains are listed in Table 1 (27-29). Plasmid pADCl (30) was obtained from Catherine F. Clarke. Plasmid pADCl-*LYS4-HA*, a high copy plasmid carrying an epitope-tagged homoaconitase gene was prepared as follows. A 2-kb fragment containing the *LYS4* gene was obtained by PCR of genomic DNA isolated from yeast strain S288C using PCR primers 5'-TCGCGTCGAC ATGCTACGAT CAACCACATT TACTCG and 5'-AGATATTTCG GGCCGCCCTA GTTGGGATTT GACCCAACCT TCC. This fragment was digested with SalI and NotI and cloned into the polylinker site of plasmid pADCl, which contains a hemagglutinin (HA) epitope tag, creating plasmid pADCl-*LYS4-HA*. This plasmid was transformed into the wild type yeast strains EG103, MO-59-13c, and X3356-1B using standard yeast transformation procedures. To reintroduce the *LEU2* gene, strains EG103 and EG118 were transformed with plasmid YIp351 (31) to create EG103L (wild type *LEU2*) and EG118L (*sod1*Δ *LEU2*). In strain JW101 (*lys1*Δ) the *LEU2* gene was introduced as part of the deletion of *CCS1*(*LYS7*). These strains were used to examine the activity of Leu1p.

Unless otherwise stated, all experiments in liquid media were performed in synthetic medium with 2% glucose (SD) supplemented with amino acids, adenine, and uracil as described (32). In addition, a four-fold excess of the supplements tryptophan, leucine, uracil, adenine, methionine and histidine was added (SDC). Specific amino acids were dropped out as indicated (*e.g.*, SD–Leu is SDC with leucine omitted). Growth was followed by monitoring the turbidity at 600 nm (OD_{600}; OD_{600} of 1 = 1 x 10^7 cells/ml). For paraquat studies, yeast pre-cultures were grown in standard YPD media and inoculated at an initial OD_{600} of 0.05, while all other experiments used pre-cultures grown in SDC. For the Lys4p experiments, cultures were inoculated in SDC at an OD_{600} of 0.025 for EG103/EG110 and an OD_{600} of 0.05 for EG118. These cultures were allowed to grow at 30 °C, 220 rpm until mid-log phase (OD_{600} of 1-2), spun

down, washed and re-suspended in SD–Leu–Lys–Trp. This medium was used so that the effect of the absence of lysine could be measured under conditions where neither strain could grow, since removing only lysine would have allowed the wild type but not the *sod1*Δ mutant to grow. These cultures were then incubated at 30 °C, 220 rpm and collected at the indicated times. For Leu1p experiments, cultures were inoculated in SD–Leu at an OD_{600} of 0.05 for EG103L and EG118L. Yeast for aconitase experiments were inoculated in SDC medium at an OD_{600} of 0.05. The above cultures were allowed to grow at 30 ^{o}C, 220 rpm until mid-log phase (assays) or with timed OD readings as indicated.

Protein extracts. Glass bead lysis was used to prepare crude soluble extracts. Samples were processed and stored under argon or nitrogen to avoid exposure of the lysates to oxygen, preventing inactivation of the enzymes during and after lysis. For Lys4p experiments, equal volumes of concentrated cell suspension (OD_{600} 10 to 30) and 0.5 mm glass beads were mixed and subjected to six to ten rounds of 1 minute of vortexing followed by 1 minute on ice, under argon in a septum-sealed glass test tube. Particulate matter was spun out by centrifugation at 3080 x g in a Jouan CR422 centrifuge. For Leu1p and aconitase assays, lysis was performed under nitrogen in a septum-sealed test tube using 0.5 mm glass beads with a ratio of 30 sec vortexing followed by 30 sec on ice, six times.

Enzymatic assays. Lysine levels were determined by measuring the oxidation of NADH to NAD^{+} coupled to the conversion of lysine to saccharopine by saccharopine dehydrogenase as described by Nakatani et al (33). Lys4p activity was determined as described by Broquist (34,35) using homoisocitrate as a substrate and measuring spectrophotometrically the production of homoaconitate which absorbs at 240 nm. Briefly, the absorbance at 240 nm of 1 nmol of homoisocitrate in 0.33 M potassium phosphate, pH 8.5, was followed for 10 min at 30 °C. The activity of the extracts was taken as the difference between the slopes obtained with and without

added substrate. Leu1p activity was determined spectrophotometrically in a similar manner as described by Kohlhaw (36), by watching the disappearance of citraconate (Aldrich) at 235 nm. Briefly, a 0.5 ml assay mixture containing 4 mM potassium phosphate, pH 7.0, 0.4 mM citraconate, and 100 - 300 μg of protein was assayed for 3 min in a 2 mm path length cuvette. Aconitase activity was determined spectrophotometrically as described by Gardner *et al.* by monitoring the formation of NADPH at 340 nm (37). Briefly, the assay mixture contained 50 mM Tris-HCl, pH 7.5, 5 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM $NADP^+$, 2 units of $NADP^+$ isocitrate dehydrogenase, and 50-100 μg of protein. The absorbance change was measured for 5 min and the slope was calculated from the linear portion.

Isolation of mitochondria and localization of homoaconitase. Mitochondria were isolated from EG103, LL101, and EG118, and purified on linear Nycodenz density gradients as described by Glick and Pon (38). The partial lysis experiment described in Figure 5 was performed as described (39,40) except that trypsin was used, since CuZnSOD is resistant to proteinase K under the conditions of the experiment. Briefly, samples of purified mitochondria were diluted 10-fold into 20 mM K-HEPES pH 7.4 containing 0.1 mg/ml trypsin and sorbitol such that the final sorbitol concentrations ranged from 0.6 M (no change in sorbitol concentration to maintain whole mitochondria) to 0.06 M (10-fold decrease in sorbitol concentration to rupture the outer membrane, forming mitoplasts and releasing the intermembrane space proteins into solution where they are digested by the trypsin). Mitochondria/mitoplasts were then pelleted, and samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by western blotting to determine subcellular localization of the proteins. An ECL kit (Amersham Pharmacia Biotech) was used for detection. Polyclonal antibodies raised against yeast Sod1p were affinity purified using cyanogen bromide-activated sepharose covalently linked to the appropriate protein (41). Polyclonal antibodies against cytochrome b_2 (Cyt b_2), Hsp60, and alpha-ketoglutarate

dehydrogenase (KGD1) were kindly provided by C. Koehler (UCLA). Affinity-purified anti-HA antibody was purchased from Sigma.

EPR analysis. Mitochondrial samples for EPR were prepared by placing 150 µl of 25 mg/ml purified mitochondria (see above) into a 4 mm EPR tube (Wilmad), flash frozen in dry ice/ethanol, and stored at -80 ^{o}C. "Free iron" (high spin rhombic Fe(III), g = 4.3) spectra were collected on a Bruker X-band spectrophotometer at 96 K using a variable temperature nitrogen gas setup with a Wilmad quartz Dewar insert. Parameters were as follows: center field, 1560 G; sweep width, 500 G; frequency 9.33 GHz; microwave power, 31.8 mW; modulation amplitude, 20 G; modulation frequency, 100 kHz; receiver gain, 1.1×10^5; time constant, 81.92 ms. Each spectrum is representative of an average of 8 scans. "Free iron" quantification was done as described (42). All EPR data processing was performed with Bruker WINEPR software.

Metal analysis: Total mitochondrial iron levels were determined on a Thermo-Jarrel Ash Iris 1000 inductively coupled plasma atomic emission spectrometer (ICP-AE). 1 mg of purified mitochondria was placed in an eppendorf tube, allowed to dry at 95 ^{o}C for 4 hours, dissolved in 1 ml of 20% nitric acid Optima (Fisher) and incubated at 95 ^{o}C for 18 hours in a sealed tube. Digestion volume was confirmed to be constant and the full 1 ml was added to 3.5 ml of metal-free water. A blank (1 ml of nitric acid to 3.5 ml metal-free water) was also included and the value was subtracted from each sample.

Results

The leaky leucine auxotrophy of CuZnSOD delete yeast

A classic phenotype of yeast lacking CuZnSOD (*sod1*Δ) is their inability to grow aerobically in the absence of the amino acids lysine and methionine (or cysteine) in their growth medium (43). These auxotrophies are very tight—there is little to no growth in air without the addition of lysine and methionine to the *sod1*Δ strain (Figure 1B) (3) or to the *ccs1*Δ strain (Figure 1C). Note that the *sod1*Δ and *ccs1*Δ strains grow well in complete medium or in medium lacking threonine.

Although there is a 4Fe-4S cluster protein in the leucine biosynthetic pathway—Leu1p—that is potentially superoxide-sensitive, no defects in leucine synthesis have been previously reported for *sod1*Δ yeast. Such an auxotrophy may have been overlooked in the earlier studies because many of the parental strains utilized carried a *leu2* mutation and were therefore unable to synthesize leucine, regardless of the status of Leu1p (43). Therefore, we constructed *LEU2*$^{+}$ strains EG103L and EG118L by transforming EG103 and EG118 strains with an integrating plasmid carrying the LEU2 gene. We then examined the ability of EG103L and EG118L to grow aerobically in medium lacking leucine. These experiments showed that there is a leaky auxotrophy for leucine in strains lacking CuZnSOD activity—strains with *sod1*Δ or *ccs1*Δ mutations grew much more slowly without leucine (Figure 1). Similar results were obtained with *sod1*Δ and *ccs1*Δ mutants derived from the *S. cerevisiae* strain BY4741 (data not shown). As with the methionine and lysine auxotrophies, the leucine auxotrophy was not observed in *sod1*Δ cells grown anaerobically or in strains lacking the matrix enzyme MnSOD (*SOD1*$^{+}$ *sod2*Δ) (data not shown).

Induced amino acid auxotrophies in wild type yeast exposed to paraquat

The bipyridyl herbicide paraquat (methyl viologen) is known to give rise to intracellular superoxide production, and *sod1*Δ yeast are exquisitely sensitive to it. Elevated concentrations of paraquat in wild type yeast are expected to replicate the phenotype of *sod1*Δ strains assuming enough superoxide is generated to overtax the cells ability to cope with it. Wild type cells can grow in complete medium with concentrations of paraquat as high as 5 or 10 mM (28). However, 0.25 mM paraquat induced amino acid auxotrophies for lysine, methionine and leucine (Figure 2), indicating that the pathways producing these amino acids are especially sensitive to superoxide. Indeed, the observed pattern is quite similar to that seen in *sod1*Δ yeast—paraquat causes almost complete growth inhibition of the wild type strain in media lacking lysine or methionine and partial growth inhibition in medium lacking leucine. These data support the conclusion that the three amino acid auxotrophies of *sod1*Δ yeast are specifically due to the presence of superoxide and prompted us to look for inactivation of specific 4Fe-4S cluster proteins involved in these amino acid biosynthetic pathways.

The activity of 4Fe-4S cluster enzymes.

We analyzed the activity of the three aconitase-like 4Fe-4S cluster enzymes in our wild type and *sod1*Δ strains in order to determine whether their activities are affected by the absence of Sod1p.

Aconitase is the classic example of an enzyme that is inactivated by superoxide-mediated oxidation of its 4Fe-4S cluster (13). Aconitase has been reported to be inactivated in yeast lacking either MnSOD (*sod2*Δ) (14) or CuZnSOD (44) and we found similar results under the conditions used in the present study (Figure 3A). The addition of 1 mM paraquat to the wild type strain also caused a decrease in aconitase activity. Thus aconitase is susceptible to inactivation

by superoxide produced by both natural and artificial means. Since aconitase is not a rate limiting enzyme, decreases in its activity do not necessarily affect growth or respiration rate. MnSOD and aconitase are both located in the mitochondrial matrix, so it is perhaps not surprising that aconitase activity is decreased in *sod2*Δ strains. The fact that aconitase activity was also affected in *sod1*Δ yeast is harder to explain, since the two proteins are not present in the same cellular compartment.

The activity of Lys4p, the 4Fe-4S cluster containing enzyme in the lysine biosynthetic pathway, was measured in the *sod1*Δ, the *sod2*Δ, and wild type yeast that had been grown for twelve hours in medium lacking lysine, tryptophan, and leucine. We observed a dramatic difference between wild type and the mutants, although the specific activity of Lys4p was low even in wild type cells (Figure 3B). This result is somewhat surprising, as the *sod2*Δ mutant does not exhibit a lysine auxotrophy. To address this point, we measured free lysine levels in the three strains and found that there was 12.5 ± 2 nmol per mg protein in wild type, 5.0 ± 0.8 nmol per mg protein in *sod1*Δ, and 16.0 ± 2 nmol per mg protein in *sod2*Δ. The level of free lysine in the *sod1*Δ mutant was less than half that of wild type, indicating that the inactivation of Lys4p in this strain affected the amount of available lysine and providing a good explanation for the observed lysine auxotrophy. The *sod2*Δ strain actually had a slightly higher level of lysine than the wild type strain, consistent with the fact that *sod2*Δ yeast do not exhibit a lysine auxotrophy. It remains unclear why the *sod2*Δ strain can still make wild type levels of lysine with the drastically lowered enzyme activity that we observed.

To test whether the inactivation of the cytosolic 4Fe-4S cluster protein Leu1p could be the cause of the leaky leucine auxotrophy, we measured its activity in *sod1*Δ, *sod2*Δ, and wild type yeast, as well as wild type yeast exposed to paraquat. In *sod1*Δ and wild type plus paraquat, the Leu1p activity was approximately half that of the wild type control (Figure 3C). The

moderate level of inactivation correlates well with the observed leakiness of the leucine auxotrophy—enough leucine is produced to support some growth, but at a lower rate. Since Leu1p and Sod1p are both cytosolic proteins, it seems likely that Sod1p exerts a direct protective effect. The *sod2*Δ strain had the same Leu1p activity as wild type, indicating that the matrix-localized MnSOD plays little or no role in protecting this cytosolic protein.

Restoration of 4Fe-4S cluster enzyme activity by low oxygen

When cells are grown anaerobically, the lysine (and leucine) auxotrophies of the *sod1*Δ mutant are not observed (43). We therefore sought to determine whether anaerobiosis affected the Lys4p and Leu1p activities. After twelve hours of aerobic growth in SDC–Leu–Lys–Trp (Lys4p) or in SDC–Leu (Leu1p), cultures were divided in two. One of these cultures was placed in low aeration conditions (either under 3% oxygen with no shaking or under 100% nitrogen), while the other was returned to normal culture conditions, shaking with ambient oxygen conditions (high aeration). As before, under high aeration the activities of these enzymes were reduced, but three hours in low oxygen conditions fully restored the Lys4p activity in both *sod1*Δ and *sod2*Δ cells (Figure 4A) and the Leu1p activity in *sod1*Δ (Figure 4C). Restoration of activity under low oxygen conditions was independent of protein synthesis—the increase occurred even if cycloheximide was added to the culture medium at the time of the split (Figure 4B and 4D). These data are consistent with a reactivation event that involves repair or re-synthesis of the 4Fe-4S cluster prosthetic group in existing protein molecules. In other words, Lys4p and Leu1p are inactivated but not degraded under superoxide stress.

Localization of homoaconitase

Lys4p has been reported to be a mitochondrial enzyme (19) and it contains a potential mitochondrial localization signal similar to aconitase as suggested by hydrophobicity plots (data not shown). Because it is inactivated in *sod1*Δ mutants, we thought it possible that Lys4p shared a location with Sod1p, *i.e.* that it was located in the IMS. To test this possibility, we constructed a hemagglutinin (HA) tagged version of the enzyme. The HA-tagged protein complemented the lysine growth defect of two different *lys4* mutant strains (M0-59-13c and X3356-1B) indicating that it was properly processed and active *in vivo* (data not shown).

Mitochondria purified from wild type yeast (LL101) expressing HA-tagged Lys4p were used in a partial lysis experiment to distinguish matrix proteins from IMS proteins. Samples of mitochondria were incubated in buffer containing trypsin and various concentrations of sorbitol, ranging from 0.6 M, where intact mitochondria are preserved, to 0.06 M, where complete lysis of the outer membrane occurs, leaving mitoplasts but releasing intermembrane space proteins into solution where they are degraded by the trypsin. As can be seen on the western in Figure 5, the bands for the matrix marker proteins Hsp60 and Kgd1p remain constant across the entire range of sorbitol concentrations, indicating that they are located in the mitoplasts (*i.e.* in the matrix), while the intensity of the IMS marker Cyt b_2 band decreases dramatically as the sorbitol concentration decreases, indicating a location in the IMS. The HA-tagged Lys4p band remains constant at all sorbitol concentrations indicating that it is located in the mitochondrial matrix. In support of previous reports (1), we found that CuZnSOD is present in the IMS. Thus, Lys4p, like aconitase, is present in the matrix and yet is dependent on Sod1p, a protein located in a different cellular compartment, for its full protection.

Total and "free" mitochondrial iron

Previous results from this lab indicate that *sod1*Δ mutant yeast exhibit alterations in iron metabolism, including a slight increase in total iron (45) and a large (3- to 4-fold) increase in "free" iron, or iron detectable by EPR at $g = 4.3$ (4). Large increases (10-fold) in total mitochondrial iron (22-24) not bound to proteins or heme (23) have been reported for several mutations that affect Fe-S cluster biosynthesis. Therefore, we measured total and "free" iron in purified mitochondria from wild type and *sod1*Δ yeast to see if our earlier whole cell results were indicative of a mitochondrial iron processing defect that could be affecting Fe-S cluster biosynthesis. Total iron, as measured by ICP-AE, was slightly increased in mitochondria from *sod1*Δ cells (Figure 6A), similar to what we previously observed in whole cells but unlike the large increases observed in mutants defective in Fe-S cluster biosynthesis. "Free" iron as measured by EPR at $g = 4.3$ was not significantly increased in *sod1*Δ mitochondria (Figure 6B), in contrast to what is observed in whole cells or in mitochondria defective in Fe-S biosynthesis. Both the ICP-AE and EPR data indicate that *sod1*Δ does not have a severe defect in mitochondrial iron metabolism, suggesting that Fe-S cluster production mechanisms are grossly intact.

Discussion

Superoxide is produced by a number of cellular processes, the predominant source being leakage from the electron transport chain. Superoxide and its downstream products, including hydrogen peroxide and hydroxyl radical, are collectively known as reactive oxygen species (ROS). The toxic effects of ROS are numerous and have been implicated in a number of damage paradigms and human diseases (9,10,46). However, damage due specifically to superoxide itself is much more limited. The best established target for superoxide is the exposed 4Fe-4S cluster in the active sites of many dehydratase enzymes, the best known of which is aconitase. Attack by superoxide oxidizes the cluster and leads to loss of the labile iron resulting in a 3Fe-4S cluster and enzyme inactivation (12-17).

In the present study, we explored the molecular basis for the aerobic lysine auxotrophy of yeast lacking CuZnSOD and found a previously unreported air-dependent leaky auxotrophy for leucine (Figure 1). These same auxotrophies are induced in wild type yeast by treatment with the superoxide generating drug paraquat (Figure 2), indicating that oxidative stress specifically leads to the auxotrophies. This stress acts on 4Fe-4S cluster enzymes in each amino acid's metabolic pathway, as evidenced by their inactivation in *sod1*Δ yeast grown aerobically (Figure 3). Reactivation of these 4Fe-4S cluster enzymes occurred in low oxygen even in the presence of the protein synthesis inhibitor cycloheximide (Figure 4), implying that such reactivation is due to repair of the cluster and not through synthesis of new protein. Thus, we have found two more targets of superoxide mediated inactivation, Lys4p (homoaconitase) and Leu1p (isopropylmalate dehydratase).

Leu1p and CuZnSOD are both located in the cytosol, so it is logical that Sod1p provides protection to Leu1p by decreasing the local concentration of superoxide. We were more puzzled

by the ability of CuZnSOD to provide protection to the mitochondrial enzyme Lys4p. We considered three possible explanations.

We first considered the possibility that Lys4p was located in the IMS and thus shared a common location with CuZnSOD. Upon exploring the sub-mitochondrial localization of Lys4p, however, we found Lys4p to be in the matrix (Figure 5), while Sod1p is in the IMS. While this result was somewhat surprising, it is not completely unprecedented, as the activity of aconitase, also a matrix enzyme, is decreased when Sod1p is absent (Figure 3 and (44)). The activity of the matrix enzyme succinate dehydrogenase (SDH), which contains several non-exposed Fe-S clusters and which is inactivated in yeast and mice lacking Sod2p, was also observed to decrease in *sod1*Δ yeast (data not shown). These results fit the general emerging picture—CuZnSOD protects some Fe-S clusters in the mitochondrial matrix from its location in (an)other compartment(s).

Second, we considered the possibility that disruptions in iron metabolism could be the cause of the lowered activity of these Fe-S cluster proteins. Such disruptions could conceivably lead to two different manifestations—low or high mitochondrial iron. Since our previous work indicated that *sod1*Δ yeast feel iron-starved (45), we considered it possible that, due to oxidizing conditions outside of the matrix in mutants without active CuZnSOD, iron was failing to reach the mitochondria and that their mitochondrial iron was consequently low. Alternatively, many disruptions in Fe-S cluster synthesis pathways cause large increases (up to 10-fold or more) in mitochondrial iron (22-24), in a non-heme, non-iron sulfur state (23). To test these hypotheses, we measured total and "free" iron in mitochondria purified from wild type and *sod1*Δ mutant yeast (Figure 6). We observed only a very small increase in total mitochondrial iron in *sod1*Δ cells, an increase similar in magnitude to what we have observed in whole cells (45), but different from that observed in, for example, *yfh1* mutants (22) that are defective in Fe-S

synthesis. We found no difference in "free" iron (iron detectable by EPR at $g = 4.3$) between wild type and *sod1*Δ yeast in purified mitochondria. We thus conclude that the *sod1*Δ mutation does not severely reduce iron levels in the mitochondria, nor does it have a global effect on the synthesis of Fe-S clusters. This conclusion is supported by the fact that respiration, which requires the action of Fe-S centers and cytochromes, is not decreased in *sod1*Δ mutants (unpublished results). Under the growth conditions used in these experiments, whole *sod1*Δ cells show a four- to five-fold increase in "free" iron, so the absence of such iron in mitochondria indicates that excess "free" iron is located elsewhere in the cell.

Thus we are left with the third possible explanation—the specific sensitivity of exposed 4Fe-4S clusters to superoxide—as the most likely explanation for the lysine and leucine auxotrophies. As mentioned above, understanding the protection of Leu1p by Sod1p is straightforward since both enzymes are present in the cytosol. It is more difficult to account for the cross-compartmental protection of Lys4p and aconitase by Sod1p.

We believe movement of superoxide into the matrix is likely in yeast based on our data and on the following considerations. The pK_a of superoxide is 4.8, so that at physiological pH (around 7 in the cytosol) it will exist mainly as the anion. However, at the outer surface of the inner mitochondrial membrane, the local pH may be substantially lower due to the export of protons by respiration to form the pH gradient. In the absence of Sod1p, excess superoxide may become protonated in this local environment, diffuse across the inner membrane, and become trapped in the matrix where the pH is typically higher (above 7.5) (47,48). Such behavior has been documented in the case of acetate/acetic acid with isolated mitochondria (49,50). In both pairs, O_2^-/HO_2 and $CH_3CO_2^-/CH_3CO_2H$, the conjugate acid is lipid soluble and can diffuse freely across membranes, while the conjugate base is anionic and relatively membrane impermeant. In both cases, the pK_a of the acid is approximately 4.8. This model is diagrammed in Figure 7.

At this point a question arises as to why Sod2p, which is present in the matrix, does not protect Lys4p and aconitase in *sod1*Δ yeast. It is possible that Sod2p is not present in the correct location or in sufficient quantity to successfully combat the influx of superoxide across the inner membrane of *sod1*Δ yeast. In this case, CuZnSOD activity outside of the matrix would be required to prevent an overwhelming inward flow of superoxide. In addition, it is known that MnSOD is subject to reversible product inhibition by H_2O_2, rendering it less active at high superoxide concentrations (51). This inhibition is quite severe in the human enzyme, less so in bacterial ones (52), and is apparently an evolutionarily conserved trait since a MnSOD mutant engineered to have reduced product inhibition (and thus be a more efficient dismutase) was strongly growth inhibitory when expressed in cultured cells (53). Such product inhibition in the yeast MnSOD could help explain its inability to compensate for loss of CuZnSOD.

Apparently, superoxide originating from the matrix does not cause lysine auxotrophy, since it is not observed in *sod2*Δ mutants. This fact remains especially puzzling since Lys4p activity is decreased in *sod2*Δ mutants (Figure 3B), although the level of lysine itself remains normal in these cells. The lysine level is greatly decreased in *sod1*Δ cells. These data fit the observed phenotypes but do little to explain them. A number of observations support the idea that MnSOD is less important than CuZnSOD in the overall protection of yeast growing in glucose media (2,3). The total amount of CuZnSOD is ten (2) to fifty (54) times greater than that of MnSOD in yeast. Furthermore, overexpression of MnSOD in the matrix of various cell types enhances their ability to combat oxidative stress (55-57) indicating that MnSOD is expressed at a level that is just sufficient to protect against normal superoxide levels. The production of lysine in *sod2*Δ yeast cells despite the inactivation of Lys4p could be explained if cluster repair is more efficient in s*od2*Δ than in *sod1*Δ cells.

The methionine auxotrophy of *sod1*Δ has been proposed to result from a depletion of NADPH in the cell by oxidative stress leading to a lowered synthesis of methionine since its biosynthetic pathway requires a great deal of NADPH (58,59). This model is supported by the observation that stimulation of NADPH production by overexpression of transketolase, an enzyme of the pentose phosphate pathway, rescues the methionine auxotrophy of the *sod1*Δ mutant but not the lysine auxotrophy (59). The methionine auxotrophy is inducible in a wild type strain exposed to the redox-cycling drug paraquat (Figure 2) confirming that this auxotrophy is in fact due to an increase in oxidative stress. However, it is interesting to note that sulfite reductase (YSiR), the enzyme that carries out the NADPH-dependent six-electron reduction of sulfite (SO_3^{2-}) to sulfide (S^{2-}) in the methionine biosynthetic pathway, contains a 4Fe-4S cluster (60,61), and it catalyzes the step at which methionine production is interrupted. Inactivation of this enzyme could result in a methionine auxotrophy directly, and could lead to further toxicity by preventing the removal of sulfite, a toxin to which *sod1*Δ mutants are particularly susceptible (58).

We conclude that the role of CuZnSOD in *S. cerevisiae* lysine and leucine metabolism is to protect the 4Fe-4S cluster enzymes Lys4p and Leu1p from direct attack by superoxide on their Fe-S clusters. We find no indication that the primary defect is an alteration in mitochondrial iron metabolism. Our data also indicate that superoxide is able to pass through the mitochondrial inner membrane of yeast and damage Fe-S proteins in the matrix despite the presence of MnSOD, so that Sod1p plays an important role in protecting mitochondrial matrix proteins, as well as cytoplasmic proteins, from superoxide-mediated damage.

Acknowledgements

This work was supported by Grant DK46828 from the National Institutes of Health (to JSV), by U.S. Public Health Service National Research Service Award GM08496 the Chemistry-Biology Interface Predoctoral Training Grant (MAW and MHSC), and by the UC Toxic Substances Research and Teaching Program, Lead Campus Program in Toxic Mechanisms (MAW). LLL was a Howard Hughes Predoctoral Fellow. Many thanks to Dr. Jnanendra K. Bhattacharjee and Dr. Catherine Clarke for strains and plasmids, to Drs. Carla Koehler, Greg Payne, and Catherine Clarke for antibodies, and to Dr. Carla Koehler and Sean Curran for purified mitochondria. Also, many thanks to Dr. Carla Koehler, Sean Curran, and Dr. Catherine Clarke for advice and helpful discussions.

Keywords

CuZnSOD, MnSOD, iron-sulfur cluster inactivation, Lysine, Leucine, Homoaconitase, Aconitase, Isopropylmalate isomerase, mitochondria, yeast.

References

1. Sturtz, L. A., Diekert, K., Jensen, L. T., Lill, R., and Culotta, V. C. (2001) *J Biol Chem* **276**, 38084-38089
2. Gralla, E. B., and Kosman, D. J. (1992) *Adv Genet* **30**, 251-319
3. Gralla, E. B. (1997) in *Cold Spring Harbor Monograph Series, 34. Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (Scandalios, J. G., ed), pp. 495-525, Cold Spring Harbor Laboratory Press, Plainview, NY
4. Srinivasan, C., Liba, A., Imlay, J. A., Valentine, J. S., and Gralla, E. B. (2000) *J Biol Chem* **275**, 29187-29192
5. Wei, J. P., Srinivasan, C., Han, H., Valentine, J. S., and Gralla, E. B. (2001) *J Biol Chem* **276**, 44798-44803
6. Hassan, H. M., and Moody, C. S. (1982) *Can J Physiol Pharmacol* **60**, 1367-1373
7. Bilinski, T., Litwinska, J., and Blaszczynski, M. (1985) *Acta Microbiol Pol* **34**, 15-17
8. Culotta, V. C., Klomp, L. W., Strain, J., Casareno, R. L., Krems, B., and Gitlin, J. D. (1997) *J Biol Chem* **272**, 23469-23472
9. Costa, V., and Moradas-Ferreira, P. (2001) *Mol Aspects Med* **22**, 217-246
10. Jamieson, D. J. (1998) *Yeast* **14**, 1511-1527
11. Beinert, H., Holm, R. H., and Munck, E. (1997) *Science* **277**, 653-659
12. Flint, D. H., Tuminello, J. F., and Emptage, M. H. (1993) *J Biol Chem* **268**, 22369-22376
13. Gardner, P. R., and Fridovich, I. (1991) *J Biol Chem* **266**, 19328-19333
14. Longo, V. D., Liou, L. L., Valentine, J. S., and Gralla, E. B. (1999) *Arch Biochem Biophys* **365**, 131-142
15. Gardner, P. R., and Fridovich, I. (1991) *J Biol Chem* **266**, 1478-1483

16. Fridovich, I. (1997) *J Biol Chem* **272**, 18515-18517

17. Fridovich, I. (1995) *Annu Rev Biochem* **64**, 97-112

18. Irvin, S. D., and Bhattacharjee, J. K. (1998) *J Mol Evol* **46**, 401-408

19. Bhattacharjee, J. K. (1985) *Crit Rev Microbiol* **12**, 131-151

20. Flint, D. H., and Allen, R. M. (1996) *Chem Rev* **96**, 2315-2334

21. Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999) *Embo J* **18**, 3981-3989

22. Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M., and Kaplan, J. (1997) *Science* **276**, 1709-1712

23. Kispal, G., Csere, P., Guiard, B., and Lill, R. (1997) *FEBS Letters* **418**, 346-350

24. Voisine, C., Cheng, Y. C., Ohlson, M., Schilke, B., Hoff, K., Beinert, H., Marszalek, J., and Craig, E. A. (2001) *Proc Natl Acad Sci U S A* **98**, 1483-1488

25. Muhlenhoff, U., Richter, N., Gerber, J., and Lill, R. (2002) *J Biol Chem*

26. Lange, H., Kispal, G., and Lill, R. (1999) *J Biol Chem* **274**, 18989-18996

27. Liu, X. F., Elashvili, I., Gralla, E. B., Valentine, J. S., Lapinskas, P., and Culotta, V. C. (1992) *J Biol Chem* **267**, 18298-18302

28. Gralla, E. B., and Valentine, J. S. (1991) *J Bacteriol* **173**, 5918-5920

29. Wang, L., Okamoto, S., and Bhattacharjee, J. K. (1989) *Curr Genet* **16**, 7-12

30. Spain, B. H., Koo, D., Ramakrishnan, M., Dzudzor, B., and Colicelli, J. (1995) *J Biol Chem* **270**, 25435-25444

31. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) *Yeast* **2**, 163-167

32. Kaiser, C., Michaelis, S., and Mitchell, A. (eds) (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

33. Nakatani, Y., Fujioka, M., and Higashino, K. (1972) *Anal Biochem* **49**, 225-231

34. Broquist, H. P. (1971) *Methods Enzymol* **17B**, 112-129

35. Strassman, M., and Ceci, L. N. (1966) *J Biol Chem* **241**, 5401-5407

36. Kohlhaw, G. B. (1988) *Methods Enzymol* **166**, 423-429

37. Gardner, P. R., Raineri, I., Epstein, L. B., and White, C. W. (1995) *J Biol Chem* **270**, 13399-13405

38. Glick, B. S., and Pon, L. A. (1995) *Methods Enzymol* **260**, 213-223

39. Koehler, C. M., Merchant, S., Oppliger, W., Schmid, K., Jarosch, E., Dolfini, L., Junne, T., Schatz, G., and Tokatlidis, K. (1998) *Embo J* **17**, 6477-6486

40. Glick, B. S., Brandt, A., Cunningham, K., Muller, S., Hallberg, R. L., and Schatz, G. (1992) *Cell* **69**, 809-822

41. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1998) *Current Protocols in Molecular Biology*. 4 vols., John Wiley & Sons, Inc., New York, NY

42. Srinivasan, C., and Gralla, E. B. (2002) *Methods Enzymol* **349**, 173-180

43. Bilinski, T., Krawiec, Z., Liczmanski, A., and Litwinska, J. (1985) *Biochem Biophys Res Commun* **130**, 533-539

44. Strain, J., Lorenz, C. R., Bode, J., Garland, S., Smolen, G. A., Ta, D. T., Vickery, L. E., and Culotta, V. C. (1998) *J Biol Chem* **273**, 31138-31144

45. De Freitas, J. M., Liba, A., Meneghini, R., Valentine, J. S., and Gralla, E. B. (2000) *J Biol Chem* **275**, 11645-11649

46. Halliwell, B., and Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine*, 3 Ed., Oxford University Press, Oxford

47. Liu, S. S. (1999) *J Bioenerg Biomembr* **31**, 367-376

48. Guidot, D. M., Repine, J. E., Kitlowski, A. D., Flores, S. C., Nelson, S. K., Wright, R. M., and McCord, J. M. (1995) *J Clin Invest* **96**, 1131-1136

49. Nicholls, D. G., and Ferguson, S. J. (2002) *Bioenergetics 3*, Academic Press, London

50. Rasmussen, H., Chance, B., and Ogata, E. (1965) *Proc Natl Acad Sci U S A* **53**, 1069-1076

51. Hearn, A. S., Fan, L., Lepock, J. R., Luba, J. P., Greenleaf, W. B., Cabelli, D. E., Tainer, J. A., Nick, H. S., and Silverman, D. N. (2004) *J Biol Chem* **279**, 5861-5866

52. Hsu, J. L., Hsieh, Y., Tu, C., O'Connor, D., Nick, H. S., and Silverman, D. N. (1996) *J Biol Chem* **271**, 17687-17691

53. Davis, C. A., Hearn, A. S., Fletcher, B., Bickford, J., Garcia, J. E., Leveque, V., Melendez, J. A., Silverman, D. N., Zucali, J., Agarwal, A., and Nick, H. S. (2004) *J Biol Chem* **279**, 12769-12776

54. Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., O'Shea, E. K., and Weissman, J. S. (2003) *Nature* **425**, 737-741

55. St Clair, D. K., Oberley, T. D., and Ho, Y. S. (1991) *FEBS Lett* **293**, 199-203

56. Wenk, J., Brenneisen, P., Wlaschek, M., Poswig, A., Briviba, K., Oberley, T. D., and Scharffetter-Kochanek, K. (1999) *J Biol Chem* **274**, 25869-25876

57. Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M., and Inze, D. (1991) *Embo J* **10**, 1723-1732

58. Chang, E. C., and Kosman, D. J. (1990) *J Bacteriol* **172**, 1840-1845

59. Slekar, K. H., Kosman, D. J., and Culotta, V. C. (1996) *J Biol Chem* **271**, 28831-28836

60. Yoshimoto, A., and Sato, R. (1968) *Biochim Biophys Acta* **153**, 555-575

61. Crane, B. R., Siegel, L. M., and Getzoff, E. D. (1997) *Biochemistry* **36**, 12101-12119

Footnotes:

[1]**Abbreviations:** CuZnSOD or Sod1p, copper-zinc superoxide dismutase; IMS, mitochondrial intermembrane space; MnSOD or Sod2p, manganese superoxide dismutase; *sod1*Δ, yeast lacking the CuZnSOD gene; *sod2*Δ, yeast lacking the MnSOD gene; *ccs1*Δ, yeast lacking the *CCS1* (*LYS7*) gene; Aco1p, aconitase; Lys4p, homoaconitase; Leu1p, isopropylmalate dehydratase; Fe-S, iron sulfur (cluster); WT, wild type; PQ, paraquat. ICP-AE, inductively coupled plasma atomic emission spectrometer; EPR, electron paramagnetic resonance.

[2]This gene was first identified as part of the lysine biosynthetic pathway, hence the name *LYS7*. Since then, its true function—insertion of copper into CuZnSOD—has been discovered. We agree with a recent proposal to rename the yeast gene *CCS1* so that its name better reflects its function and agrees with the names of similar genes in other organisms and are accordingly using the name *CCS1* herein.

Figure legends

Figure 1. Leaky leucine auxotrophy. Strains were made *LEU2*$^+$ by integration of plasmid YIp351 and tested for growth without various amino acids. A) EG103L (WT), B) EG118L (*sod1*Δ), and C) JW101 (*ccs1*Δ) yeast were grown in complete medium or in the absence of leucine, methionine, lysine, or threonine. The optical density at 600nm was measured every two hours over a 14 hour period. After 24 hours (not shown), *sod1*Δ and *ccs1*Δ attained densities comparable to WT in medium lacking leucine, but never exhibited growth in media lacking methionine or lysine. Open circles, SD complete medium; filled circles, SD–Thr; open triangles, SD–Leu; filled triangles, SD–Met; open squares, SD–Lys. Points shown are averages of 8 samples (separate cultures).

Figure 2. Paraquat addition to wild type induces auxotrophies. EG103L was grown for 12 hrs in the presence (grey bars) or absence (black bars) of 0.25mM paraquat (methyl viologen) in SDC medium (Comp), SD–Thr (-Thr), SD–Leu (-Leu), SD–Lys (-Lys), or SD–Met (-Met). Paraquat treatment resulted in a 24%, 79%, and 75% growth inhibition in media lacking leucine, lysine, and methionine respectively, while no significant effect was observed in complete medium or in medium lacking threonine. Data are averages of 8 separate cultures. Asterisks (*) indicate a significant difference between treated and untreated samples in the same medium ($P<0.05$ by the Student's t-test).

Figure 3. 4Fe-4S cluster enzyme inactivation. Activities of Leu1p, Lys4p, and aconitase were measured on extracts from EG103 (WT), EG103 + 1mM paraquat (PQ), EG118 (*sod1*Δ), and EG110 (*sod2*Δ) yeast strains. Extracts were prepared under argon or nitrogen to minimize enzyme inactivation, as Fe-S clusters are very sensitive to oxidation. (A) Aconitase activity was assayed on extracts of cells grown in SDC medium to an OD_{600} of 1.5. (B) Homoaconitase (Lys4p) activity was obtained after growth in SDC to an OD_{600} of 1.0 and subsequent incubation in SD–Lys–Leu–Trp medium for 12 hrs to induce Lys4p activity and to prevent further growth. (C) Isopropylmalate isomerase (Leu1p) activity was measured on extracts from cells grown in SD–Leu medium to an OD_{600} of 1.5. EG103L and EG118L were used to restore leucine biosynthetic competency. Asterisks (*) indicate a significant difference from WT ($P<0.05$ by the Student's t-test). Values are averages of 5 to 8 separate cultures.

Figure 4. Reactivation of Lys4p and Leu1p. EG103 (WT), EG118 (*sod1*Δ), and EG110 (*sod*2Δ) yeast were grown as described in Figure 3 and switched to SD–Lys–Leu–Trp medium to induce amino acid biosynthetic pathways and to prevent further growth. Lys4p activity (A, B) and Leu1p activity (C, D) were determined. (A, C) Cultures were divided into two aliquots and incubated at 30°C for 3 hr, with one aliquot shaken at 220 rpm under ambient oxygen (high aeration, H) and the other aliquot with no shaking under 3% oxygen (A) or nitrogen (C) (low aeration, L). (B, D) Cells were switched to SD–Lys–Leu–Trp under low aeration in the absence or presence of cycloheximide (Cyclohex) to inhibit protein synthesis. An asterisk (*) indicates a significant difference between the high and low aeration condition ($P<0.05$ by the Student's t-test). One sample has no detectable activity (N.D.).

Figure 5. Localization of Lys4p and CuZnSOD. Mitochondria from LL101 cells expressing HA-tagged Lys4p were isolated and fractionated as described in Experimental Procedures to incrementally release IMS proteins as sorbitol concentration decreased. Immunoblotting was performed using antibodies that recognize Sod1p (IMS), Cyt b2 (IMS), HA (Lys4 tag), Kgd1p (matrix), and Hsp60 (matrix). Trypsin was used to degrade proteins not protected by a membrane, and triton was used to completely solubilize all membranes and expose all proteins to trypsin. Lys4p was determined to be localized in the matrix.

Figure 6. Iron levels in isolated mitochondria. Mitochondria were purified from EG103 (WT) and EG118 (*sod1*Δ) yeast that were grown in SDC to an OD_{600} of 6.0. A) Total mitochondrial iron was measured by ICP-AE and is reported in nmol Fe/mg mitochondrial protein. B) EPR measurements looking at "free" iron were done at g = 4.3 in liquid nitrogen, quantitated by comparison to an iron standard and reported as pmol Fe/mg mitochondrial protein. Results shown are averages of data from four separate preparations.

Figure 7. **Model for the effect of the absence of Sod1p on Fe-S proteins in the cytosol and matrix.** During aerobic growth, superoxide is generated on the inner and outer surfaces of the inner mitochondrial membrane, as well as in the cytoplasm. In wild type cells, Sod1p in the IMS and cytoplasm intercepts superoxide generated in those locations, and the 4Fe-4S cluster enzymes Aco1p, Lys4p, and Leu1p remain active. When Sod1p is absent (*sod1*Δ) the superoxide concentration increases in the IMS and cytosol. Superoxide anion (pKa = 4.8) in the IMS near the inner membrane becomes protonated due to the local low pH created by the respiratory proton gradient. This uncharged form of superoxide is then able to diffuse across the inner membrane into the matrix where it deprotonates and inactivates Aco1p and Lys4p. Leu1p is inactivated by superoxide in the cytosol. In either case, Sod2p continues to intercept superoxide made by the respiratory chain at the inside (matrix) surface of the inner membrane, but it does not react with superoxide that enters the matrix by direct diffusion, either because it is not correctly positioned, because it is not present in sufficient quantity, or because it is product-inhibited at higher superoxide concentration. OM, outer mitochondrial membrane; IM, inner membrane; IMS, inter membrane space. Inverted text is used to indicate Fe-S enzymes in an inactive state. Single-sided arrows indicate movement of a single species, regular arrows indicate a chemical reaction, and bars indicate inhibition. The pores that make the OM nonspecifically permeable to small ions are indicated by gaps; there are no such pores in the IM and only uncharged hydrophobic molecules can penetrate into the matrix in the absence of a specific transporter protein. At high superoxide concentration and relatively low pH, spontaneous disproportionation can occur, as indicated by the dashed arrow.

Table 1
Yeast strains used in this study

Strain	Relevant genotype	Complete genotype	Source
EG103	Wild type	*MATα leu2 his3 trp1 ura3*	(28)
EG103L	Wild type/ LEU2	EG103 with YIp351	This work
EG118	*sod1*Δ	*MATα leu2 his3 trp1 ura3 sod1*Δ::URA3	(28)
EG110	*sod2*Δ	*MATα leu2 his3 trp1 ura3 sod2*Δ::TRP1	(27)
EG118L	*sod1*Δ/ LEU2	EG118 with YIp351	This work
LL101	EG103/ LYS4-HA	EG103 with pADCL-LYS4-HA	This work
JW101	*ccs1*Δ (*lys7*Δ)	*MATα leu2 his3 trp1 ura3 ccs1*Δ::LEU2	(5)
BY4741	Wild type	*MATα leu2 his3 met15 ura3*	Purchased
BY4741	*sod1*Δ (#6913)	*MATα leu2 his3 met15 ura3 sod1*Δ::KAN^R	Purchased
BY4741	*ccs1*Δ (#614)	*MATα leu2 his3 met15 ura3 ccs1*Δ::KAN^R	Purchased
MO-59-13c	*lys4*Δ	*lys4*	(29)
X3356-1B	*lys4*Δ	*MATα lys4 gal2*	Bhattacharjee

Figure 1

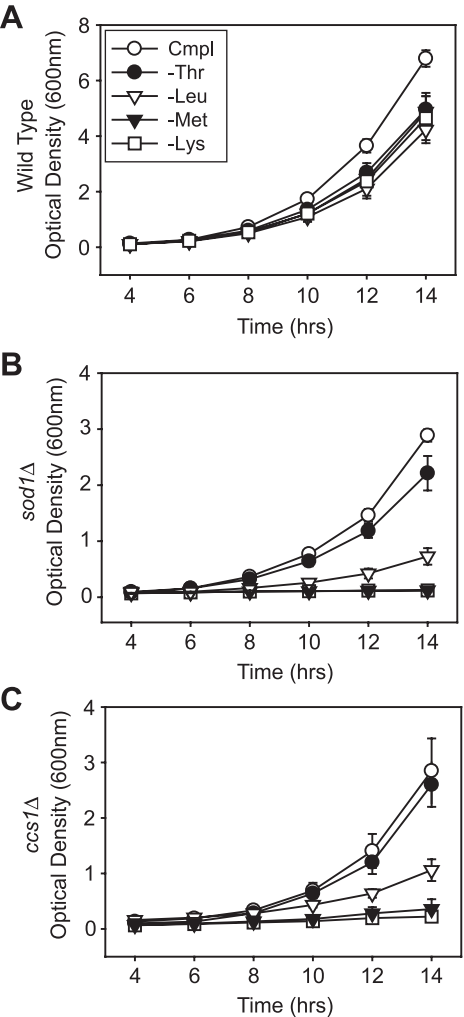

A
Wild Type
Optical Density (600nm)
Cmpl
-Thr
-Leu
-Met
-Lys
Time (hrs)
B
sod1Δ
Optical Density (600nm)
Time (hrs)
C
ccs1Δ
Optical Density (600nm)
Time (hrs)

Figure 2

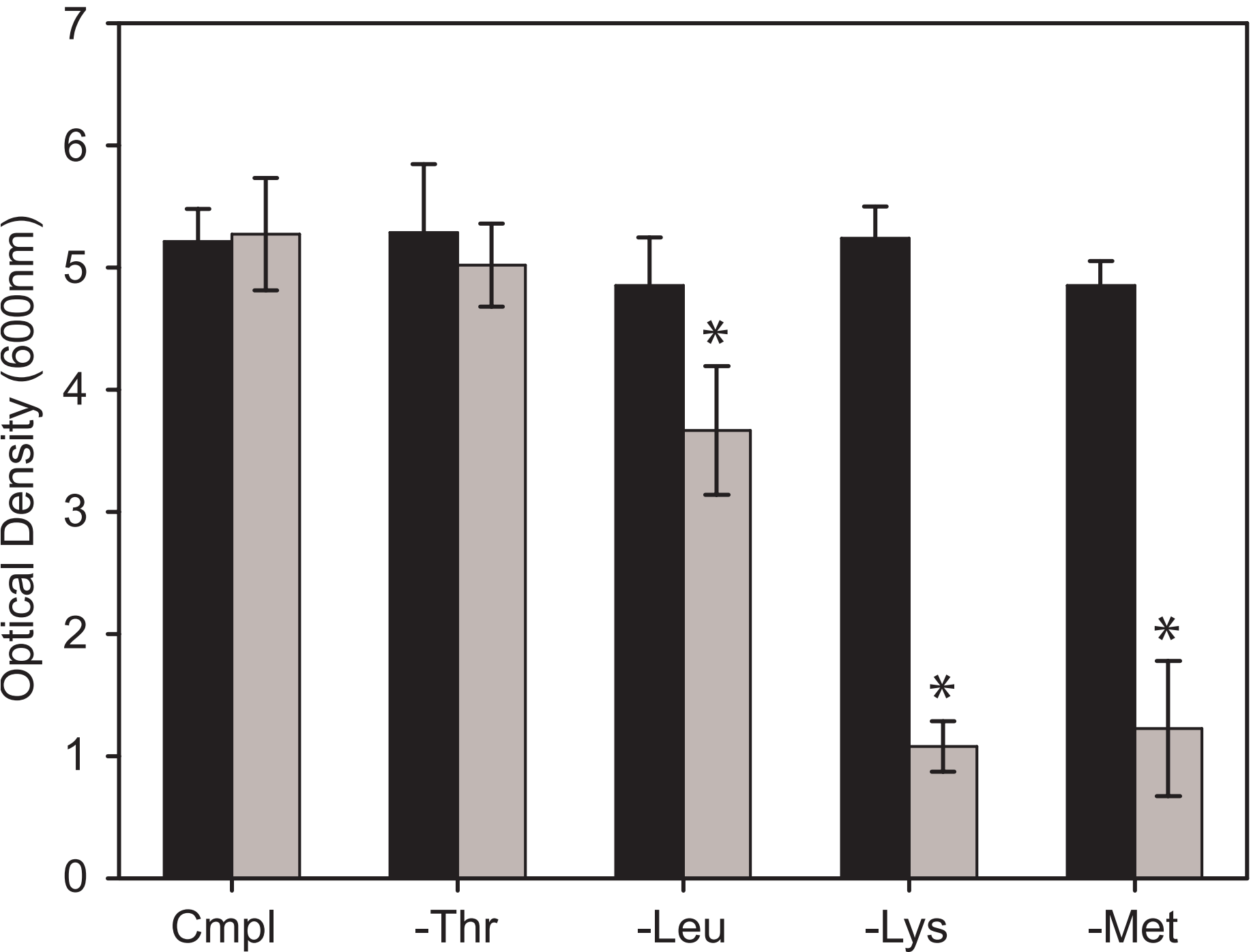


Figure 3

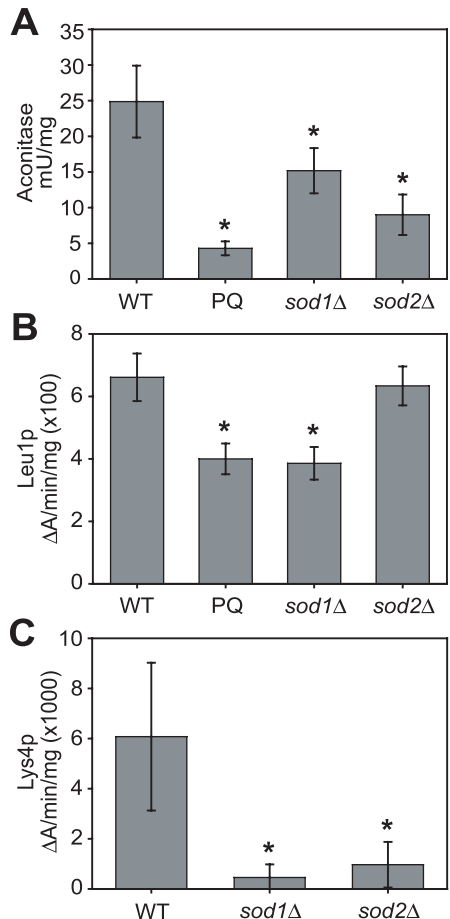

A
Aconitase mU/mg
35
30
25
20
15
10
5
0
*
*
*
WT
PQ
sod1Δ
sod2Δ
B
Leu1p ΔA/min/mg (x100)
8
6
4
2
0
*
*
WT
PQ
sod1Δ
sod2Δ
C
Lys4p ΔA/min/mg (x1000)
10
8
6
4
2
0
*
*
WT
sod1Δ
sod2Δ

Figure 4

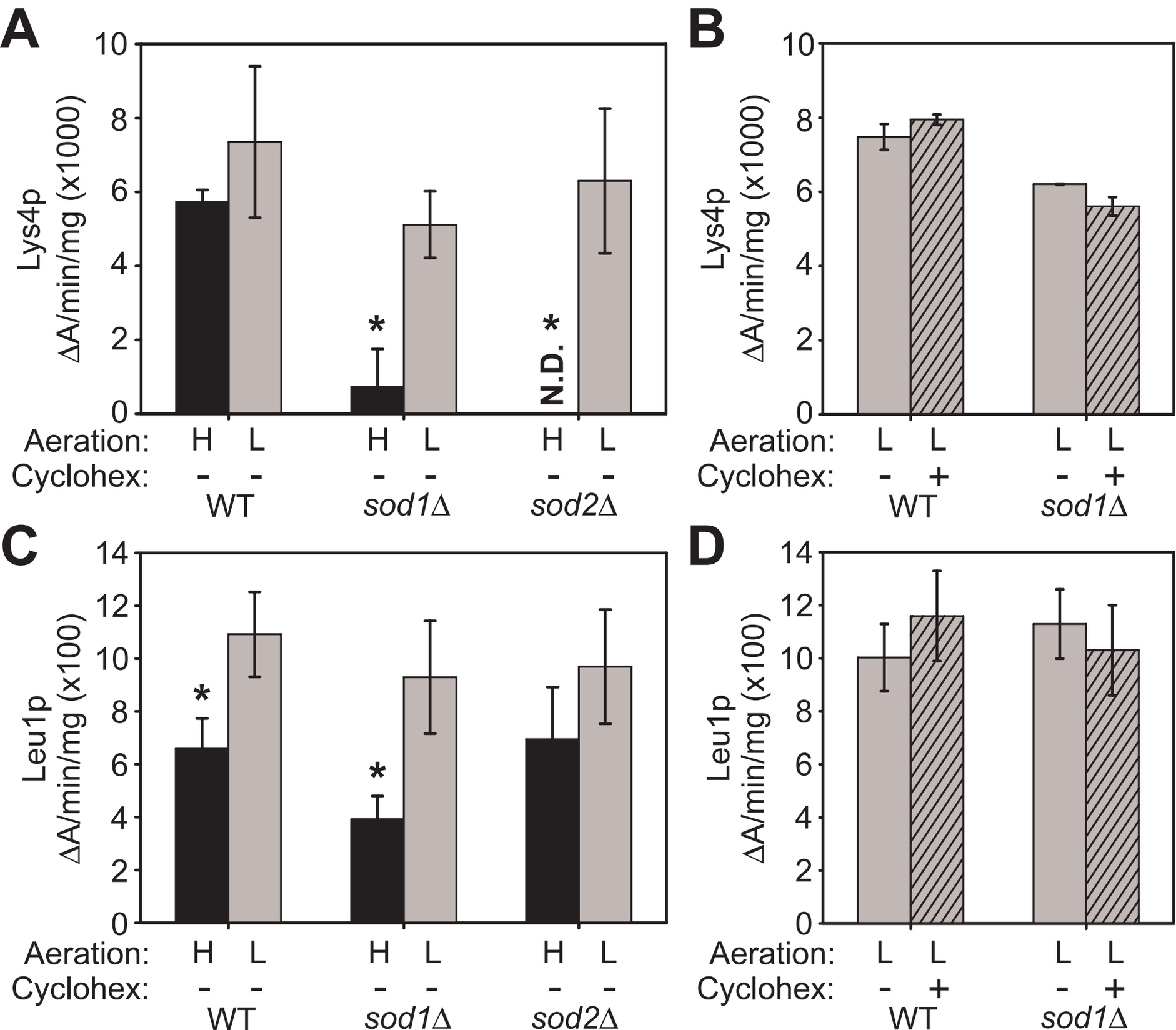

A
Lys4p
ΔA/min/mg (x1000)
10
8
6
4
2
0
*
N.D. *
Aeration: H L H L H L
Cyclohex: - - - - - -
WT
sod1Δ
sod2Δ
B
Lys4p
ΔA/min/mg (x1000)
Aeration: L L L L
Cyclohex: - + - +
WT
sod1Δ
C
Leu1p
ΔA/min/mg (x100)
14
12
10
8
6
4
2
0
*
*
Aeration: H L H L H L
Cyclohex: - - - - - -
WT
sod1Δ
sod2Δ
D
Leu1p
ΔA/min/mg (x100)
Aeration: L L L L
Cyclohex: - + - +
WT
sod1Δ

Figure 5

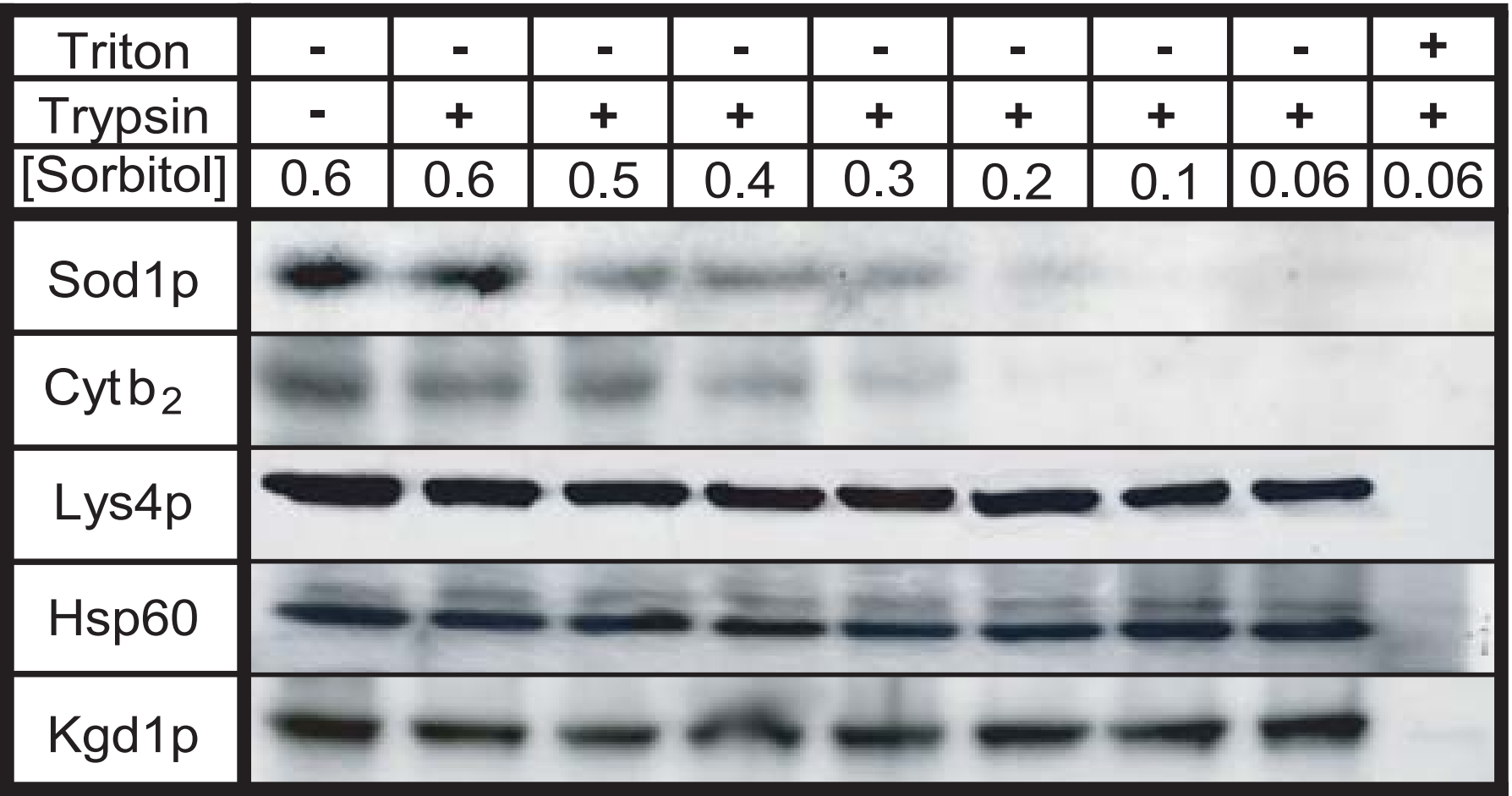

Triton - - - - - - - - +
Trypsin - + + + + + + + +
[Sorbitol] 0.6 0.6 0.5 0.4 0.3 0.2 0.1 0.06 0.06
Sod1p
Cyt b2
Lys4p
Hsp60
Kgd1p

Figure 6

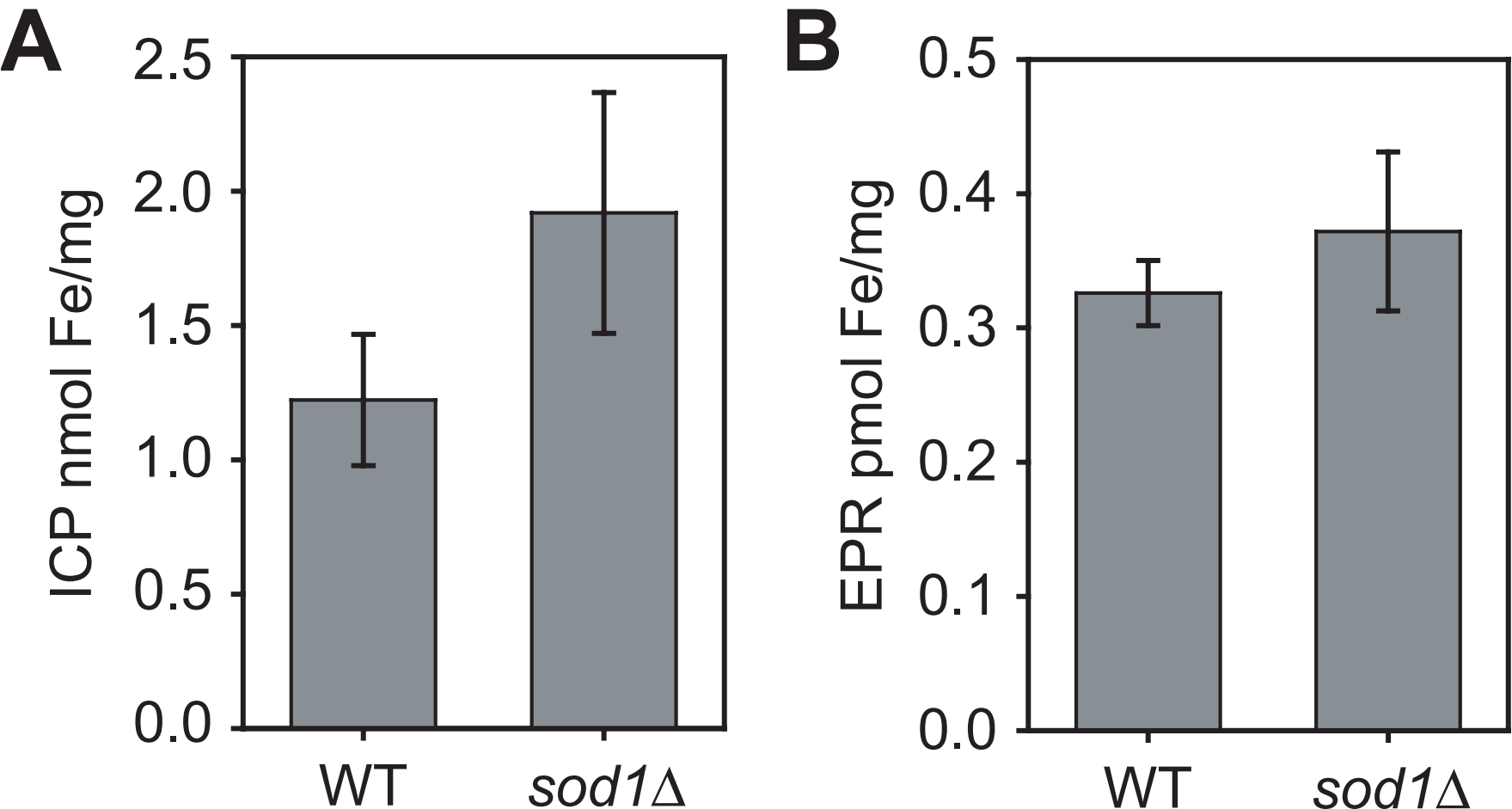
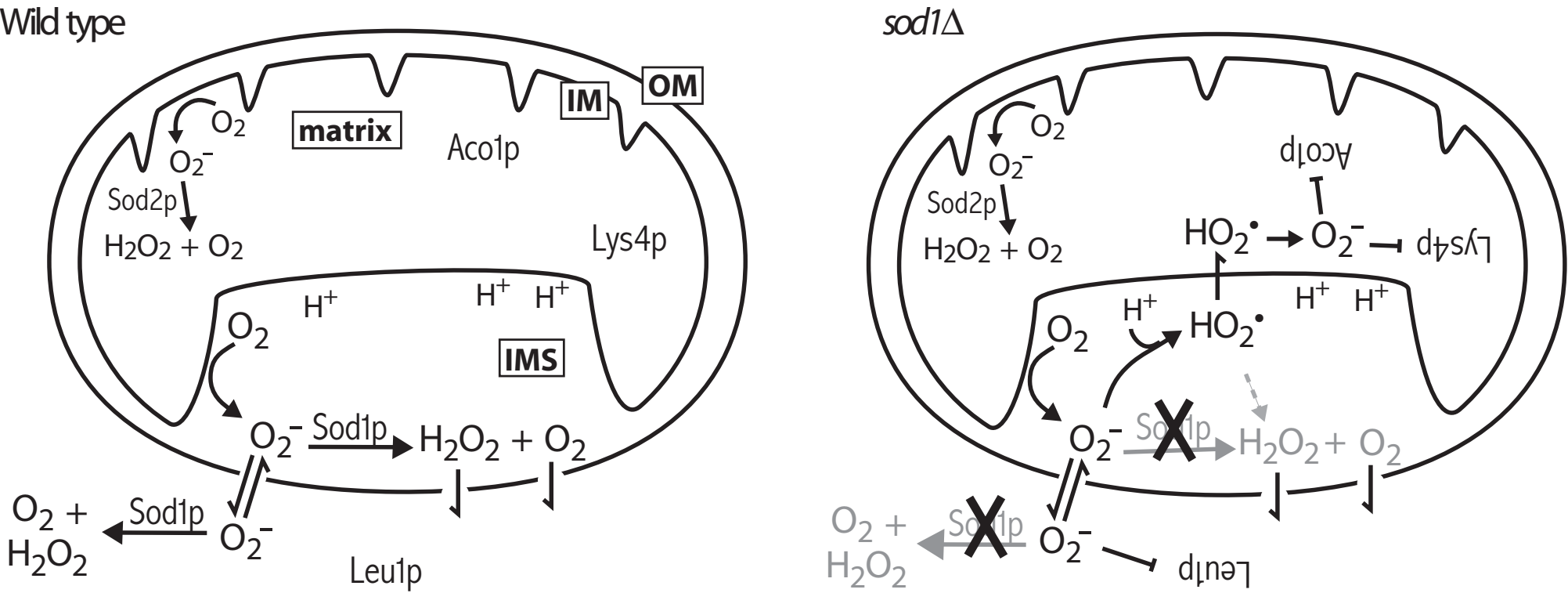

A
ICP nmol Fe/mg
2.5
2.0
1.5
1.0
0.5
0.0
WT
sod1Δ
B
EPR pmol Fe/mg
0.5
0.4
0.3
0.2
0.1
0.0
WT
sod1Δ

Figure 7


Wild type
matrix
IM
OM
Aco1p
Lys4p
IMS
Sod2p
$H_2O_2 + O_2$
O_2
O_2^-
H^+
Sod1p
Leu1p
$O_2 + H_2O_2$
sod1Δ
$HO_2^\bullet$

Superoxide inhibits 4Fe-4S cluster enzymes involved in amino acid biosynthesis: cross-compartment protection by CuZnSOD

Matthew Alan Wallace, Lee-Loung Liou, Jacob Martins, Matthew H. S. Clement, Sasaneh Bailey, Valter D. Longo, Joan Selverstone Valentine and Edith Butler Gralla

J. Biol. Chem. published online May 27, 2004
