The EMBO Journal (2006) 25, 1775–1783 | © 2006 European Molecular Biology Organization | All Rights Reserved 0261-4189/06
www.embojournal.org

THE EMBO JOURNAL

The effects of mitochondrial iron homeostasis on cofactor specificity of superoxide dismutase 2

Mei Yang[1], Paul A Cobine[2,3], Sabine Molik[4], Amornrat Naranuntarat[1], Roland Lill[4], Dennis R Winge[2,3] and Valeria C Culotta[1,*]

[1]Division of Toxicological Sciences, Department of Environmental Health Sciences, The Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, USA, [2]Department of Medicine, University of Utah Health Sciences Center, Salt Lake City, Utah, USA, [3]Department of Biochemistry, University of Utah Health Sciences Center, Salt Lake City, Utah, USA and [4]Institut fur Zytobiologie, Philipps-Universitat Marburg, Germany

Many metalloproteins have the capacity to bind diverse metals, but in living cells connect only with their cognate metal cofactor. In eukaryotes, this metal specificity can be achieved through metal-specific metallochaperone proteins. Herein, we describe a mechanism whereby *Saccharomyces cerevisiae* manganese superoxide dismutase (SOD2) preferentially binds manganese over iron based on the differential bioavailability of these ions within mitochondria. The bulk of mitochondrial iron is normally unavailable to SOD2, but when mitochondrial iron homeostasis is disrupted, for example, by mutations in *S. cerevisiae mtm1, ssq1* and *grx5*, iron accumulates in a reactive form that potently competes with manganese for binding to SOD2, inactivating the enzyme. Studies in *mtm1* mutants indicate that iron inactivation of SOD2 involves the Mrs3p/Mrs4p mitochondrial carriers and iron-binding frataxin (Yfh1p). A small pool of SOD2-reactive iron also exists under normal iron homeostasis conditions and binds SOD2 when mitochondrial manganese is low. The ability to control this reactive pool of iron is critical to maintaining SOD2 activity and has important potential implications for oxidative stress in disorders of iron overload.

The EMBO Journal (2006) **25,** 1775–1783. doi:10.1038/sj.emboj.7601064; Published online 6 April 2006
Subject Categories: cellular metabolism
Keywords: Aft1p; iron; manganese; mitochondria; SOD2

Introduction

Heavy metals such as zinc, copper, iron and manganese are widely used in biology as enzymatic cofactors and structural determinants in proteins. These ions all accumulate in cells at concentrations well above the extracellular mileu (Outten and O'Halloran, 2001; Finney and O'Halloran, 2003), and all bind polypeptides via coordination to thiol, imidazole and carboxylic side chains. In spite of the seemingly diverse choice of candidate cofactors, metalloproteins generally bind only their cognate metal *in vivo*. In some cases, metal specificity is inherent to the polypeptide sequence, where the protein only accommodates a metal with a particular binding geometry (Finney and O'Halloran, 2003). However, most metalloproteins seem more flexible, for example, a copper-requiring enzyme may also bind zinc or cobalt *in vitro*, but is only active when copper-bound. Metal ion selectivity in this case may be achieved *in vivo* through the action of accessory proteins known as metallochaperones (Pufahl *et al*, 1997). Metallochaperones capture a specific metal ion in spite of the absence of free intracellular metals (Rae *et al*, 1999; Outten and O'Halloran, 2001; Finney and O'Halloran, 2003), and directly transfer their cargo to metalloprotein targets. Metallochaperones have been well characterized for copper (O'Halloran and Culotta, 2000; Bartnikas and Gitlin, 2001; Finney and O'Halloran, 2003) and while the metallochaperone concept has been expanded to include other metals (Colpas and Hausinger, 2000; O'Halloran and Culotta, 2000; Anderson *et al*, 2005), information on chaperones for metals other than copper is quite sparse.

In bacteria, there are rare instances of metal ion misincorporation. The manganese-containing superoxide dismutase (MnSOD) of *Escherichia coli* can also bind iron, and iron binds to MnSOD with similar metal binding geometries and affinities as manganese (Beyer and Fridovich, 1991; Privalle and Fridovich, 1992; Whittaker, 2003; Mizuno *et al*, 2004). Isolates of MnSOD from *E. coli* contain a mixture of iron- and manganese-bound molecules, and under anaerobic conditions, the enzyme becomes virtually all iron-bound (Beyer and Fridovich, 1991; Privalle and Fridovich, 1992). Iron binding inactivates the SOD enzyme, due to an aberrant redox potential at the active site (Vance and Miller, 1998) and a possible block in substrate access (Whittaker, 2003).

Eukaryotes express a highly homologous MnSOD (SOD2) in the mitochondrial matrix that is predicted to exhibit similar inactivation by iron based on comparative structural analyses (Borgstahl *et al*, 1992; Wintjens *et al*, 2004). However, there has been no documentation of iron misincorporation in SOD2, other than a trace amount of iron-association with the yeast enzyme (Ravindranath and Fridovich, 1975). Eukaryotes have therefore evolved with means for ensuring cofactor specificity in MnSOD.

We have been employing bakers yeast as a model system to explore the mechanism by which Sod2p specifically acquires manganese. (In this article, Sod2p refers to the *Saccharomyces cerevisiae* polypeptide; all other eukaryotic versions of the enzyme are denoted SOD2). Manganese activation requires a mitochondrial localization for Sod2p, and the protein unfolding step associated with mitochondrial import is thought to drive metal insertion (Luk *et al*, 2005). In our search for proteins that facilitate manganese insertion

*Corresponding author. Division of Toxicological Sciences, Department of Environmental Health Sciences, The Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD 21205, USA.
Tel.: +1 410 955 3029; Fax: +1 410 955 0116;
E-mail: vculotta@jhsph.edu

Received: 24 November 2005; accepted: 7 March 2006; published online: 6 April 2006

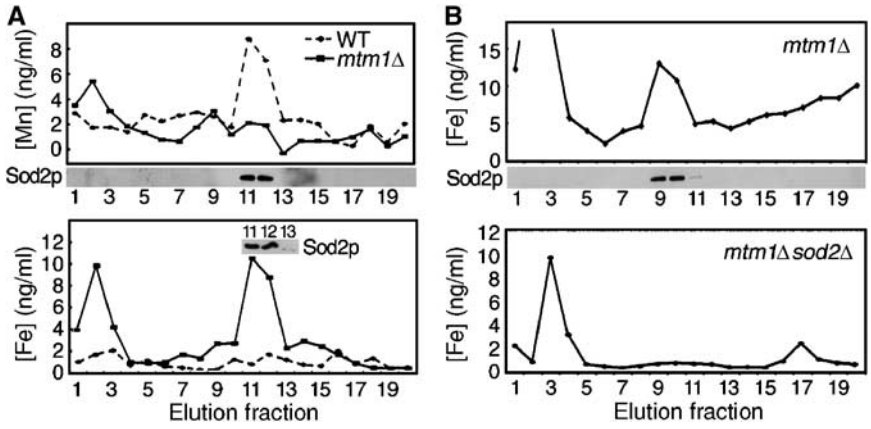


Figure 1 Sod2p associates with iron in *mtm1* mutants. The soluble fraction from gradient-purified mitochondria (see Materials and methods) was loaded onto a Mono Q anion exchange column. Fractions 1–4 represent flow-through fractions containing positive and uncharged molecules that do not bind to the anion exchange resin under these conditions. Fractions 5–20 represent bound molecules eluted with a 0–100% gradient of 1 M NaCl. Fractions were analyzed by ICP-OES for metals. Concentrations of manganese (A, top) and iron (A, bottom; B) are shown. Fractions were also analyzed for Sod2p by immunoblot. (**A**) Immunoblots represent fractions 1–20 of the WT strain (middle strip) and fractions 11–13 of the *mtm1* mutant (inset). With the same column injection volume, Sod2p from WT and *mtm1* cells elute at the identical fraction number. (**B**) Immunoblot represents fractions 1–20 of the *mtm1* mutant. The injection volume was smaller (12 versus 15 ml) for the column in (B) compared to (A) explaining the earlier fractionation of Sod2p in case. Strains utilized: WT, BY4741; *mtm1*Δ, MY019; *mtm1*Δ *sod2*Δ, MY020.

into Sod2p, we identified *S. cerevisiae* Mtm1p, a member of the mitochondrial carrier family of transporters (Luk *et al*, 2003). Yeast *mtm1* mutants accumulate inactive Sod2p in the mitochondrial matrix and enzyme activity can be restored by growing cells in the presence of very high concentrations of manganese (Luk *et al*, 2003). However, Mtm1p is not a manganese transporter, as *mtm1*Δ mitochondria are not deprived of manganese (Luk *et al*, 2003). Interestingly, our analysis of mitochondrial metals revealed elevated iron levels in *mtm1*Δ mitochondria (Luk *et al*, 2003). This uncovering of high iron in *mtm1* mutants prompted our recent investigations into the manganese versus iron selectivity of Sod2p *in vivo*.

Herein, we demonstrate that loss of Sod2p activity in *mtm1* mutants is due to misincorporation of iron into Sod2p rather than manganese. Such iron inactivation of Sod2p is not unique to *mtm1* mutants, and was observed in other yeast mutants affected in mitochondrial iron metabolism. Our studies provide evidence for the existence of at least two pools of mitochondrial iron: an 'SOD2-inert' pool that predominates with normal iron homeostasis, and an 'SOD2-reactive' iron form that effectively competes with mitochondrial manganese for binding to SOD2. A small pool of SOD2-reactive iron also exists in the absence of iron-related defects, and this iron pool readily associates with Sod2p when mitochondrial manganese is low. Overall, these studies demonstrate that metallation of mitochondrial SOD2 is not as manganese-specific as originally thought. The differential bioavailability of manganese versus iron in the mitochondria plays an important role in determining the metal ion specificity of this enzyme.

Results

Sod2p associates with iron in mtm1 mutants

We explored the relationship between the high mitochondrial iron and low Sod2p activity in *S. cerevisiae mtm1* mutants. Loss of Sod2p activity *per se* does not lead to high mitochondrial iron since *sod2*Δ mutants do not hyperaccumulate mitochondrial iron (Yang and Culotta, unpublished). We therefore tested whether the reverse was true and whether mitochondrial iron was interacting with, and inhibiting Sod2p.

The metal that associates with Sod2p in wild-type (WT) versus *mtm1* mutants was analyzed in a mitochondrial fractionation study. Soluble components of gradient-purified mitochondria were subjected to Mono Q anion exchange and size exclusion chromatography. Sod2p fractions were identified by immunoblot, and manganese and iron profiles analyzed by ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy). Sod2p elutes from Mono Q in a single peak over two fractions with both WT cells (immunoblot of Figure 1A) and *mtm1* mutants (immunoblot of Figure 1B). Metal analysis of WT mitochondria revealed a single manganese-containing peak that coeluted with Sod2p during anionic exchange (Figure 1A top). Manganese and Sod2p continued to co-elute during subsequent chromatography by size exclusion (not shown). With WT cells, the bulk of soluble manganese in the mitochondria is bound to Sod2p.

Unlike WT cells, there was no anionic peak of soluble manganese with *mtm1* mutants (Figure 1A, top). Instead *mtm1*Δ mitochondria have two major iron-containing components that were absent in WT cells (Figure 1A bottom and 1B top). The first represents soluble material that did not bind Mono Q (fractions $\approx$1–4) and the second represents an anionic bound fraction. In all cases, this second iron-peak of *mtm1* mutants co-eluted with Sod2p as monitored by immunoblot (Figure 1A, bottom and Figure 1B top). Iron and Sod2p continued to co-elute during subsequent size exclusion chromatography (not shown). Among the soluble iron-containing proteins of yeast mitochondria, only the iron–Sod2p peak was visible. Other mitochondrial iron proteins possess high p*I* values or are of lower abundance (e.g., Yhb1p, Cyc1p, Bio2p and Lys4p) and could easily escape

 ©2006 European Molecular Biology Organization

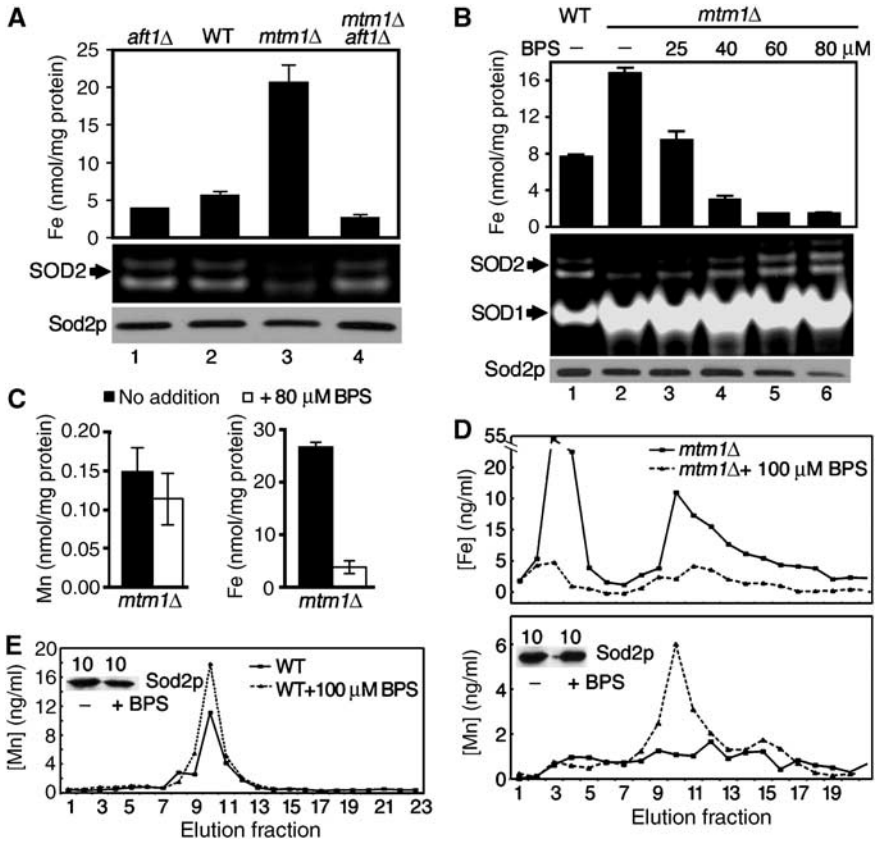


Figure 2 Low mitochondrial iron rescues the Sod2p defect in *mtm1* mutants. The designated strains were grown in YPD media containing where indicated (B–E), the specified concentrations of the iron chelator BPS (bathophenanthrolinedisulfonate). (**A–C**): Bar graphs represent mitochondrial levels of iron and manganese as determined by atomic absorption spectrometry. Data represent averages of four readings obtained from two independent mitochondrial preps; error bars indicate standard deviation. (A–B): Whole-cell lysates were analyzed for SOD activity by native gel electrophoresis and nitroblue tetrazolium staining (top gel), and for Sod2p polypeptide levels by immunoblot (bottom gel). 'SOD2' and 'SOD1' indicate activity of Mn Sod2p and Cu/Zn Sod1p. On native gels, Sod2p activity typically resolves into 2–3 distinct bands, especially with BPS treatment. These bands all represent Sod2p, as determined by native gel immunoblot (not shown) and may correspond to different metal occupation states of the tetrameric enzyme. Combined analysis of Sod2p activity and protein levels for the various experimental trials of part (A) can be found in Supplementary Figure S1. (**D, E**) Anion exchange chromatography analysis of mitochondrial fractions was conducted as in Figure 1 for *mtm1*Δ(*D*) or WT (E) cells grown in the absence (solid lines) or presence (dashed lines) of 100 μM BPS. Insets are the immunoblots of Sod2p from fraction 10 of these experiments. (E) The Sod2p of BPS-treated cells exhibits a ≈30–50% increase in manganese association that was reproducible over three experimental trials (see Supplementary Figure S2). Strains utilized: WT, BY4741; *mtm1*Δ, MY019; *mtm1*Δ *sod2*Δ, MY020; *aft1*Δ, 4438; *mtm1*Δ *aft1*Δ, MY030.

detection by anionic exchange. Furthermore, the labile 4Fe–4S cluster of aconitase may be disrupted during the aerobic processing of mitochondria. In any case, the anionic iron peak of *mtm1* mutants clearly represents Sod2p, as this peak is absent in *mtm1*Δ *sod2*Δ double mutants lacking Sod2p (Figure 1B).

Iron inactivation of Sod2p

To test whether iron inactivates Sod2p in *mtm1*Δ mutants, we sought to reduce mitochondrial iron levels through mutations in *AFT1,* encoding an iron-sensing transcription factor (Yamaguchi-Iwai *et al*, 1995). A number of iron transporters rely on Aft1p for expression and *aft1*Δ mutants of yeast accumulate low iron (Yamaguchi-Iwai *et al*, 1996; Yun *et al*, 2000; Rutherford *et al*, 2003). As seen in Figure 2A top, the high mitochondrial iron of *mtm1*Δ cells was not observed in an *mtm1*Δ *aft1*Δ double mutant. Effects on Sod2p activity were monitored by the native gel assay. In these assays, active Sod2p often migrates as two distinct bands (for description, see legend to Figure 2). Mutants of *mtm1* exhibit low Sod2p activity (Figure 2A, lane 3) and this was reversed in the double *mtm1*Δ *aft1*Δ mutant (lane 4). These results indicate that mitochondrial iron of *mtm1* mutants inactivates Sod2p.

To further monitor the effects of mitochondrial iron, we used the impermeable iron chelator, bathophenanthrolinedisulfonate (BPS). As seen in Figure 2B top, the level of mitochondrial iron in *mtm1* mutants was reduced in accordance with increasing concentrations of BPS in the growth medium. There was no toxicity to the cell up to 100 μM BPS (not shown) and BPS did not greatly alter mitochondrial levels of manganese (Figure 2C). In the native gel assay, Sod2p activity in *mtm1* mutants increased as mitochondrial iron levels dropped (Figure 2B, lanes 3–6). By analysis of mitochondrial anionic fractions, manganese, but not iron, was seen to associate with Sod2p when *mtm1* mutants were treated with BPS (Figure 2D). It is important to note that this manganese binding is not due to an exchange of metals in pre-existing Fe–Sod2p, but rather reflects manganese insertion into newly synthesized Sod2p molecules. Metal insertion

©2006 European Molecular Biology Organization

only occurs with newly synthesized Sod2p molecules that are freshly imported into mitochondria (Luk *et al*, 2005).

Lowering of mitochondrial iron by BPS not only rescued Sod2p in *mtm1* mutants but also slightly improved Sod2p activity in WT cells. WT cells consistently exhibited an ≈30–50% increase in manganese association with Sod2p during BPS treatment (Figure 2E, also see Supplementary Figure S2). These results indicate that a small fraction of Sod2p associates with iron even in WT cells.

The mitochondrial carriers Mrs3/Mrs4p contribute to iron inactivation of Sod2p

We explored the mitochondrial factors that may contribute to iron inactivation of Sod2p in *mtm1* mutants. Mrs3p and Mrs4p are transporters of the mitochondrial carrier family that localize to the mitochondrial inner membrane (Wiesenberger *et al*, 1991). Although the precise substrate of transport by Mrs3p/Mrs4p has not been clarified, these transporters contribute to mitochondrial iron levels (Foury and Roganti, 2002; Muhlenhoff *et al*, 2003b; Li and Kaplan, 2004) and facilitate synthesis of iron sulfur clusters and heme (Muhlenhoff *et al*, 2003b; Zhang *et al*, 2005). As seen in Figure 3A, a double *mrs3 mrs4* deletion resulted in a partial lowering of mitochondrial iron in *mtm1* mutants. In spite of this seemingly small effect on iron, Sod2p activity increased (Figure 3A, lane 3; also see Supplementary, Figure S1). Mitochondrial iron is also controlled by yeast *MMT1* and *MMT2*, encoding members of the cation diffusion facilitator family that localize to the mitochondria (Li and Kaplan, 1997; Jensen *et al*, 2004). Compared to the effects of *mrs3*Δ *mrs4*Δ mutations, there was no restoration of Sod2p activity with *mmt1*Δ *mmt2*Δ mutations (Figure 3B, lane 3; also see Supplementary, Figure S1). The iron relevant to Mrs3/4p seems more bioavailable to inactivate Sod2p.

Effects of mitochondrial iron overload on Sod2p activity

Do elevations in mitochondrial iron always inactivate Sod2p or is this a special case of *mtm1* mutants? A number of yeast mutants have been identified that accumulate high mitochondrial iron and many of these are defective in Fe/S cluster biogenesis. *SSQ1* and *GRX5* encode a molecular chaperone and glutaredoxin, respectively, that are required to assemble Fe/S clusters on target proteins (Knight *et al*, 1998; Rodriguez-Manzaneque *et al*, 2002; Muhlenhoff *et al*, 2003a). In the experiment of Figure 3C, *ssq1* and *grx5* mutants accumulated elevated levels of mitochondrial iron and also exhibited an impairment in Sod2p activity (Figure 3C). Sod2p activity was restored by BPS treatment (Figure 3D, also see Supplementary Figure S1), demonstrating a role for iron in Sod2p inactivation. Therefore, iron inactivation of Sod2p is not unique to *mtm1* mutants and can be observed with other defects in mitochondrial iron homeostasis.

However, we observed two instances where elevated mitochondrial iron did not correlate with Sod2p inactivation. First, culturing WT yeast cells in the presence of high extracellular iron effectively increased mitochondrial iron, yet Sod2p activity was normal (Figure 4A, lane 2). The enzyme only associated with manganese in iron-treated WT cells (Figure 4C). We also tested the effects of mitochondrial iron

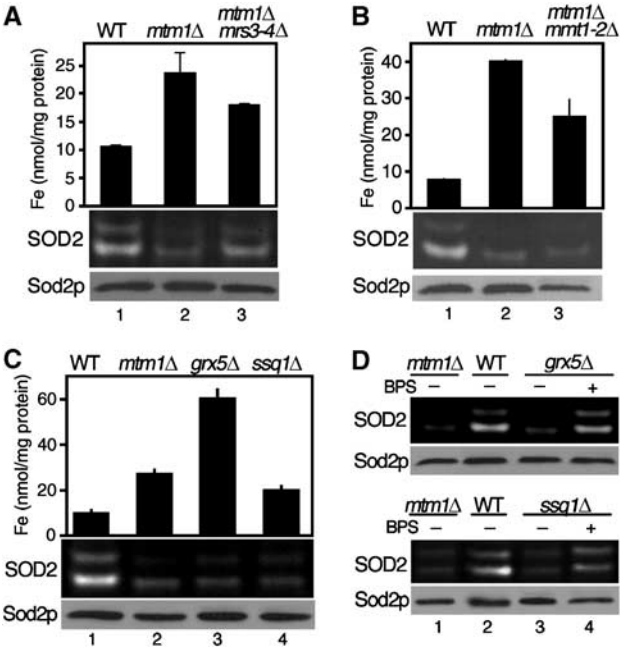


Figure 3 Mitochondrial proteins that contribute to iron inactivation of Sod2p. The indicated yeast strains were tested for mitochondrial iron and for Sod2p activity and polypeptide levels as described in Figure 2A. Analysis of the various experimental trials can be found in Supplementary Figure S1. Where indicated in (D), cells were cultured in the presence of 80 μM BPS. Yeast strains utilized—(**A**, **C**, **D**): WT, BY4741; *mtm1*Δ, MY019; *mtm1*Δ *mrs3-4*Δ, the triple *mtm1*Δ *mrs3*Δ *mrs4*Δ strain VC111; *ssq1*Δ, 5278; *grx5*Δ, 2769; (**B**): WT, 1783; *mtm1*Δ*mmt1-2*Δ, the triple *mtm1*Δ *mmt1*Δ *mmt2*Δ strain VC112.

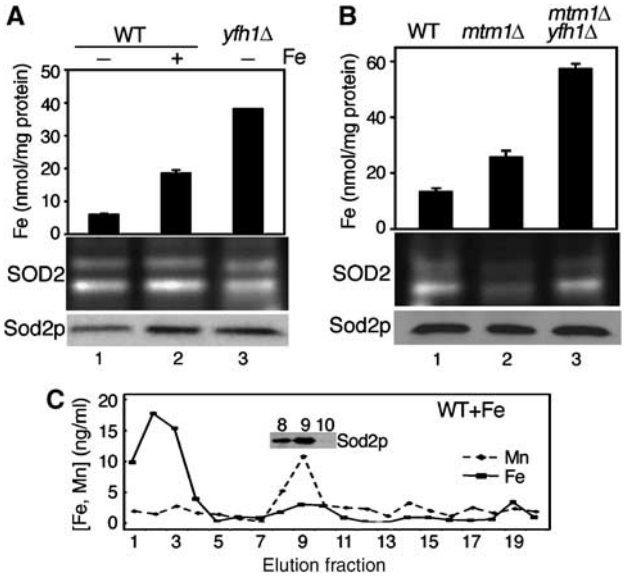


Figure 4 Sod2p activity in WT and *yfh1*Δ strains is not inhibited by mitochondrial iron. (**A**, **B**) The indicated yeast strains were analyzed for Sod2p activity and protein levels and for mitochondrial iron as in Figure 2. Where indicated (Fe: +), cells were cultured in the presence of 1.0 mM ammonium iron (III) citrate. Analysis of the various experimental trials can be found in Supplementary Figure S1. (**C**) Mitochondrial anion exchange fractions from WT cells cultured in 100 μM iron (II) chloride (which effects an ∼5-fold increase in total mitochondrial iron; not shown) were analyzed for iron (solid lines) and manganese (dashed lines) as in Figure 1. Inset is immunoblot analysis of fractions 8–10 showing elution of Sod2p in fractions 8–9 of this experiment. Strains used: WT, BY4741; *yfh1*Δ, MY036; *mtm1*Δ, MY019; *mtm1*Δ *yfh1*Δ, MY038.

 ©2006 European Molecular Biology Organization

overload in *yfh1* mutants. *YFH1* encodes the iron-binding frataxin homologue defective in Friedreich's ataxia (Babcock *et al*, 1997; Foury and Cazzalini, 1997). In *S. cerevisiae,* loss of *yfh1* has been associated with high mitochondrial iron and defects in Fe/S cluster biogenesis (Babcock *et al*, 1997; Foury and Cazzalini, 1997; Chen *et al*, 2002; Muhlenhoff *et al*, 2002, 2003a). We observed that in spite of high mitochondrial iron, *yfh1*Δ mutants exhibit normal Sod2p activity (Figure 4B, lane 3). The differential Sod2p activity results obtained with *yfh1*Δ versus *ssq1*Δ and *grx5*Δ mutants seemed surprising since all three have been implicated in Fe/S cluster biogenesis. As one possibility, Yfh1p itself may participate in iron inactivation of Sod2p. To test this, we created an *mtm1*Δ *yfh1*Δ double mutant. As seen in Figure 4C lane 3, Sod2p activity was normal in this mutant in spite of the very high levels of mitochondrial iron (also see Supplementary Figure S1). Yfh1p contributes to iron inactivation of Sod2p in *mtm1* mutants.

Effects of mtm1 mutations on iron homeostasis

Yeast mutants defective in Fe/S cluster biogenesis (e.g., *yfh1, ssq1, grx5*) are known to activate Aft1p, the iron regulatory transcription factor (Foury and Talibi, 2001; Belli *et al*, 2004; Chen *et al*, 2004; Rutherford *et al*, 2005). Since *mtm1* mutations phenocopy *grx5* and *ssq1* mutants with regard to mitochondrial iron and Sod2p, we addressed whether *mtm1* mutations likewise induce Aft1p and block Fe/S cluster synthesis.

To test for Aft1p activation, an *FET3-lacZ* reporter was used (Jensen and Culotta, 2002). In WT cells, *FET3-lacZ* was induced by Aft1p during iron starvation with BPS, and repressed somewhat by iron supplements (Figure 5A). Yet in *mtm1* mutants, *FET3-lacZ* was expressed at high levels even with iron supplements, and BPS treatment only moderately increased expression (Figure 5A). Aft1p appears constitutively active in *mtm1*Δ cells.

We next addressed whether Mtm1p is needed for Fe/S cluster synthesis. As with mutants known to affect Fe/S biogenesis (e.g., *yfh1, ssq1* and *grx5* (Lill and Kispal, 2000)), *mtm1*Δ cells accumulate mitochondrial DNA mutations (Luk *et al*, 2003) which can potentially interfere with Fe/S enzyme activities (Kaut *et al*, 2000). As such, an Mtm1p depletion study was conducted in which *MTM1* under the *GAL1-10* promoter was repressed by glucose, and immediate effects on mitochondrial iron and Fe/S proteins was monitored. This identical *GAL1* depletion approach has been used to study the roles of various mitochondrial ISC (iron sulfur cluster) components in Fe/S cluster biogenesis (Muhlenhoff *et al*, 2003a). In the case of Mtm1p, *GAL-MTM1* cells exhibited a substantial rise in mitochondrial iron following 4 days of Mtm1p depletion by growth in glucose (Figure 5B). This effect on mitochondrial iron is similar to what is seen with null mutations in *mtm1* (Figure 2A and B). However, unlike *mtm1*Δ cells, the Mtm1p-depleted cells did not loose mitochondrial DNA over this time frame (not shown). In spite of the rise in mitochondrial iron, Mtm1p-depeleted cells exhibited no major defect in Fe/S proteins. Aco1p and Leu1p are mitochondrial and cytosolic 4Fe–4S proteins and the activity of these enzymes remained normal in Mtm1p-depleted cells (Figure 5C). The Fe/S succinate dehydrogenase (SDH) also remained enzymatically active with Mtm1p depletion (Figure 5C). We additionally monitored the *de novo* assembly of Aco1p and Leu1p by an ^{55}Fe radiolabeling assay (Kispal *et al*, 1999). The rate of ^{55}Fe incorporation into the apoproteins was only slightly affected by Mtm1p depletion (Figure 5D). By comparison, the analogous depletions of Yfh1p, Ssq1p, Grx5p (and other members of the Fe/S cluster machinery) are known to dramatically inhibit ($\geqslant$4-fold) ^{55}Fe labeling of Fe/S proteins as well as activities of

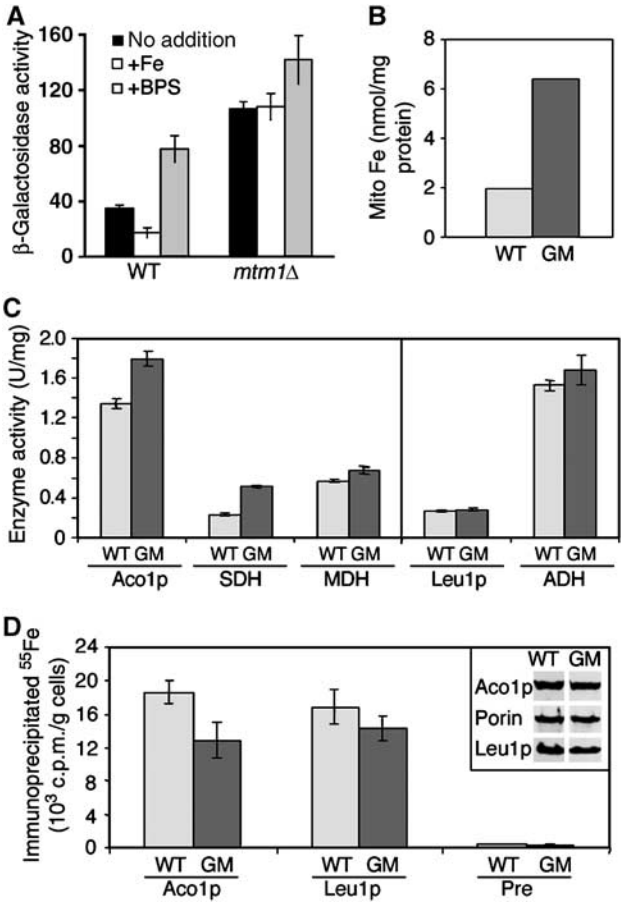


Figure 5 Aft1p activity and Fe/S cluster assembly in *mtm1* mutants. (**A**) The WT BY4741 or *mtm1*Δ mutant MY019 were transformed with the *FET3-lacZ* reporter plasmid pAR1 and were cultured in LF-SD medium that was supplemented where indicated with 1.6 mM ammonium iron (III) citrate ('+Fe') or 100 μM of the iron chelator BPS ('+BPS'). *β*-Galactosidase activity was measured as described in Materials and methods. Results represent the averages of four readings obtained from cultures of two independent single colonies, and error bars represent standard deviation. (**B–D**) The WT (strain W303) and Gal-*MTM1* (GM) cells were cultured for 4 days in glucose-containing medium to deplete Mtm1p. (B) The non-heme, non-Fe/S iron content of isolated mitochondria was measured by the bathophenantroline method (Li *et al*, 1999). (C) Mitochondria were isolated and analyzed for the enzyme activities of the Fe/S proteins aconitase (Aco1p) and succinate dehydrogenase (SDH) as well as the non-Fe/S protein malate dehydrogenase (MDH) as a control. The activity of the cytosolic Fe/S protein isopropylmalate isomerase (Leu1p) and the control enzyme alcohol dehydrogenase (ADH) was measured in total cell lysates. (D) Cells were cultured in LF-SD medium supplemented with glucose for 16 h prior to radiolabeling with ^{55}Fe for 2 h. Whole-cell extracts were used for immunoprecipitation with specific antibodies against Aco1p and Leu1p as well as with a preimmune serum (Pre). The amount of ^{55}Fe that was co-precipitated was quantified by liquid scintillation counting. The inset shows the amounts of Aco1p and Leu1p and as a control, porin detected in WT and Gal-*MTM1* cells by immunostaining of the corresponding cell extracts.

©2006 European Molecular Biology Organization

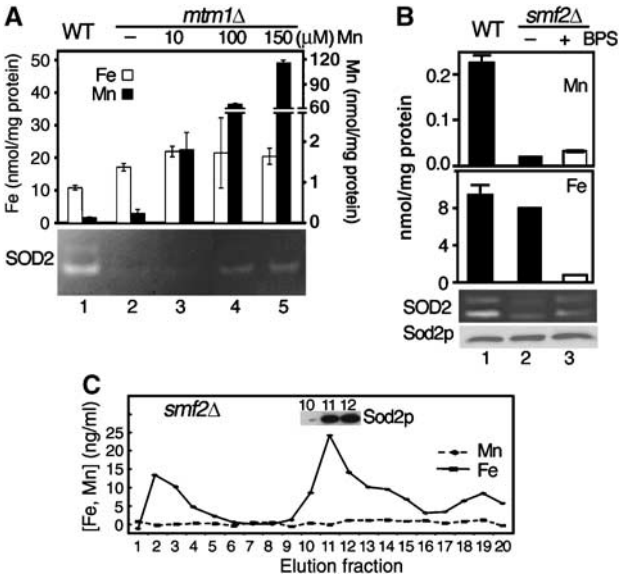


Figure 6 Competition between iron and manganese for binding to Sod2p *in vivo*. (**A, B**) The indicated yeast strains were grown in YPD medium supplemented where indicated with 10, 100 or 150 μM $MnCl_2$ (A) or 80 μM BPS (B). Mitochondrial iron and manganese contents and Sod2p activity (A, B) and Sod2 protein (B) were assayed as in Figure 2. Note the different scales for iron and manganese, including the broken scale for manganese in (A). Analysis of the various experimental trials can be found in Supplementary Figure S1. (C) Soluble mitochondrial components from *smf2*Δ mutant yeast were fractionated by anion exchange, and metal profiles determined (iron (solid) and manganese (dashed)) as in Figure 1. Inset is immunoblot of fractions 10–12 for Sod2p. Strains utilized: WT, BY4741; *mtm1*Δ, MY019; *smf2*Δ, 1878.

Fe/S enzymes (Kaut *et al*, 2000; Lange *et al*, 2000; Muhlenhoff *et al*, 2003a). If Mtm1p has any role in Fe/S biogenesis, this is minor in comparison to known factors for Fe/S biogenesis.

Mitochondrial manganese and iron inactivation of Sod2p

Our studies clearly show that Sod2p activity can be influenced by mitochondrial iron homeostasis. Can mitochondrial manganese counteract the effects of iron? We have previously shown that Sod2p activity of *mtm1* mutants can be recovered by supplementing the growth medium with manganese (Luk *et al*, 2003). However, the levels of manganese required were quite high (>100 μM) and approached toxic quantities (Luk *et al*, 2003). We now show that this translates to a ~200–400-fold increase in mitochondrial manganese with no change in mitochondrial iron (Figure 6A, lanes 4–5). In *mtm1* cells treated with just 10 μM manganese, mitochondrial manganese levels rise by nearly 10-fold; however, this is not sufficient to restore Sod2p activity (Figure 6A, lane 3 and Luk *et al*, 2003). The iron of *mtm1* mutants is remarkably bioavailable and competes effectively with mitochondrial manganese for binding to Sod2p.

We also explored the effects of manganese starvation on iron–Sod2p interactions. Manganese starvation can be achieved in yeast cells through a deletion in *SMF2*, encoding an Nramp transporter for manganese (Luk and Culotta, 2001). There is a cell wide manganese deficiency in *smf2* mutants, and mitochondrial manganese is very low (Figure 6B top) (Luk and Culotta, 2001). By comparison, *smf2*Δ cells exhibit no change in mitochondrial iron (Figure 6B). Sod2p has low activity in *smf2* mutants (Figure 6B, lane 2 and Luk and Culotta, 2001) and as expected, there is an absence of detectable manganese association with Sod2p (Figure 6C). However, rather than being apo (no metal bound to Sod2p), the Sod2p of these manganese-deficient cells was found to be associated with iron (Figure 6C). Iron binding to Sod2p inhibits the enzyme, because activity was increased upon lowering mitochondrial iron with BPS (Figure 6B, lane 3; also see Supplementary Figure S1). Hence, when manganese is low, Sod2p becomes vulnerable to iron inactivation. Even in the absence of an iron homeostasis defect, there exists a pool of mitochondrial iron that is capable of inactivating Sod2p.

Discussion

Using bakers yeast as a model system, we provide evidence that eukaryotic manganese SOD2 can associate with iron *in vivo*. Iron binding precludes insertion of the correct manganese ion cofactor and destroys SOD2 enzymatic activity. Iron inactivation of yeast Sod2p can be promoted when mitochondrial iron homeostasis is disrupted or when cells are starved for manganese.

This is the first report of eukaryotic SOD2 inactivation by mitochondrial iron. While iron inactivation of SOD2 may be rare, iron commonly inhibits bacterial MnSOD *in vivo* (Beyer and Fridovich, 1991; Privalle and Fridovich, 1992; Mizuno *et al*, 2004). Bacteria generally express a second, non-manganese SOD (i.e., FeSOD) in the same compartment, but eukaryotic SOD2 is the *only* SOD of the mitochondrial matrix. SOD2 is an extremely important antioxidant enzyme and its loss can lead to neonatal lethality (Li *et al*, 1995; Lebovitz *et al*, 1996) and reduced lifespan (Duttaroy *et al*, 2003), making it critical to prevent iron interactions with the enzyme.

Some, but not all, cases of mitochondrial iron overload lead to Sod2p inactivation in yeast. Our studies have differentiated mitochondrial iron into two classes in terms of bioavailability to Sod2p. A portion of the mitochondrial iron in *mtm1*, *grx5* and *ssq1* yeast mutants is bioavailable to yeast Sod2p ('SOD2-reactive'), but mitochondrial iron in WT cells and in *yfh1* frataxin mutants is largely 'SOD2-inert'. Grx5p, Ssq1p and Yfh1p all participate in a sequential pathway of Fe/S cluster assembly (Lill and Muhlenhoff, 2005; Rouault and Tong, 2005). Yfh1p acts upstream to donate iron to Fe/S scaffolds, while Ssq1p and Grx5p act downstream to transfer the clusters from these scaffolds to Fe/S proteins (Muhlenhoff *et al*, 2003a; Lill and Muhlenhoff, 2005). The frataxin-derived iron that is normally employed for Fe/S proteins may be shunted towards Sod2p when downstream components of Fe/S cluster assembly are blocked. In fact, our studies implicate a role for frataxin in iron inactivation of Sod2p, since the *mtm1* defect in Sod2p is not seen in a double *mtm1 yfh1* mutant (Figure 4B). Frataxin may help to increase the bioavailability of iron not only for Fe/S clusters but inadvertently for Sod2p as well.

Frataxin works in an iron pathway also involving the mitochondrial carriers Mrs3p and Mrs4p (Muhlenhoff *et al*, 2003b; Lill and Muhlenhoff, 2005; Rouault and Tong, 2005; Zhang *et al*, 2005). We show here that these same two

 ©2006 European Molecular Biology Organization

carriers contribute to iron inactivation of Sod2p in *mtm1* mutants (Figure 3A). By comparison, the Mmt1p/Mmt2p diffusion cation transporters of the mitochondria do not affect Sod2p activity (Figure 3B). This differential effect of Mmt1p/Mmt2p versus Mrs3p/Mrs4p indicates that SOD2-inert and SOD2-reactive iron pools may utilize distinct mitochondrial transport systems. Alternatively, distinct mitochondrial localizations of Mmt1p/Mmt2p and Mrs3p/Mrs4p could account for the differential effects of these transporters. Currently, the precise nature of SOD2-reactive versus SOD2-inert iron is not clear, but may represent different oxidation states or ligand-binding properties of the metal.

SOD2-reactive iron is not unique to disruptions in mitochondrial iron homeostasis. A small fraction of Sod2p from *S. cerevisiae* is normally found iron-bound (Ravindranath and Fridovich, 1975), and treating WT cells with iron chelators increases manganese binding to Sod2p (Figure 2E). Normally, this small pool of SOD-reactive iron cannot compete well with mitochondrial manganese, but when mitochondrial manganese levels drop, the pool of reactive iron gains access to Sod2p (Figure 6B and C). These results also indicate that mitochondrial Sod2p cannot readily accumulate as an 'apo' enzyme totally devoid of metals, but prefers to exist in either the manganese or iron bound state.

Although Mtm1p was originally proposed to act in manganese activation of Sod2p (Luk *et al*, 2003), our more recent studies suggest either that Mtm1p has a primary function in mitochondrial iron metabolism or alternatively, *mtm1* mutations alter levels of a particular metabolite within the mitochondrial matrix that stabilizes iron in a bioavailable form. As a transport carrier for mitochondria, Mtm1p may exchange a solute that directly or indirectly affects iron homeostasis. We show here that cells respond to a loss in *mtm1* through activation of the iron regulator Aft1p. However, Aft1p activation by itself is not responsible for the high mitochondrial iron of *mtm1* mutants. Expression of a constitutively active *AFT1-1up* allele in a WT cell does not cause high mitochondrial iron (Babcock *et al*, 1997). We also find that *AFT1-1up* expression does not lead to a loss of Sod2p activity in WT cells (Supplementary Figure S3). Activation of Aft1p, as seen in *mtm1* mutants, has been a hallmark of mutants defective in Fe/S cluster biogenesis (Chen *et al*, 2004; Lesuisse *et al*, 2005; Rutherford *et al*, 2005). However, *mtm1* mutants exhibit no major loss of either mitochondrial or cytosolic Fe/S clusters (Figure 5). Therefore, in *mtm1* mutants, Aft1p is sensing a perturbation in mitochondrial iron homeostasis independent of the mitochondrial ISC assembly and export systems (Lill and Muhlenhoff, 2005).

How does SOD2 normally choose manganese over iron? SOD2 generally binds manganese, but with certain defects in mitochondrial iron homeostasis (e.g., *mtm1*, *ssq1* and *grx5* mutants), there is an expansion in the pool of SOD2-reactive iron. This iron is so reactive with yeast Sod2p that it takes a rise in mitochondrial manganese levels of >200-fold to restore Sod2p activity (Figure 6A). We therefore propose a model of 'differential metal bioavailability' for metal ion selectivity in SOD2. SOD2 normally binds manganese simply because available manganese levels out-compete the small amount of SOD2-reactive iron. Under conditions where SOD2-reactive iron increases or when mitochondrial manganese levels drop, SOD2 shifts to the Fe-bound state. If SOD2 does require a metallochaperone, this factor is expected to bind either iron or manganese depending on which ion is more bioavailable. Alternatively, separate chaperone-like molecules may be involved in delivery of iron versus manganese.

In human disorders of iron overload, it is believed that iron can cause oxidative stress to cells mainly through Fenton chemistry, but this study suggests a new aspect of iron toxicity: inactivation of an important mitochondrial antioxidant enzyme. Although iron may not be largely SOD2-reactive in cases of Friedreich's ataxia where frataxin is defective (Anderson *et al*, 2005; Seznec *et al*, 2005), other disorders of iron overload should be considered, for example, neuroferritinopathy and sideroblastic anemia (reviewed in Rouault, 2001; Napier *et al*, 2005). In this regard, it is noteworthy that SOD2 deficiency itself leads to a sideroblastic anemia-like disorder in mice (Friedman *et al*, 2004).

Materials and methods

Yeast strains, plasmids and culture conditions

Most of the yeast strains used in this study are isogenic to BY4741 (*MAT*a, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*, *his3*Δ*1*), including the *sod2*Δ::*kanMX4* (6605), *aft1*Δ::*kanMX4* (4438), *ssq1*Δ::*kanMX4* (5278), *grx5*Δ::*kanMX4* (2769) and *smf2*Δ::*kanMX4* (1878) mutants purchased from Research Genetics (Huntsville, AL). The double *mrs3*Δ::*kanMX4 mrs4*Δ::*kanMX4* mutant (781-1A) was a gift from Dr Andrew Dancis (University of Pennsylvania). Disruptions of *MTM1* in BY4741 and the corresponding *sod2*Δ, *aft1*Δ and *mrs3*Δ*mrs4*Δ strains were generated using the *mtm1*Δ::*LEU2* plasmid *pVC257*Δ (Luk *et al*, 2003), resulting in strains MY019 (*mtm1*Δ), MY020 (*mtm1*Δ *sod2*Δ), MY030 (*mtm1*Δ *aft1*Δ) and VC111 (*mtm1*Δ *mrs3*Δ *mrs4*Δ). Disruption of *YFH1* in BY4741 and in the *mtm1*Δ strain MY019, generating MY036 and MY038, was accomplished using the *yfh1*Δ::*URA3* plasmid pJS406 (Strain *et al*, 1998). The *yfh1*Δ *mtm1*Δ double mutant was cultured on iron-limited plates, prepared by mixing agarose (GIBCO) with iron-limited selecting minimal synthetic medium (Jensen and Culotta, 2002). The *mmt1*Δ *mmt2*Δ double mutant LJ224 was derived from parental strain 1783 (*MATa*, *leu2-3*, *112*, *ura3-52*, *trp1-1*, *his4* and *can1*^{r}) (Jensen *et al*, 2004). Disruptions of *MTM1* in 1783 and LJ224 were generated using the *mtm1*Δ::*TRP1* plasmid pVC257Δ-T, resulting in strains VC110 (from 1783) and VC112 (*mtm1*Δ *mmt1*Δ *mmt2*Δ). The pVC257Δ-T plasmid was created by mobilizing the *MTM1* containing disruption insert from pVC257 by *Bam*HI and *Sal*I digestion, and inserting the cassette into the integrating *TRP1* vector pRS404 digested with the same enzymes. To test for effects of active Aft1p expression, the *AFT1-1up* plasmid, AFT1-1up-313 (Jensen and Culotta, 2002), was used. Creation of the *Gal-MTM1* strain for depleting Mtm1p involved strain W303-1A (*MATa, ura3-1, ade2-1, trp1-1, his3-11,15* and *leu2-3,112*). The promoter of *MTM1* was exchanged for the galactose-inducible *GAL1* promoter by PCR-mediated DNA replacement (http://mips.gsf.de/proj/yeast/CYGD/db/index.html). In brief, PCR fragments carrying the *HIS3* gene and the *GAL1-10* promoter region of vector pTL26 flanked by region -230 to -180 and $+1$ to $+50$ of the *MTM1* gene were used to transform strain W303-1A. All gene replacements and gene deletions were verified by PCR.

Yeast cells were propagated at 30°C either in enriched yeast-peptone-based medium with 2% (w/v) glucose (YPD) or in an iron-limited selecting minimal synthetic medium (LF-SD) (Jensen and Culotta, 2002). *Gal-MTM1* cells were depleted of Mtm1p to physiologically critical levels by preincubating for 4 days on glucose-containing solid-rich medium.

Chromotographic mitochondrial fractionations

Purified mitochondria were prepared by loading crude mitochondria onto a discontinuous Nycodenz gradient (16% on 22%). Intact mitochondria were recovered from the gradient interface after centrifugation at 150 000 *g* for 1 h. The intact mitochondria were washed by dilution into isotonic buffer followed by centrifugation at 12 000 *g*. Mitochondria were lysed by three 30 s pulses of sonication

©2006 European Molecular Biology Organization

at 50% output of a microtip (Ultrasonic, W-380). The soluble fraction was separated from the insoluble fraction by centrifugation at 15 000 *g*. The soluble fraction was diluted into Buffer A (20 mM Tris–HCl pH 7.2) and filtered with a 0.45 μm syringe filter. The sample was then loaded onto a HR10/10 MonoQ column (Amersham Biosciences) equilibrated in 10 column volumes (CV) of Buffer A. The unbound proteins were washed from the column with the injection volume and an additional 5 CV of Buffer A and collected as fractions 1–4 (5 ml per fraction). A 25 CV gradient (0–100%) of Buffer B (20 mM Tris–HCl pH 7.2, 1 M NaCl) was then initiated. Fractions (1 ml) were collected for the gradient and all fractions were then analyzed by ICP-OES (Perkin Elmer, Optima 3100XL) versus buffer blanks after dilution in 10% Nitric acid. Concentrations were determined from a standard curve constructed with serial dilutions of commercially available mixed metal standards. Two metal mixes were used: one contained Ca, Cd, Co, Cu, Fe, Ni and Zn and a second mix contained Mg and Mn (Optima). Blanks of Nitric acid or buffer samples with and without 'metal-spikes' were analyzed to ensure reproducibility.

Analysis of Sod2p activity and polypeptide levels

Cultures (50 ml) of *S. cerevisiae* strains were typically grown without shaking at 30°C for 17–18 h to a final OD_{600} of 2–4.0 as described (Luk *et al*, 2003). In experiments using the slow-growing *yfh1*Δ *mtm1*Δ mutant, all cultures including the WT and *mtm1*Δ controls were grown to a final OD_{600} of 0.5–0.7. Cell lysates were prepared by glass bead homogenization (Luk and Culotta, 2001) and 60.0 μg of lysate protein was analyzed for both SOD activity by native gel electrophoresis and nitroblue tetrazolium staining (Luk *et al*, 2003) and for SDS–PAGE and immunoblotting as described (Luk and Culotta, 2001; Luk *et al*, 2003). To prevent SDS-mediated precipitation of Sod2p, samples for electrophoresis were not boiled.

Miscellaneous biochemical analyses

Atomic absorption analysis of mitochondrial metals employed crude mitochondria preparations obtained from 200 ml cultures according to published methods (Luk and Culotta, 2001). To monitor activation of Aft1p, yeast cells transformed with a *FET3-lacZ* reporter plasmid (Jensen and Culotta, 2002) were grown to an $A_{600\,nm}$ of ~1.0 in LF-SD medium supplemented as needed with 100 μM BPS or 1.6 mM ammonium iron (III) citrate. Cell extracts prepared by glass bead homogenization were then assayed for β-galactosidase as previously described (Jensen and Culotta, 2002). *In vivo* labeling of yeast cells with radioactive iron ($^{55}FeCl_3$) and measurement of ^{55}Fe incorporation into mitochondrial or cytosolic Fe/S proteins by immunoprecipitation and liquid scintillation counting was carried out as described previously (Kispal *et al*, 1999). The anti-Aco1p antibody was raised against purified preparations of Aco1 protein. For analysis of mitochondrial Fe/S enzyme activities, mitochondria were isolated as described (Daum *et al*, 1982) and enzyme activities of malate dehydrogenase, aconitase, alcohol dehydrogenase, isopropylmalate isomerase (Leu1p) (Kispal *et al*, 1997) and succinate dehydrogenase were carried out according to published procedures (Robinson *et al*, 1991; Robinson and Lemire, 1995). The standard error of the determination of enzyme activities was between 5 and 15%.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Andrew Dancis for the 781-1A strain, Matt Hall for critical review of this manuscript and Laran Jensen for the LJ224 strain and for assistance with atomic absorption spectrophotometry and *lacZ* reporter assays. This research was supported by the JHU NIEHS center and by NIH grants ES 08996 (awarded to VCC) and CA61286 (awarded to DRW) and by grants of the Deutsche Forschungsgemeinschaft (SFB 593 and Gottfried-Wilhelm Leibniz program) and Fonds der Chemischen Industrie (awarded to RL). MY is supported by NIH post-doctoral fellowship F32 GM 074402 and by NIEHS training grant ES 07141.

References

Anderson PR, Kirby K, Hilliker AJ, Phillips JP (2005) RNAi-mediated suppression of the mitochondrial iron chaperone, frataxin, in *Drosophila*. *Hum Mol Genet* **14:** 3397–3405

Babcock M, Silva Dd, Oaks R, Davis-Kaplan S, Jiralerspong S, Montermini L, Pandolfo M, Kaplan J (1997) Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. *Science* **276:** 1709–1712

Bartnikas TB, Gitlin JD (2001) How to make a metalloprotein. *Nat Struct Biol* **8:** 733–734

Belli G, Molina MM, Garcia-Martinez J, Perez-Ortin JE, Herrero E (2004) *Saccharomyces cerevisiae* glutaredoxin 5-deficient cells subjected to continuous oxidizing conditions are affected in the expression of specific sets of genes. *J Biol Chem* **279:** 12386–12395

Beyer WF, Fridovich I (1991) *In vivo* competition between iron and manganese for occupancy of the active site region of the manganese-superoxide dismutase of *Escherichia coli*. *J Biol Chem* **266:** 303–308

Borgstahl GE, Parge HE, Hickey MJ, Beyer Jr WF, Hallewell RA, Tainer JA (1992) The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* **71:** 107–118

Chen OS, Crisp RJ, Valachovic M, Bard M, Winge DR, Kaplan J (2004) Transcription of the yeast iron regulon does not respond directly to iron but rather to iron–sulfur cluster biosynthesis. *J Biol Chem* **279:** 29513–29518

Chen OS, Hemenway S, Kaplan J (2002) Inhibition of Fe–S cluster biosynthesis decreases mitochondrial iron export: evidence that Yfh1p affects Fe–S cluster synthesis. *Proc Natl Acad Sci USA* **99:** 12321–12326

Colpas GJ, Hausinger RP (2000) *In vivo* and *in vitro* kinetics of metal transfer by the *Klebsiella aerogenes* urease nickel metallochaperone, UreE. *J Biol Chem* **275:** 10731–10737

Daum G, Bohni PC, Schatz G (1982) Import of proteins into mitochondria. Cytochrome *b2* and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J Biol Chem* **257:** 13028–13033

Duttaroy A, Paul A, Kundu M, Belton A (2003) A Sod2 null mutation confers severely reduced adult life span in *Drosophila*. *Genetics* **165:** 2295–2299

Finney LA, O'Halloran TV (2003) Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science* **300:** 931–936

Foury F, Cazzalini O (1997) Deletion of the yeast homologue of the human gene associated with Friedreich's ataxia elicits iron accumulation in mitochondria. *FEBS Lett* **411:** 373–377

Foury F, Roganti T (2002) Deletion of the mitochondrial carrier genes *MRS3* and *MRS4* suppresses mitochondrial iron accumulation in a yeast frataxin-deficient strain. *J Biol Chem* **277:** 24475–24483

Foury F, Talibi D (2001) Mitochondrial control of iron homeostasis: a genome wide analysis of gene expression in a yeast frataxin deficient strain. *J Biol Chem* **276:** 7762–7768

Friedman JS, Lopez MF, Fleming MD, Rivera A, Martin FM, Welsh ML, Boyd A, Doctrow SR, Burakoff SJ (2004) SOD2-deficiency anemia: protein oxidation and altered protein expression reveal targets of damage, stress response, and antioxidant responsiveness. *Blood* **104:** 2565–2573

Jensen LT, Culotta VC (2002) Regulation of *S. cerevisiae FET4* gene expression by iron and oxygen. *J Mol Biol* **318:** 251–260

Jensen LT, Sanchez RJ, Srinivasan C, Valentine JS, Culotta VC (2004) Mutations in *Saccharomyces cerevisiae* iron–sulfur cluster assembly genes and oxidative stress relevant to Cu, Zn superoxide dismutase. *J Biol Chem* **279:** 29938–29943

Kaut A, Lange H, Diekert K, Kispal G, Lill R (2000) Isa1p is a component of the mitochondrial machinery for maturation of cellular iron–sulfur proteins and requires conserves cysteine residues for function. *J Biol Chem* **275:** 15955–15961

Kispal G, Csere P, Guiard B, Lill R (1997) The ABC transporter Atm1p is required for mitochondrial iron homeostasis. *FEBS Lett* **418:** 346–350

 ©2006 European Molecular Biology Organization

Kispal G, Csere P, Prohl C, Lill R (1999) The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. *EMBO J* **18:** 3981–3989

Knight SAB, Sepuri NBV, Pain D, Dancis A (1998) Mt-Hsp70 Homolog, Ssc2p, required for maturation of yeast frataxin and mitochondrial iron homeostasis. *J Biol Chem* **273:** 18389–18393

Lange H, Kaut A, Kispal G, Lill R (2000) A mitochondrial ferredoxin is essential for biogenesis of cellular iron–sulfur proteins. *Proc Natl Acad Sci USA* **97:** 1050–1055

Lebovitz RM, Zhang H, Vogel H, Cartwright Jr J, Dionne L, Lu N, Huang S, Matzuk MM (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc Natl Acad Sci USA* **93:** 9782–9787

Lesuisse E, Knight SA, Courel M, Santos R, Camadro JM, Dancis A (2005) Genome-wide screen for genes with effects on distinct iron uptake activities in *Saccharomyces cerevisiae*. *Genetics* **169:** 107–122

Li J, Kogan M, Knight SA, Pain D, Dancis A (1999) Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution. *J Biol Chem* **274:** 33025–33034

Li L, Kaplan J (1997) Characterization of two homologous yeast genes that encode mitochondrial iron transporters. *J Biol Chem* **272:** 28485–28493

Li L, Kaplan J (2004) A mitochondrial–vacuolar signaling pathway in yeast that affects iron and copper metabolism. *J Biol Chem* **279:** 33653–33661

Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, Wallace DC, Epstein CJ (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismtuase. *Nat Genet* **11:** 376–381

Lill R, Kispal G (2000) Maturation of cellular Fe–S proteins: an essential function of mitochondria. *Trends Biochem Sci* **25:** 352–356

Lill R, Muhlenhoff U (2005) Iron–sulfur–protein biogenesis in eukaryotes. *Trends Biochem Sci* **30:** 133–141

Luk E, Carroll M, Baker M, Culotta VC (2003) Manganese activation of superoxide dismutase 2 in *Saccharomyces cerevisiae* requires *MTM1*, a member of the mitochondrial carrier family. *Proc Natl Acad Sci USA* **100:** 10353–10357

Luk E, Culotta VC (2001) Manganese superoxide dismutase in *S. cerevisiae* acquires its metal co-factor through a pathway involving the Nramp metal transproter, Smf2p. *J Biol Chem* **276:** 47556–47562

Luk E, Yang M, Jensen LT, Bourbonnais Y, Culotta VC (2005) Manganese activation of superoxide dismutase 2 in the mitochondria of *Saccharomyces cerevisiae*. *J Biol Chem* **280:** 22715–22720

Mizuno K, Whittaker MM, Bachinger HP, Whittaker JW (2004) Calorimetric studies on the tight-binding metal interactions of *Escherichia coli* manganese superoxide dismutase. *J Biol Chem* **279:** 27339–27344

Muhlenhoff U, Gerber J, Richhardt N, Lill R (2003a) Components involved in assembly and dislocation of iron–sulfur clusters on the scaffold protein Isu1p. *EMBO J* **22:** 4815–4825

Muhlenhoff U, Richhardt N, Ristow M, Kispal G, Lill R (2002) The yeast frataxin homolog Yfh1p plays a specific role in the maturation of cellular Fe/S proteins. *Hum Mol Genet* **11:** 2025–2036

Muhlenhoff U, Stadler JA, Richhardt N, Seubert A, Eickhorst T, Schweyen RJ, Lill R, Wiesenberger G (2003b) A specific role of the yeast mitochondrial carriers MRS3/4p in mitochondrial iron acquisition under iron-limiting conditions. *J Biol Chem* **278:** 40612–40620

Napier I, Ponka P, Richardson DR (2005) Iron trafficking in the mitochondrion: novel pathways revealed by disease. *Blood* **105:** 1867–1874

O'Halloran TV, Culotta VC (2000) Metallochaperones: an intracellular shuttle service for metal ions. *J Biol Chem* **275:** 25057–25060

Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **292:** 2488–2492

Privalle CT, Fridovich I (1992) Transcriptional and maturation effects of manganese and iron on the biosynthesis of manganese-superoxide dismutase in *Escherichia coli*. *J Biol Chem* **267:** 9140–9145

Pufahl R, Singer C, Peariso KL, Lin SJ, Schmidt P, Fahrni C, Culotta VC, Penner-Hahn JE, O'Halloran TV (1997) Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278:** 853–856

Rae TD, Schmidt PJ, Pufhal RA, Culotta VC, O'Halloran TV (1999) Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* **284:** 805–808

Ravindranath SD, Fridovich I (1975) Isolation and characterization of a manganese-containing superoxide dismutase from yeast. *J Biol Chem* **250:** 6107–6112

Robinson KM, Lemire BD (1995) Flavinylation of succinate: ubiquinone oxidoreductase from *Saccharomyces cerevisiae*. *Methods Enzymol* **260:** 34–51

Robinson KM, von Kieckebusch-Guck A, Lemire BD (1991) Isolation and characterization of a *Saccharomyces cerevisiae* mutant disrupted for the succinate dehydrogenase flavoprotein subunit. *J Biol Chem* **266:** 21347–21350

Rodriguez-Manzaneque MT, Tamarit J, Belli G, Ros J, Herrero E (2002) Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Mol Biol Cell* **13:** 1109–1121

Rouault TA (2001) Iron on the brain. *Nat Genet* **28:** 299–300

Rouault TA, Tong WH (2005) Iron–sulphur cluster biogenesis and mitochondrial iron homeostasis. *Nat Rev Mol Cell Biol* **6:** 345–351

Rutherford JC, Jaron S, Winge DR (2003) Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. *J Biol Chem* **278:** 27636–27643

Rutherford JC, Ojeda L, Balk J, Muhlenhoff U, Lill R, Winge DR (2005) Activation of the iron regulon by the yeast Aft1/Aft2 transcription factors depends on mitochondrial but not cytosolic iron–sulfur protein biogenesis. *J Biol Chem* **280:** 10135–10140

Seznec H, Simon D, Bouton C, Reutenauer L, Hertzog A, Golik P, Procaccio V, Patel M, Drapier JC, Koenig M, Puccio H (2005) Friedreich ataxia: the oxidative stress paradox. *Hum Mol Genet* **14:** 463–474

Strain J, Lorenz CR, Bode J, Smolen GA, Garland SA, Vickery LE, Culotta VC (1998) Suppressors of superoxide dismutase (SOD1) deficiency in *Saccharomyces cerevisiae*: identification of proteins predicted to mediate iron–sulfur cluster assembly. *J Biol Chem* **273:** 31138–31144

Vance CK, Miller AF (1998) A simple proposal that can explain the inactivity of metal-substituted superoxide dismutases. *J Am Chem Soc* **120:** 461–467

Whittaker JW (2003) The irony of manganese superoxide dismutase. *Biochem Soc Trans* **31:** 11318–11321

Wiesenberger G, Link TA, Ahsen U, Waldherr M, Schweyen RJ (1991) *MRS3* and *MRS4*, two suppressors of mtRNA splicing defects in yeast, are new members of the mitochondrial carrier family. *J Mol Biol* **217:** 23–37

Wintjens R, Noel C, May AC, Gerbod D, Dufernez F, Capron M, Viscogliosi E, Rooman M (2004) Specificity and phenetic relationships of iron- and manganese-containing superoxide dismutases on the basis of structure and sequence comparisons. *J Biol Chem* **279:** 9248–9254

Yamaguchi-Iwai Y, Dancis A, Klausner R (1995) AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *EMBO J* **14:** 1231–1239

Yamaguchi-Iwai Y, Stearman R, Dancis A, Klausner RD (1996) Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. *EMBO J* **15:** 3377–3384

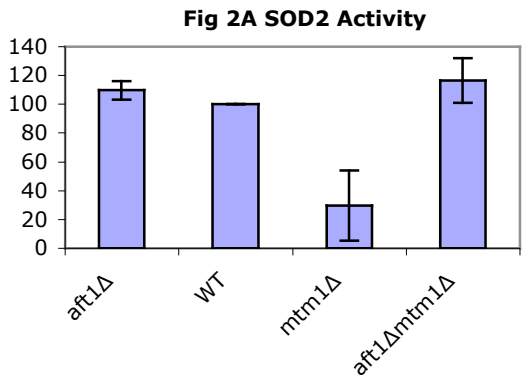
Yun CW, Ferea T, Rashford J, Ardon O, Brown PO, Botstein D, Kaplan J, Philpott CC (2000) Desferrioxamine-mediated iron uptake in *Saccharomyces cerevisiae*. Evidence for two pathways of iron uptake. *J Biol Chem* **275:** 10709–10715

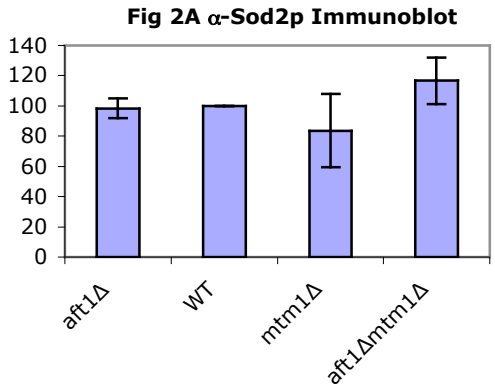
Zhang Y, Lyver ER, Knight SA, Lesuisse E, Dancis A (2005) Frataxin and mitochondrial carrier proteins, Mrs3p and Mrs4p, cooperate in providing iron for heme synthesis. *J Biol Chem* **280:** 19794–19807

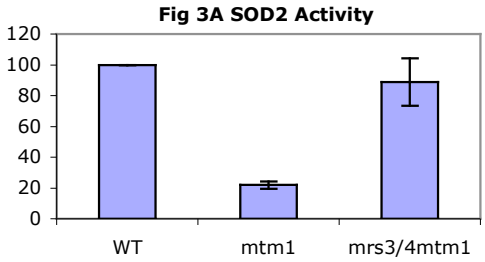
©2006 European Molecular Biology Organization

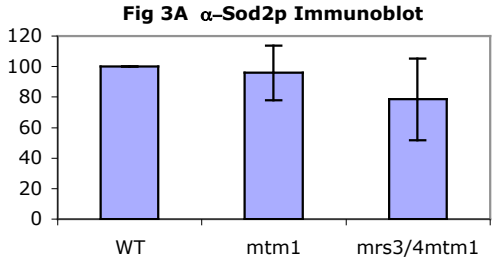
Supplementary Information

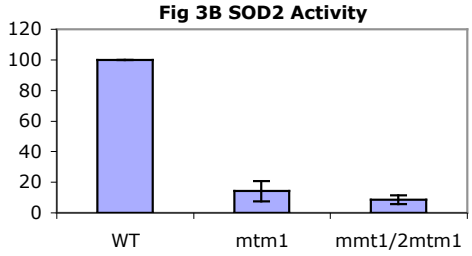
Fig S1. Densitometric tracings of *S. cerevisiae* Sod2p activity gels and α–Sod2p immunoblots. Both bands corresponding to Sod2p activity (nondenaturing gels) and the single band corresponding to the Sod2 polypeptide (immunoblots) were scanned by NIH IMAGE software. The integrated density of the bands was measured relative to the background and then normalized to the value of WT cells = 100. Shown are the averages of 2-4 independent experimental trials where error bars indicate range of values obtained.

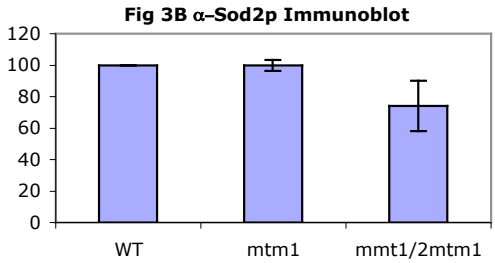












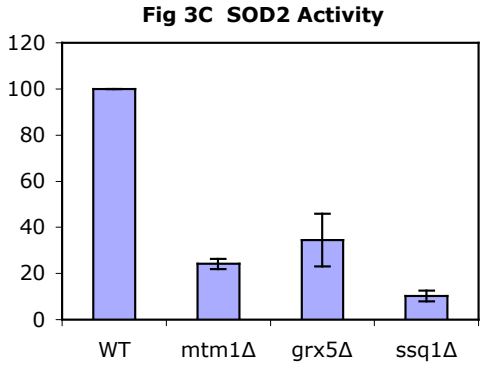


Fig 3C SOD2 Activity
120
100
80
60
40
20
0
WT
mtm1Δ
grx5Δ
ssq1Δ

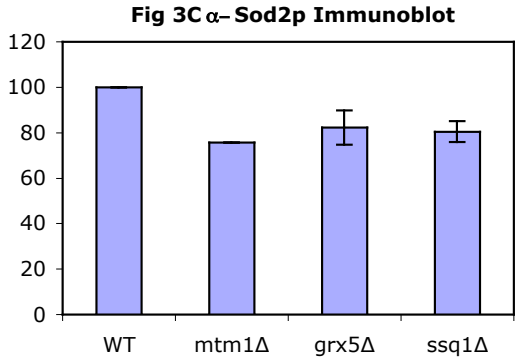


Fig 3C α– Sod2p Immunoblot
120
100
80
60
40
20
0
WT
mtm1Δ
grx5Δ
ssq1Δ

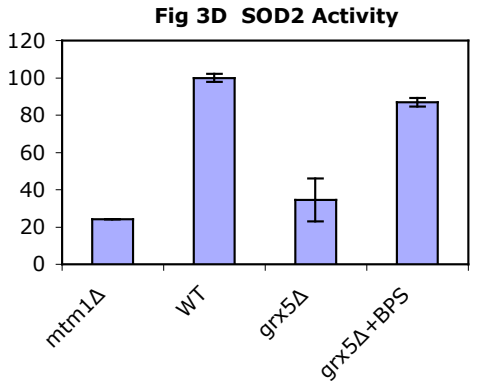


Fig 3D SOD2 Activity
120
100
80
60
40
20
0
mtm1Δ
WT
grx5Δ
grx5Δ+BPS

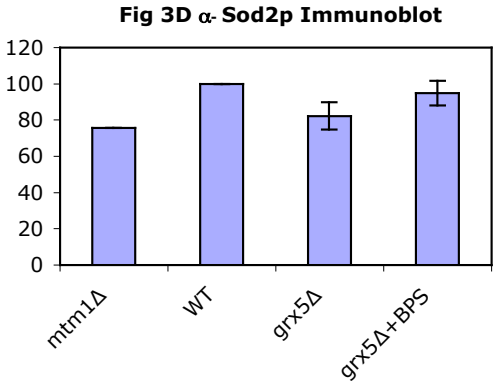


Fig 3D α- Sod2p Immunoblot
120
100
80
60
40
20
0
mtm1Δ
WT
grx5Δ
grx5Δ+BPS

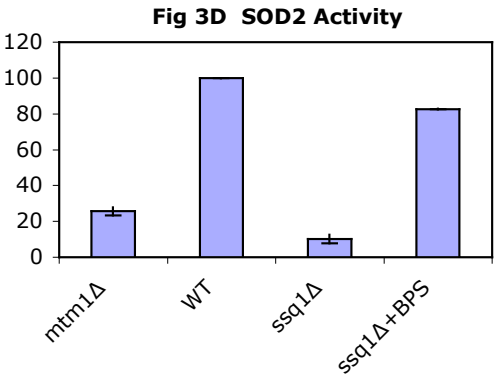


Fig 3D SOD2 Activity
120
100
80
60
40
20
0
mtm1Δ
WT
ssq1Δ
ssq1Δ+BPS

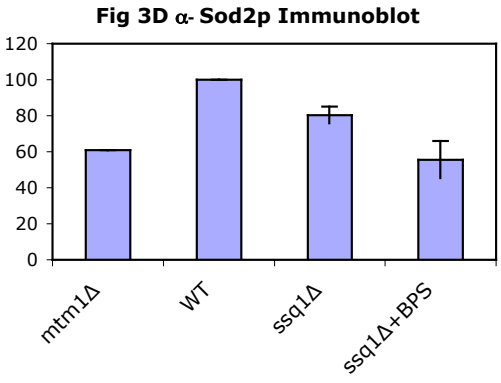


Fig 3D α- Sod2p Immunoblot
120
100
80
60
40
20
0
mtm1Δ
WT
ssq1Δ
ssq1Δ+BPS

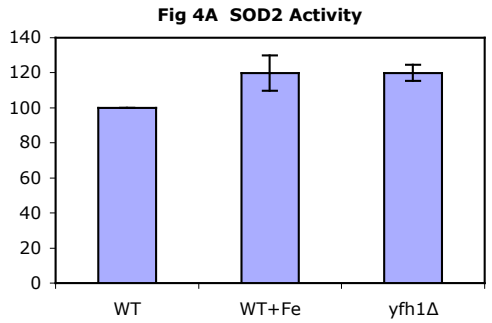


Fig 4A SOD2 Activity
140
120
100
80
60
40
20
0
WT
WT+Fe
yfh1Δ

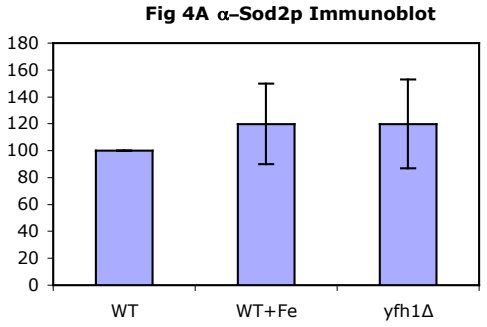


Fig 4A α–Sod2p Immunoblot
180
160
140
120
100
80
60
40
20
0
WT
WT+Fe
yfh1Δ

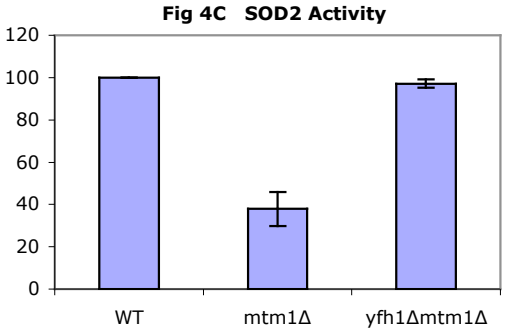


Fig 4C SOD2 Activity
120
100
80
60
40
20
0
WT
mtm1Δ
yfh1Δmtm1Δ

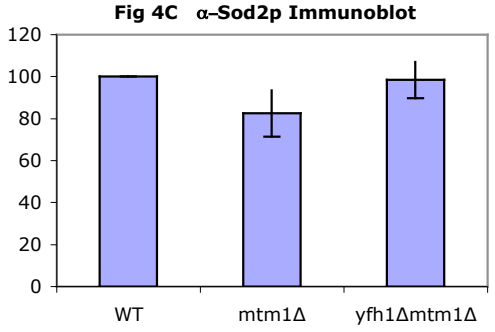


Fig 4C α–Sod2p Immunoblot
120
100
80
60
40
20
0
WT
mtm1Δ
yfh1Δmtm1Δ

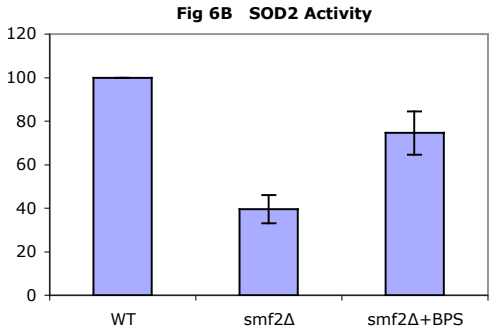


Fig 6B SOD2 Activity
120
100
80
60
40
20
0
WT
smf2Δ
smf2Δ+BPS

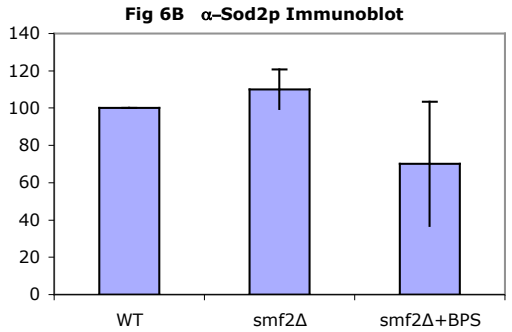


Fig 6B α–Sod2p Immunoblot
140
120
100
80
60
40
20
0
WT
smf2Δ
smf2Δ+BPS

Fig S2. Reproducible effects of BPS on manganese association with Sod2p in WT cells. Two additional trials of the experiment in Fig. 2E are shown. In the three trials, BPS treatment resulted in a 44% (Fig. 2E), 56% (A) and 33% (B) increase in manganese association with Sod2p.

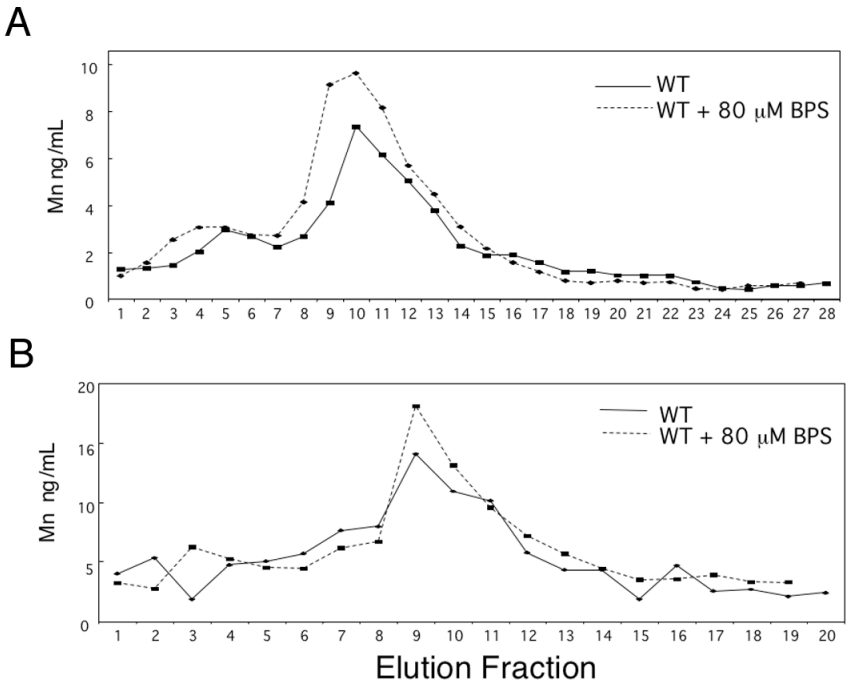
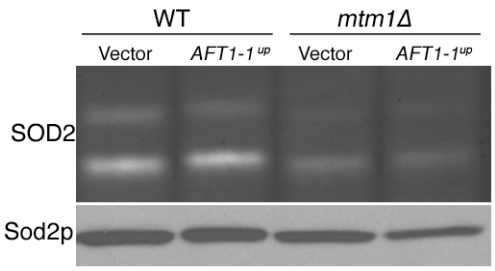



Fig S3. Expressing *AFT1-1^{up}* allele in wild type (WT) cells did not repress SOD2 activity. The BY4741 (WT) and the *mtm1Δ* mutant cells were transformed with the *AFT1-1^{up}* expressing plasmid as described in *Materials and Methods* or with the empty vector (pRS313). The indicated yeast strains were assayed for Sod2p enzymatic activity and Sod2p protein as in Fig 2.