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Insights into the iron-ome and manganese-ome of *mtm1* *Saccharomyces cerevisiae* Mitochondria

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Abstract

Biophysical spectroscopies and LC-ICP-MS were used to evaluate the iron-ome and manganese-ome of mitochondria from *mtm1* yeast cells. Deleting the mitochondrial carrier gene *MTM1* causes Fe to accumulate in mitochondria and Mn superoxide dismutase (SOD2) activity to decline. One explanation for this is that some accumulated Fe misincorporates into apo-Sod2p. Mössbauer spectroscopy revealed that most of the accumulated Fe was Fe^{III} nanoparticles which are unlikely to misincorporate into apo-Sod2p. Under anaerobic conditions, Fe did not accumulate yet SOD2 activity remained low, suggesting that the two phenomena are independent. Mn concentrations were two-fold higher in *mtm1* mitochondria than in WT mitochondria. Soluble extracts from such samples were subjected to size-exclusion LC and fractions were analyzed with an on-line ICP-MS. Two major Mn peaks were observed, one due to MnSod2p and the other to a Mn species with a mass of 2–3 kDa (called Mn₂₋₃). Mn₂₋₃ may deliver Mn into apo-Sod2p. Most Mn in WT mitochondria was associated with MnSod2p, whereas most Mn in *mtm1* mitochondria was associated with Mn₂₋₃. The [Mn₂₋₃] increased in cells grown on high MnCl₂ while the MnSod2p concentration remained unchanged. Corresponding Fe traces showed numerous peaks, including a complex of ~ 3 kDa which may be the form of Fe that misincorporates, and an Fe peak with the molecular mass of Sod2p that may correspond to FeSod2p. The intensity of this peak suggests that deleting *MTM1* probably diminishes SOD2 activity by some means other than Fe misincorporation. A portion of Sod2p in *mtm1* mitochondria might be unfolded or immature. Mtm1p may import a species required for apo-Sod2p maturation, activity or stability.

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[†]Electronic Supplementary Information (ESI) available: Fig. S1, Mössbauer spectrum of anaerobically grown WT mitochondria; Fig. S2, HMM chromatograms of mitochondrial soluble fractions from Experiment 1 of the first group of LC studies; Fig. S3, same as S2, but for Experiment 3 of the first group of LC studies; Fig. S4, SOD2 activity gel and Western blot images of isolated mitochondria and pooled fractions eluted from HMM LC studies; Table S1, a summary of Mössbauer simulation parameters; Table S2, a summary of EPR simulation results; Table S3, characterization of the mitochondrial soluble fractions used for the first group of LC-ICP-MS experiments; Table S4, peak analysis of HMM chromatograms; Table S5, peak analysis of LMM chromatograms.. See DOI: 10.1039/b000000x/

Introduction

The Mtm1p protein found in the budding yeast *Saccharomyces cerevisiae* is a member of the mitochondrial carrier family.¹⁻² Located in the mitochondrial inner membrane (IM), members of this family transport low-molecular-mass (LMM) species between the cytosol and the matrix of the organelle. The substrate transported by Mtm1p has not been identified and so the exact cellular function of the protein is unknown. Carriers can be organized according to whether their substrates are nucleotides, carboxylates, or amino acids, with Mtm1p in the last group.³ Substrates tend to be negatively charged and carriers often catalyze an exchange in which protons move in opposition to substrates.

Deleting the *MTM1* gene partially inactivates Mn superoxide dismutase (MnSod2p),^{1,4} suggesting that Mtm1p might be involved in Mn metabolism. During maturation, apo-Sod2p is sent to the mitochondrial matrix where Mn is installed during folding, suggesting that Mtm1p either imports Mn or is a chaperone for that installation. However, the Mn concentration within *mtm1* mitochondria is elevated relative to that of WT mitochondria,¹ discounting the possibility that Mtm1p imports Mn.

Attention shifted to Fe metabolism when Sod2p was found to be inactivated in association with an elevation of Fe in *mtm1* cells.^{1,4} Genetic and chromatographic results suggest that an *Apo-Sod2p-Reactive* (ASR) pool of Fe in the matrix (referred to as Fe_{ASR}) competes with Mn ions for installation into the active-site of apo-Sod2p. Indeed, Fe misincorporates into superoxide dismutases, a process that prevents the apo-enzyme from being activated by Mn.⁵ The size of the Mn pool in yeast mitochondria varies with the concentration of Mn in the growth medium.¹ The mitochondrial Fe concentration increases under Mtm1p-deficient conditions.

The extent of Fe misincorporation appears related to the Fe concentration in the growth medium, in that Fe-deficient conditions (created by adding the chelator bathophenanthroline disulfonate) prevent both Fe accumulation and loss of SOD2 activity in *mtm1* mitochondria.⁴ Misincorporation is also prevented by additionally deleting *MRS3/4*, the genes encoding high-affinity Fe transporters on the mitochondrial IM.⁴ This suggests that the Fe_{ASR} pool enters the mitochondria through Mrs3p/4p.

A single Mn species, associated with MnSod2p, was identified in fractions obtained by passing WT mitochondrial extracts through an anion-exchange column;⁴ no such species was observed with *mtm1* extracts. Replacing this peak in chromatograms of *mtm1* mitochondrial extracts was a dominating Fe peak that co-eluted with Sod2p. This peak, attributed to FeSod2p, was absent in extracts of a *mtm1 sod2* double-deletion mutant. This was the only Fe-containing peak evident in soluble *mtm1* extracts, and the peak was absent in WT extracts. This suggested that the majority of the soluble Fe in *mtm1* mitochondrial extracts that eluted through the column was misincorporated into apo-Sod2p.

The situation is actually more complicated in that a deficiency of Mtm1p is not unique in causing Fe to accumulate or misincorporate.⁴ Depleting the cell of Ssq1p, Grx5p, and Atm1p also causes Fe to accumulate in mitochondria and Sod2p to partially inactivate. These proteins are involved in mitochondrial Fe metabolism, but they have distinct

functions. Ssq1p is a soluble Hsp70-family chaperone that helps install nascent Fe/S clusters into recipient apo-Fe/S proteins.⁶ Atm1p is a mitochondrial IM ABC transporter that exports an unknown sulfur-containing species required for cytosolic Fe/S cluster assembly.⁷ Grx5p is a monothiol glutaredoxin that binds the scaffold proteins Isa1p/Isa2p.⁸ Based on these diverse functions, it would appear that neither apo-Sod2p-misincorporation nor Fe accumulation in mitochondria are *primary* effects of Mtm1p deficiency. Rather, they appear to be *secondary* effects arising in mitochondria lacking Mtm1p, Grx5p, Ssq1p, or Atm1p.

Another complexity is that Fe accumulation and misincorporation appear to be independent of each other. The absence of certain mitochondrial proteins, e.g. yeast frataxin homolog Yfh1p⁹ and the scaffold protein Isu1p, cause Fe accumulation but not misincorporation. Interestingly, the proteins which, when depleted in cells, cause Fe to accumulate but NOT misincorporate (e.g. Yfh1p and Isu1p) help *build* Fe/S clusters. In contrast, proteins which, when lacking, cause both Fe accumulation AND misincorporation (i.e. Atm1p, Grx5p, and Ssq1p) may help *transfer* assembled clusters into recipient apo-proteins.¹⁰ This correlation implies that Mtm1p functions in cluster transfer, not assembly. The misincorporated Fe has been proposed to originate from the Fe/S cluster donated by Isu1p. This is consistent with the increased level of Isu1p in *mtm1* cells relative to in WT cells^{10–11} in that the cell may compensate for a deficiency of Fe/S clusters by increasing Isu1p levels.

Another complexity in understanding the *mtm1* phenotype is that the *type* of Fe that accumulates in Atm1p-depleted mitochondria (and in *yfh1* and *Yah1p*-depleted mitochondria) is ferric oxyhydroxide (phosphate or polyphosphate) nanoparticles.^{12–14} This insoluble form of Fe seems unlikely to insert into apo-Sod2p; we would expect that soluble Fe^{II} ions insert. Also, the size of the Fe_{ASR} pool is unknown. It should enlarge in strains lacking Mtm1p, Atm1p, Grx5p, and Ssq1p, relative to in WT cells, but there is no certainty that the majority of the Fe that accumulates in the mitochondria of these strains is that particular pool of Fe.

To address this question, X-ray absorption spectroscopy (XAS) was performed on *mtm1* mitochondria as well as on mitochondria from a control mutant (*rho*[−]) that also accumulates Fe but exhibits normal SOD2 activity when the growth medium is supplemented with 2 mM Fe.¹⁰ No difference in the properties of the accumulated Fe, including oxidation state, geometry, or ligand environment, could be discerned. This suggests that the Fe_{ASR} pool that misincorporates into apo-Sod2p must be quite *small* – so small that it is undetectable by XAS.

Mtm1p has also been suggested to function in heme biosynthesis. One of the five mitochondrial proteins that coexpresses with other proteins involved in heme biosynthesis in zebrafish is an ortholog of yeast Mtm1p.¹¹ Silencing this gene prevented Fe incorporation into protoporphyrin IX. This suggests that this Mtm1p-like protein is involved *early* in heme biosynthesis, i.e. prior to insertion by ferrochelatase, perhaps by regulating *d*-aminolevulinate synthase, the committed step in heme biosynthesis.

Previously, we studied the iron-ome of mitochondria from WT and mutant strains related with mitochondrial Fe/S and heme biosynthesis.^{12,14–15} The iron-ome of fermenting WT

mitochondria is mainly composed of Fe/S clusters, heme centers, HS Fe^{II/III}, and Fe^{III} nanoparticles. However, mutant mitochondria commonly exhibit a dominant Fe^{III} nanoparticle feature, and are largely devoid of intact Fe/S clusters and heme centers. In this paper, we describe the iron-ome of mitochondria from *mtm1* yeast using biophysics and liquid chromatography (LC) in conjunction with an on-line inductively coupled plasma mass spectrometer (ICP-MS). Similarly to the previous studies, most of the accumulated Fe is present as Fe^{III} oxyhydroxide nanoparticles, which is unlikely to misincorporate into apo-Sod2p. LMM Mn and Fe species were observed in mitochondrial extracts that could potentially represent the sought-after pools. An Fe-containing protein comigrated approximately with MnSOD-containing fractions, but the relative level of this protein was similar in both WT and *mtm1* mitochondria. We conclude that deleting *MTM1* diminishes SOD2 activity in a manner that does not involve Fe misincorporation, and suggest alternative cellular functions for this protein.

Experimental Procedures

Yeast Strains and Media

The *mtm1* mutant strain used was derived from WT strain W303 (*MAT α* , *ura3-1*, *ade2-1*, *trp1-1*, *his3-11,15*, *leu2-3,112*). Gal-YAH1 is a derivative of W303 containing a galactose-inducible *YAH1* promoter.¹⁶ W303 and *mtm1* cells were grown on standard rich medium with 2% (w/v) glucose and 40 mg/L adenine hemisulfate dihydrate (YPAD) agar plates for 3–4 days. Gal-YAH1 cells were grown on standard rich medium with 2% (w/v) galactose and 40 mg/L adenine sulfate agar plates for 3–4 days. Generally a colony taken from the agar plate was inoculated in Fe-deficient minimal medium containing 2% (w/v) glucose, supplemented with 100 mg/L leucine, 40 mg/L adenine hemisulfate dihydrate, 20 mg/L uracil, 20 mg/L histidine, 50 mg/L tryptophan, and 1 μ M copper sulfate. ⁵⁷Fe-citrate was added to minimal medium at 20 μ M final concentration. For manganese supplementation, 200 μ M MnCl₂ was added to minimal medium (the [Mn] in unsupplemented minimal medium was ~ 2 μ M).

Cell Growth and Mitochondria Isolation

Cell stocks were prepared in 15% glycerol and frozen at –80 °C. As needed, frozen cells were scraped with a sterile wooded stick, and spread onto an agar plate. Single colonies were used to inoculate 50 mL of medium. Once grown, the cultures were used to inoculate 1 L of minimal medium (YPAD medium for *mtm1* cells). When OD₆₀₀ reached ~ 1, cultures were transferred into 24 L of minimal medium at 30 °C in a stirred glass fermenter. Solutions were purged with O₂ or Ar (~ 1 L/min) to achieve aerobic and anaerobic growth conditions, respectively. Cells were harvested at OD₆₀₀ ~ 1.

Mitochondria were isolated in a refrigerated Ar-atmosphere glovebox as described.¹² Solutions used under anaerobic conditions were degassed on a Schlenk line and then brought into the box. Isolated mitochondria were packed into EPR tubes or Mössbauer cups either at 10,000×g for 1 hr or at 18,000×g for 30 min in an ultracentrifuge with a swinging-bucket rotor. Samples were frozen in liquid N₂ for further analysis. A batch of isolated *mtm1* mitochondria was packed in Mössbauer cup, treated with 1% sodium deoxycholate and then

10 mM sodium dithionite prior to freezing. Mössbauer and EPR spectra were acquired as described.¹⁴

SOD Activity Assays

Cells were grown on 200 mL of minimal medium containing 20 μ M Fe-citrate. For 12 different batches, WT and *mtm1* cells were harvested at OD₆₀₀ between 0.6 – 1.8, matched to within 0.2 absorbance units for each pair of batches. Cells were harvested and lysed by glass-bead agitation.¹ For anaerobic growth, the medium was purged with Ar, and cells were harvested in the glovebox and processed under an Ar atmosphere. Cells were resuspended in lysis buffer containing 10 mM sodium phosphate (pH 7.8), 0.1% (v/v) Triton X-100, 5 mM EDTA, 5 mM EGTA, and 50 mM NaCl. An equivalent volume of 0.5 mm glass beads (Biospec Inc.; 0.5 mm diameter), 1 mM PMSF and 1% (v/v) protease inhibitor cocktail (P8340, Sigma) were added to cell suspensions. Cells were agitated by vortex action for 1 min and cooled on ice for 1 min; this procedure was repeated 6 more times. Lysate was clarified by centrifugation at 16,000 $\times$ g for 10 min at 4 °C. The supernatant was passed through a 0.45 μ m centrifuge tube filter (Millipore), and the filtrate was stored at 4 °C or in the glovebox.

The SOD activity gel assay was performed as described¹⁷ except that 60 μ g of whole-cell protein was loaded onto 12% Tris-HCl gels (Bio-Rad), and native gel electrophoresis was performed at 4 °C. The gel was soaked in a stain containing 50 mM potassium phosphate (pH 8.2), 0.16 mM nitro blue tetrazolium (NBT), 0.27 mM riboflavin, 1 mM sodium cyanide, 6.7 mM TEMED (*N,N,N',N'*-tetramethylethylenediamine), and was incubated in the dark for 1 hr at 4 °C. The yellowish gel was exposed to light from a 40 W white bulb for 10 ~ 15 min to develop a blue background with colorless SOD bands. Gels were imaged by a Fujifilm LAS-4000 mini imaging system. The density of each band corresponding to SOD2 activity was quantified by using NIH ImageJ software and normalized to aerobically grown W303 activity.

Sod2p solution activity was measured by monitoring the reduction of NBT by a photochemical flux of O₂⁻.¹⁸ The reaction mixture was prepared in 330 μ L of 50 mM potassium phosphate buffer at pH 7.8 containing 0.1 mM EDTA, by adding 10 μ L of 90 mM NaCN, 33 μ L of 1 mM NBT, 50 μ L of 0.1 M methionine, and 10 μ L of 0.16 mM riboflavin. Enzyme and water were added to a final volume of 1.0 mL. In the absence of enzyme, background formation of blue formazan was monitored at 550 nm after every 10 sec exposure to a 40 W white light bulb at the intensity required for the absorbance to increase at a rate of 0.05/min. One unit of SOD activity was defined by the amount of protein required to inhibit formazan formation by 50%.

Western Blots

Whole cell lysates and purified mitochondria were analyzed by Western blot. Lysate (60 μ g) and mitochondrial (20 μ g) proteins were resolved by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked with 1% casein and incubated with an antibody against Sod2p. Blot densities on membranes were quantified as above.

Oxyblot Assays

Mitochondrial extracts were solubilized with 12% (w/v) SDS. Solutions were reacted with 2,4-dinitrophenylhydrazine and DNP-derivatized proteins were separated by SDS-PAGE followed by Western blotting using the Oxyblot assay kit (Millipore). The primary antibody against DNP was used for visualization. The extent of oxidative damage was quantified densitometrically.

Other Enzyme Assays

Aconitase assays were performed essentially as described.¹² Isolated mitochondria were solubilized in a buffer consisting of 20 mM Tris (pH 7.4) and 0.5% (v/v) Triton-X 100. 20 μ L of solubilized mitochondria was mixed with 40 μ L of 0.1 M Tris (pH 7.4), 40 μ L of 0.5 M NaCl, and 80 μ L of deionized H₂O in a 1 mm path length quartz cuvette. 20 μ L of 10 mM sodium cis-aconitate (Sigma) was added to the mixture to initiate the reaction. The consumption of cis-aconitate was monitored by a decrease of OD₂₄₀. The reaction rate was calculated from the linear part of the slope assuming a molar extinction coefficient of 3.6 mM⁻¹ cm⁻¹.¹⁹ Activities were expressed as units per mg protein; 1 unit is defined as 1 nmol of aconitate converted per min.

Leu1p assays were performed as described.²⁰ Cells were grown on minimal medium with 30 mg/L leucine (to increase Leu1p expression level) and were harvested at OD₆₀₀ of 0.7 ~ 0.8. 50 mL of cell culture was centrifuged and the pellet was washed with ice-cold deionized water and resuspended in 0.3 mL of lysis buffer (20 mM KH₂PO₄ buffer, pH 7.2, 50 mM NaCl, 1 mM EDTA, 0.5 mM PMSF). Glass bead agitation was performed as above with 6 cycles of 30 sec-vortex and 30 sec-cooling on ice. Lysate was clarified by centrifugation at 10,000 $\times$ g for 5 min. 50 μ L of supernatant was immediately mixed with 50 μ L of 0.2 M KH₂PO₄ (pH 7.0) buffer, 395 μ L of deionized water, and 5 μ L of 0.1 M sodium citraconate (Sigma). The decrease in OD₂₃₅ was monitored for 3 min and the reaction rate was calculated from the initial slope assuming a molar extinction coefficient of 4.7 mM⁻¹ cm⁻¹.²¹ Activities are reported as units per mg protein, with 1 unit defined as 1 nmol of citraconate converted per min.

Succinate dehydrogenase (SDH) assays were performed essentially as described,²² but modified as follows. Isolated mitochondria were diluted to 7 mg/ml protein concentration with 0.6 M sorbitol and 20 mM HEPES, pH 7.4. The reaction mixture consisted of 100 mM Tris-HCl (pH 8.3), 0.5 mM EDTA, 2 mM KCN, 2 mM iodonitrotetrazolium chloride (INT) (Sigma), 12 g/L Cremophor EL, and 20 mM sodium succinate, previously adjusted at pH 8.3. The reaction was initiated by adding 10 μ L of mitochondrial suspension to 990 μ L of the reagent at 30 °C. Color development by INT-formazan was monitored at 500 nm for 6 min. The reaction rate was obtained from the linear part of the initial slope assuming a molar extinction coefficient of 19.3 mM⁻¹ cm⁻¹.²² Blank experiments were performed in the absence of sodium succinate and the resulting background rate was subtracted from the sample rates. Activities were determined as units per mg protein (1 unit is defined as 1 nmol of INT-formazan formed per min).

Protein and Iron Content Analysis

Whole cell lysate and mitochondrial protein concentrations were measured by the BCA protein assay kit (Pierce). Mitochondrial and whole cell metal concentrations were measured by ICP-MS (Agilent 7700x). Packed mitochondria and whole cells were diluted 2-fold with water. Then 50, 75 and 100 μL of the resulting suspensions were dissolved overnight in 200 μL of 30% trace-metal grade HNO_3 and 100 μL of 35% trace-metal grade HCl (Fisher Scientific). Reported protein concentrations in mitochondria and whole cells were acquired from packed mitochondria and cells, and adjusted using previously reported packing efficiencies of 0.70 for whole cells and 0.77 for mitochondria.²³

Spectroscopy

Mössbauer spectra were recorded on a Model MS4 WRC spectrometer (SEE Co., Edina, MN) and an 8 T spectrometer,¹⁴ calibrated using $\alpha\text{-Fe}$ foil as described.¹² EPR spectra were recorded on an X-band EMX spectrometer (Bruker Biospin, Billerica, MA) as described.¹⁴ Spectra were simulated with SpinCount and signal intensities were quantified relative to 1 mM CuEDTA spectrum obtained at 0.2 mW and 10 K. For UV-vis spectroscopy, isolated mitochondria were diluted 2-fold with buffer containing 0.6 M sorbitol, 20 mM HEPES-KOH (pH 7.4), and 1 mM EGTA. Spectra were collected using a 2 mm path length quartz cuvette (NSG Precision Cells, Inc.) on a Hitachi 3010 UV-vis spectrometer with a Head-On photomultiplier tube.

LC-ICP-MS Experiments

In the first group of experiments, isolated mitochondria were resuspended in 10 mM HEPES (pH 7.2) containing 1 mM PMSF, and sonicated 3 times for 30 sec each with a Branson 450 sonifier at a 60% duty load using a two-step microtip. The suspension was pelleted at $18,000\times g$ for 30 min and the soluble mitochondrial layer was collected. For the first LC-ICP-MS experiment of this group, the pellet was resuspended in buffer, and the second soluble fraction was collected after centrifugation and combined with the first soluble fraction. For the second and third experiments of this group, the second soluble fraction was not combined. Extracts (2 mL injections) were passed through a Superdex 200pg 16×600 mm column (GE Life Science) equilibrated in 25 mM Tris-HCl pH 7.7. Buffer was prepared in double-distilled deionized trace-metal-free water. The elution flow rate was 0.75 mL/min, controlled by an ÄKTA (GE Healthcare Life Science, New Jersey, USA) FPLC. The entire system was housed in a refrigerated (5°C) Ar-atmosphere glovebox (Mbraun) with $\text{O}_2 < 3$ ppm as monitored by a Teledyne Model 310 analyzer.

The second group of LC studies involved size-exclusion chromatography of LMM species. Isolated mitochondria were solubilized with 1% sodium deoxycholate in 100 mM Tris at pH 7.4, and the supernatants were filtered through 10 kDa cut-off membrane using an Amicon stirred concentrator. The filtrates were applied (0.5 mL injections) to two Superdex Peptide 16×300 mm columns (GE Life Science) connected in series and equilibrated with 25 mM Tris-HCl pH 7.7, driven by a Bio-Inert HPLC (Agilent, Tokyo, Japan). The elution flow rate was 0.375 mL/min. The entire system was located within a refrigerated glovebox. The third group of LC studies involved anion-exchange chromatography (Mono Q 4.6/100 PE GE Healthcare; 1.7 mL column volume CV) of mitochondrial extracts. Extracts were prepared

as described above for HMM size-exclusion studies involving sonication, except that the sonication buffer was 20 mM Tris pH 7.2. Buffer A (20 mM Tris pH 7.2) and buffer B (same as A but with 1 M NaCl) were used to generate the elution buffer gradient. The column was washed with 8 CVs of Buffer A. Proteins were eluted using 25 CVs of a 0 → 1 M NaCl linear gradient, followed by 8 CVs of Buffer B. For the second study, Buffer C (20 mM Tris pH 7.2 plus 0.1 M NaCl) and Buffer D (same as C but with 0.35 M NaCl) were used as the elution buffer. The column was washed with 5 CVs of Buffer A. Proteins were eluted using 3 CVs of Buffer C, followed by 25 CVs of a linear 0.1 → 0.35 M NaCl gradient, and then 5 CVs of Buffer D. The elution flow rate was 1 mL/min. The Bio-inert HPLC was used for these studies.

Aqueous Fe^{III} and many other Fe^{III} complexes are insoluble at neutral pH and tend to adhere to column media. Fe^{II} is easily oxidized to Fe^{III} in air, and even under essentially anaerobic conditions, Fe accumulated on our LC columns. Thus, after each use, the size-exclusion columns were cleaned by passing 5 CVs of a chelator cocktail containing 10 μM each of EGTA, EDTA, diethylenetriamine pentaacetate, and 2,2-bipyridine followed by 10 CVs of elution buffer. (WARNING: Early attempts to use 10 mM of the same chelator cocktail seemed to damage the column media.) This was followed by 15 CVs of elution buffer. The Mono Q column was cleaned by passing 15 CVs of 20 mM Tris plus 1 M NaCl, then 15 CVs of 10 μM EGTA and EDTA, followed by 10 CVs of 20 mM Tris (without NaCl).

The ICP-MS was used in collision cell mode (He: 4.3 mL min⁻¹) with platinum cones and skimmer. The instrument was optimized daily using manufacturer's tuning solution. The sample was introduced through a standard Micromist nebulizer (Glass Expansion, Australia). For size-exclusion chromatography, the eluent was split using a micro splitter valve (Upchurch Scientific, USA) with ~ 30% directed to ICP-MS and the remainder to the ÄKTA 950 fraction collector where 500 μL fractions were collected in Deep Well 96 well plates (Greiner, Germany). For anion exchange chromatography using the on-line ICP-MS, ~ 80% of the eluent was collected in 680 μL fractions using an 1260 Infinity Analytical-Scale Fraction Collector (Agilent, Tokyo, Japan). The remaining ~ 20% of the eluent was sent to ICP-MS. ⁵⁶Fe, ⁵⁷Fe, ⁵⁵Mn, ³¹P, and ³⁴S were monitored with a dwell time of 100 ms.

RESULTS

Mitochondria from aerobically grown WT and *mtm1* cells

Mitochondria were isolated from WT cells grown under fermenting conditions on minimal medium supplemented with 20 μM ⁵⁷Fe. The concentrations of metal ions in the organelle (Table 1) were similar to previous reports.¹⁵ The samples also exhibited a low-temperature (5 K), low-field (0.05 T) Mössbauer spectrum similar to that reported.¹⁵ The major spectral feature (Fig. 1A and Table S1) was the central doublet (CD; $\delta \approx 0.44$ mm/s, $E_Q \approx 1.2$ mm/s) due to $S = 0$ [Fe₄S₄]²⁺ clusters and low spin (LS) Fe²⁺ hemes. Two minor quadrupole doublets were evident, including one ($\delta \approx 1.4$ mm/s, $E_Q \approx 3.2$ mm/s) arising from non-heme high-spin (NHHS) Fe^{II} species and the other ($\delta \approx 0.96$ mm/s, $E_Q \approx 2.26$ mm/s) arising from high spin (HS) Fe^{II} hemes. The remaining intensity included contributions from Fe^{III} oxyhydroxide (phosphate or polyphosphate) nanoparticles and/or $S = 0$ [Fe₂S₂]²⁺ clusters, but the resolution was insufficient to distinguish these. The former

species has been observed in previous preparations of yeast mitochondria grown under similar conditions. The latter species has been identified recently in mitochondria isolated from Fe-deficient cells,²⁴ and is probably present (but more difficult to distinguish) in mitochondria from Fe-sufficient cells. The simulation that overlays the spectrum of Fig. 1A includes all of these contributions, with the percentages given in Table S1.

Mitochondria isolated from *mtm1* cells grown under similar conditions contained 4-times more Fe than did WT mitochondria (Table 1). The 5 K, 0.05 T Mössbauer spectrum of *mtm1* mitochondria (Fig. 1B) was dominated by a broad quadrupole doublet ($\delta \approx 0.52$ mm/s, $E_Q \approx 0.67$ mm/s) due to nanoparticles.

This was confirmed by the 4.2 K, 8 T spectrum (Fig. 1D) which was indistinguishable from the high-field spectra of other mutant mitochondria whose Fe contents were also dominated by nanoparticles.^{12,14} Phosphorus, most likely in the form of phosphate or polyphosphate ions, are associated with nanoparticles in *Atm1*-depleted, *Yah1*-depleted, *Ssq1*-deficient, and *Yfh1*-deficient cells,^{12–14,25} and we presume that similar species are associated with the nanoparticles in *mtm1* mitochondria.

The 5 K, 0.05 T Mössbauer spectrum of *mtm1* mitochondria also exhibited a low-intensity quadrupole doublet with a high-energy line at ~ 2.9 mm/s (Fig. 1B, blue line). The low-energy line of this doublet is buried within the nanoparticle doublet, but we estimate parameters of $\delta \sim 1.4$ mm/s and $E_Q \sim 2.9$ mm/s. These values are typical of nonheme high-spin (NHHS) Fe^{II} species. Similar doublets have been observed in spectra of *Atm1*- and *Yah1*-depleted mitochondria.^{12,14} Although this doublet corresponded to only 2% of the total spectral intensity, it represented a substantial Fe concentration (~ 70 μM) within *mtm1* mitochondria.

The dominating nanoparticle doublet prevented us from determining whether the CD is included in the spectrum of Fig. 1B. Since Fe^{III} nanoparticles in other yeast strains are reducible by sodium dithionite,^{12,14} we treated *mtm1* mitochondria with the detergent deoxycholate followed by dithionite. The resulting Mössbauer spectrum (Fig. 1C) was dominated by a NHHS Fe^{II} doublet ($\delta \approx 1.3$ mm/s, $E_Q \approx 3.4$ mm/s $\Gamma \approx 0.5$ mm/s) that represented $\sim 95\%$ of spectral intensity. The remaining $\sim 5\%$ intensity had the parameters of the CD. Some or all of the species affording the HS Fe^{II} doublet may have leached out of mitochondria and chelated by the EGTA in the buffer. We estimate that all but a few % of nanoparticle Fe^{III} ions were reduced in the process. The concentration of the CD in dithionite-reduced *mtm1* mitochondria (simulated by the dashed line in Fig. 1C) corresponded to ~ 160 μM Fe, *ca.* 3-times lower than that in WT mitochondria (~ 530 μM). We conclude that *mtm1* mitochondria contain ISCs and LS hemes that are collectively at a lower concentration than in WT mitochondria.

mtm1 mitochondria exhibited a broad EPR signal near $g \sim 2$ that displayed inverse Curie-Law behavior; i.e. under non-saturating conditions, the signal intensity increased as the temperature increased (Fig. 2, top panel, compare A to B). Such behavior reflects superparamagnetism, adding further evidence for the presence of nanoparticles in *mtm1* mitochondria.

The low-field EPR region (Fig. 2, middle panel) exhibited a signal at $g = 4.3$ that arises from mononuclear HS Fe^{III} species with rhombic symmetry. The spin intensities associated with this signal were almost identical in WT and *mtm1* mitochondria, corresponding to concentrations of 1.5 ~ 2.0 μ M (Table S2). The HS Fe^{III} heme signals, with effective g -values of 6.4, 5.3 and 6.0, were more intense in the *mtm1* mitochondrial spectrum than in the WT spectrum, suggesting a more oxidizing environment in *mtm1* mitochondria. The high-field region of both spectra included $g_{av} = 1.94$ and 1.90 signals. These signals have been assigned to $S = \frac{1}{2}$ [Fe₂S₂]¹⁺ clusters from Rieske proteins and SDH.²⁶ They were 3.5-times less intense in *mtm1* than in WT mitochondria (Fig. 2, bottom panel, compare B to A), consistent with some loss of ISCs in the mutant strain.

The presence of HS and LS Fe^{II} hemes in WT and *mtm1* mitochondria were assessed from UV-vis features in the 500–620 nm region. Under aerobic conditions, heme concentrations in *mtm1* mitochondria were 6-fold less than in WT mitochondria (Fig. 3, compare B to A). Mitochondria isolated from *mtm1* cells also had 5-times more oxidative damage relative to WT cells grown under the same conditions, as assessed using the Oxyblot method (Fig. 4a).

Effect of Anaerobicity

WT and *mtm1* mitochondria isolated from cells grown under anaerobic conditions will be called WT/Ar and *mtm1*/Ar mitochondria, respectively. The Fe concentrations of these samples were substantially lower than in mitochondria from cells grown aerobically (Table 1). The intensity of the 5 K, 0.05 T Mössbauer spectrum of *mtm1*/Ar mitochondria was also low (Fig. 1E), consistent with the lower Fe concentration of the sample. Two major features were discerned, including the CD and a NHHS Fe^{II} doublet ($\delta \approx 1.2$ mm/s, $E_Q \approx 2.7$ mm/s). A significant proportion of Fe could not be assigned due to the low S/N. However, the lack of a major nanoparticle doublet indicated that the O₂ used in cell growth is required for the formation of these nanoparticles, similar to the situation for Atm1 and Yah1 strains.^{12,14} The absolute concentration of CD-associated Fe in mitochondria obtained from cells grown under anaerobic conditions was ~ 200 μ M, about half of that present under aerobic conditions and consistent with the lower Fe concentration in the samples.

The high-energy line of the NHHS Fe^{II} doublet in spectra of anaerobically grown *mtm1* mitochondria (~2.5 mm/s) was shifted slightly (by *ca.* 0.4 mm/s) towards lower energy relative to the analogous line in aerobically-grown *mtm1* mitochondria (2.9 ~ 3.0 mm/s). Comparing the spectra from anaerobic vs. aerobic WT mitochondria suggested a similar shift (Fig. S1). These results indicate different populations of HS Fe^{II} species depending on the aerobicity of the yeast culture. However, the spectra were noisy, so further studies will be required to confirm this. In contrast, there were no distinguishing differences between the NHHS Fe^{II} species in WT vs. *mtm1* mitochondria, at least to the extent discernible by Mössbauer spectroscopy.

Both WT/Ar and *mtm1*/Ar mitochondria contained hemes at approximately equal concentrations (Fig. 3, C and D), but these concentrations were substantially less than were present in WT mitochondria isolated under aerobic conditions (Fig. 3A). Interestingly, the concentration of O₂ required for aerobic-levels of heme synthesis appears to be quite low. Just prior to harvesting, one batch of *mtm1*/Ar cells were (inadvertently) exposed to a

micro-aerobic environment by being stirred for 2 hrs in the sealed bioreactor but not purged with Ar. The heme *b* and *c* concentrations in mitochondria isolated from those cells (Fig. 3E) were almost at WT levels while heme *a* was largely absent. Thus *mtm1* cells are capable of synthesizing WT levels of heme *b* and *c* centers with minimal O₂ exposure. This strongly suggests that the low level of hemes in mitochondria isolated from *mtm1* cells grown under aerobic conditions is not due to the absence of Mtm1p as has been suggested^{12,14} and may be due to ROS-promoted damage of such centers. *mtm1* cells can synthesize such prosthetic groups if O₂ and ROS levels are modest.

EPR spectra from mitochondria isolated from both WT/Ar and *mtm1*/Ar cells exhibited $g_{\text{ave}} = 1.94$ and 1.90 signals of approximately equal low intensities (Fig. 2, C and D), suggesting that cells lacking *MTM1* are capable of synthesizing ISCs under anaerobic conditions (Table S2). Interestingly, mitochondria from both WT and *mtm1* cells exhibited significant levels (quantifying to $0.2 \sim 0.5 \mu\text{M}$) of a $g_{\text{ave}} = 2.01$ signal ($g = 2.03, 2.02, 2.00$) typical of $S = \frac{1}{2} [\text{Fe}_3\text{S}_4]^+$ clusters like that found in inactive aconitase. Such signals are generally not found in spectra of mitochondria from aerobically grown cells. Confounding this analysis were significant aconitase activities in WT/Ar and *mtm1*/Ar mitochondria (Table 2).

Enzyme Activities

Activities of other selected ISC-containing enzymes were also determined to further evaluate the *mtm1* phenotype. For cells grown under aerobic conditions, aconitase activity was substantially reduced in *mtm1* mitochondria relative to WT mitochondria (Table 1). SDH activity in *mtm1* mitochondria was about 10-fold lower than in WT mitochondria; however under anaerobic conditions, SDH activity was near WT/Ar levels. These results collectively indicate that *deleting MTM1 induces oxidative stress in the presence of O₂ that damages hemes and ISCs.*

Leu1p is an ISC-containing enzyme found in the cytosol; its activity is commonly used to monitor the activity of the cytosolic ISC assembly machine known as the CIA.⁷ In *mtm1* cells, the activity of Leu1p was $\sim 60\%$ of that in WT cells under aerobic growth conditions and $< 50\%$ of that in WT cells under anaerobic conditions. This is a different pattern than was observed for the other measured activities, which were reduced under aerobic conditions and near-WT levels under anaerobic conditions. This difference suggests that ROS is *not* responsible for the absence of Leu1 activity in *mtm1* cells. One possibility is that Mtm1p, like another mitochondrial transporter Atm1p, is required for the CIA to function. Alternatively, the expression-level of Leu1 may be reduced in *mtm1* cells vs. in WT cells. We are unable to distinguish these possibilities.

SOD2 Activity

We measured SOD2 activities and Sod2p protein levels in electrophoretic gel bands. The SOD2 activity of fermenting *mtm1* cell extracts was $26 \pm 6\%$ (avg. of 3 trials) of the WT level (Fig. 4b, left panel), whereas the Sod2p protein level in *mtm1* cell extracts was $71 \pm 12\%$ of WT (Table 1). Thus, the average SOD2 specific activity of *mtm1* cell extracts was

$42 \pm 16\%$ of the WT value. This percentage-decline is similar to data reported for SOD2 specific activities in *mtm1* vs. WT cells.⁴

We also examined SOD2 activities in cells grown under anaerobic conditions. Relative to aerobically grown WT cells, WT/Ar and *mtm1*/Ar cells respectively exhibited $66 \pm 7\%$ and $14 \pm 3\%$ of SOD2 activity (Fig. 4b, left panel, compare lanes 3 and 4 to lane 1). Sod2p protein expression level in WT/Ar and *mtm1*/Ar cells were $77 \pm 19\%$ and $36 \pm 5\%$ relative to aerobically grown WT cells. This indicates SOD2 specific activities of $102 \pm 34\%$ and $42 \pm 12\%$ for WT/Ar and *mtm1*/Ar cells, respectively, relative to the aerobic case. Thus, SOD2 specific activities of *mtm1* cells were about 40% of WT levels, *regardless of whether cells were grown aerobically or anaerobically*. For comparison, the SOD2 activity of Yah1-depleted cells was also examined; these cells exhibited $87 \pm 11\%$ of the SOD2 specific activity, relative to aerobically grown WT cells (Fig. 4b, right panel, lane 2 vs. 1).

These results indicate that SOD2 inactivation in *mtm1* cells is *not* associated with ROS damage, in contrast to the loss of hemes and ISCs which are ROS-associated. We conclude that a deficiency of Mtm1p is more closely associated with the inactivation of Sod2p or perhaps with the functioning of the cytosolic ISC assembly (CIA) machine than with the accumulation of nanoparticles, the loss of mitochondrial ISC and heme centers, or the generation of ROS.

Whole Cells

WT and *mtm1* whole yeast cells were analyzed by Mössbauer spectroscopy and ICP-MS (Tables 1 and S1). The dominant feature (Fig. 5A) was a sextet due to a HS Fe^{III} species that resides in vacuoles.²⁷ Also evident were the CD, a HS heme Fe^{II} doublet and perhaps a NHHS Fe^{II} doublet ($\delta \approx 1.3$ mm/s, $E_Q \approx 3.1$ mm/s). The latter feature was barely discernible; it corresponded to only *ca.* 13 μ M Fe (2% of 640 μ M).

The spectrum of equivalently grown *mtm1* cells (Fig. 5B) was dominated by the Fe^{III} nanoparticle doublet ($\delta \approx 0.53$, $E_Q \approx 0.58$), but it also contained a sextet arising from vacuolar mononuclear HS Fe^{III} species and a more predominant NHHS Fe^{II} doublet ($\delta \approx 1.4$, $E_Q \approx 3.0$; simulated by the blue solid line). The intensity of NHHS Fe^{II} species in *mtm1* cells was also significant, corresponding to *ca.* 540 μ M (11% of 4900 μ M), ~ 40-fold higher than in WT cells. Although the percentage of spectral intensity due to the sextet was small, the absolute concentration of the NHHS Fe^{III} species in *mtm1* cells was actually similar to that in WT cells. The same pattern was observed using Aft1-1^{up} cells,²³ where vacuolar HS Fe^{III} was present along with a dominating nanoparticle species. The pattern was different with Yah1- and Atm1-deficient cells, where only the nanoparticle doublet was present (i.e. vacuolar mononuclear Fe^{III} was absent).^{12,23} The reason for this difference is unknown, but vacuoles in Yah1- and Atm1-deficient cells (but not in Aft1- 1^{up} or *mtm1* cells) appear devoid of mononuclear HS Fe^{III}.

Mössbauer spectra of WT/Ar and *mtm1*/Ar cells (Fig. 5, C and D) were similar to each other and to spectra of aerobically grown WT cells in that few nanoparticles were present. This indicates that O₂ exposure was required for nanoparticle formation in *mtm1* cells. The spectra of WT/Ar and *mtm1*/Ar cells were different relative to that of WT/O₂ cells, in that

the percentage of Fe due to the CD was diminished and the percentage of Fe due to NHHS Fe^{II} species was enlarged. The reduced CD intensity suggests diminished mitochondrial respiratory complexes under anaerobic conditions. Interpreting the increase of NHHS Fe^{II} species in anaerobically grown cells is difficult because the location and function of these species are uncertain.

Regardless of the origin of this type of Fe, *mtm1* cells accumulated somewhat more HS Fe^{II} than WT cells under both aerobic and anaerobic growth conditions. We considered that this might represent the Fe_{ASR} pool, but the sought-after Fe must be located in the mitochondria since it would incorporate into apo-Sod2p when this protein folds in the matrix. *mtm1* mitochondria do not contain substantial HS Fe^{II} species relative to the level in WT mitochondria.

Effects of deleting MTM1 on mitochondrial manganese

Mn concentrations in mitochondria isolated from *mtm1* cells grown under both aerobic and anaerobic conditions were twofold higher than in equivalent WT mitochondria (Table 1). In contrast, *mtm1* whole cells contained about the same concentration of Mn as WT cells, again independent of aerobicity. Taken together, *these results indicate that mtm1 mitochondria accumulate cellular Mn*. Whether there was a corresponding decrease in the Mn concentration of the non-mitochondrial portions of the cell could not be determined from these data. An absolute increase of *ca.* 10 μ M Mn in *mtm1* mitochondria would have caused only an increase of *ca.* 1 μ M in the overall Mn concentration in the cell (the mitochondria occupy *ca.* 10% of the cell volume). Since this Mn concentration increase also occurred under anaerobic conditions, it does not appear to be connected to the accumulation of nanoparticles in *mtm1* mitochondria. These results raise the possibility that Mtm1p exports Mn^{II} ions from the mitochondrial matrix to the cytosol.

Soluble mitochondrial extracts

In 3 independent experiments, WT and *mtm1* mitochondrial fractions were sonicated, spun by centrifugation, and soluble fractions were collected. In the first experiment, the pellet was resuspended and resonicated, and the second soluble fraction was combined with the first. In the second experiment, the pellet of the first spin was discarded. In the third experiment the pellet from the first spin was also discarded, but more importantly, the cells were grown in medium containing 200 μ M MnCl₂.

This procedure solubilized an average of 35% $\pm$ 12% (n = 6) of the Mn in WT and *mtm1* mitochondria, with no strain dependence (Table S3). These soluble fractions contained 25% $\pm$ 13% of the protein and 25% $\pm$ 12% of the SOD2 activity in the original samples. This procedure also solubilized 21% $\pm$ 6% of the Fe in WT mitochondria, whereas only 7% $\pm$ 1% of Fe in *mtm1* cells were solubilized. This difference reflects the greater proportion of nanoparticles in *mtm1* mitochondria. Because of this low recovery, the contents of the soluble fractions may not faithfully indicate the content of the soluble portions of intact mitochondria.

LC-ICP-MS Experiments

Soluble WT and *mtm1* fractions were subjected to liquid chromatography, and the eluent was monitored for metal ions using ICP-MS. In most experiments, the ICP-MS was configured *on-line*, such that the eluent was split, with a portion flowing into fractions and the remainder to the ICP-MS in real time during the chromatography run. In off-line experiments, the entire eluent was fractionated and subsequently sampled by ICP-MS.

Since a greater proportion of SOD2 activity was in the soluble fraction of Experiment 2, we describe that experiment now (and include the results of Experiment 1 in Fig. S2). The A₂₈₀ trace of the eluent off the Superdex column (not shown) indicated that > 90% of the protein in the soluble samples eluted in the void volume. This volume was *ca.* 46 mL and corresponded to molecular masses > 600 kDa. Also eluting in the void volume was a small proportion of the Mn in the injected sample (Fig. 6, *a* and *b*). Two major Mn peaks were observed in later fractions, called Mn₁₀₀ and Mn₂₋₃, with molecular masses of *ca.* 100 kDa and < 10 kDa respectively (Fig. 6, *a* and *b*, and Table S4). Mn₁₀₀ co-eluted with active tetrameric Sod2p while Mn₂₋₃ corresponded to the LMM species.

We have sufficient information to roughly estimate the speciation of Mn in mitochondria. In WT mitos grown on normal medium, *ca.* 64% of the soluble Mn was SOD2-associated. Thus, ~ 24% (0.64×38%) of total Mn ions in the original mitochondrial sample was associated with 27% of SOD2 activity. The similarity of these two numbers suggests that the remaining 73% of MnSod2p in the pellet represented another 65% of the Mn in WT mitochondria. Thus, the majority of the Mn in these organelles (27% + 65% = 92%) is associated with MnSod2p. Mn₂₋₃ represented 32 ± 3% of the soluble Mn in our WT samples, corresponding to 12% (0.32×38%) of total WT mitochondrial Mn. These results indicate that *the Mn in WT mitochondria is distributed into two species – ca. 90% is associated with MnSod2p and ca. 10% is present as soluble LMM species.*

In *mtm1* mitochondria, about 46% of total Mn was soluble. Of this, 89% was LMM and 9% was associated with MnSod2p. Only *ca.* 4% of total Mn in *mtm1* mitochondria was in the form of soluble Sod2p, and this corresponded to 25% of total SOD2 activity. The other 75% of the Sod2p protein (in the pellet) must have included another 12% of the total Mn. Thus, 16% of total Mn in *mtm1* mitochondria (4% + 12%) was probably MnSod2p-associated. The only other species observed in the soluble fraction was Mn₂₋₃, suggesting that > 80% of the Mn in *mtm1* mitochondria arise from LMM species. As mentioned above, the concentration of Mn in *mtm1* mitochondria was about *twice* that of WT mitochondria. Our analysis indicates that most of the additional Mn in *mtm1* mitochondria was present as Mn₂₋₃. Also, the MnSod2p protein level in *mtm1* mitochondria was diminished relative to WT levels. Assuming 13 μM [Mn] in WT mitochondria, this corresponds to 12 μM MnSod2p and 1 μM Mn₂₋₃. In *mtm1* mitochondria, with 26 μM [Mn], we calculate 4 μM MnSod2p and 22 μM Mn₂₋₃. Thus, *deleting MTM1 caused a 3-fold reduction in size of the LMM Mn pool in the mitochondria.* In the third experiment, for mitochondria isolated from WT cells grown on active MnSod2p and a 22-fold increase in the medium supplemented with 200 μM MnCl₂, the Mn trace (Fig. S3a) again showed two major peaks, Mn₁₀₀ (23%) and Mn₂₋₃ (68%). In this case, 15% of the total Mn in the sample

was soluble, and of this, 23% corresponded to MnSod2p. This means that $0.23 \times 15\% = 3.5\%$ of total Mn in the sample corresponded to 10% of the SOD2 activity. The remaining 90% of the activity must have represented another 31% of total Mn. Thus, nearly 35% ($3.5\% + 31\%$) of total Mn in the sample was associated with MnSod2p. The only other species present in the chromatogram was Mn₂₋₃, suggesting that it corresponded to the remaining 65% of the total Mn in WT mitochondria grown on excess Mn. With $35 \mu\text{M} = [\text{Mn}]$, we conclude that $12 \mu\text{M}$ was MnSod2p and $23 \mu\text{M}$ was Mn₂₋₃. Thus, the MnSod2p level remained about the same while the LMM Mn pool increased dramatically. Thus, the size of the LMM Mn pool in mitochondria is sensitive to the Mn concentration in the medium. Finally, we consider the Mn distribution in mitochondria from *mtm1* cells grown on $200 \mu\text{M}$ Mn (Fig. S3b). In this case, Mn₁₀₀ represented 5% of the Mn in the chromatogram while Mn₂₋₃ was 94%. 39% of total Mn was soluble, and so $(0.05 \times 39\% =)$ 2% of total Mn corresponded to 31% of MnSOD2 activity. The remaining 69% of the SOD activity must correspond to another 4.4% of the total Mn in the sample, suggesting that 6.4% of total Mn was MnSod2p. The sample had $80 \mu\text{M}$ Mn, and so there was $5 \mu\text{M}$ (80×0.064) MnSod2p in mitochondria from *mtm1* cells grown with $200 \mu\text{M}$ Mn. The remaining $75 \mu\text{M}$ was associated with Mn₂₋₃. This analysis indicates that the concentration of MnSod2p did not change with Mn supplementation in the growth medium, but that the LMM Mn pool concentration increased more than 2-fold. The results of these calculations are visually summarized in Fig. 7.

The corresponding Fe traces exhibited 7 distinguishable features, with significant concentrations in the void volume (Fe_H), 5 minor-intensity peaks in the central region (Fig. 6, *a* and *b*; Fe₂₀₀, Fe₁₀₀, Fe₆₀, Fe₃₅, and Fe₂₀), and an intense LMM feature (Fe₃) that has a slightly higher mass than Mn₂₋₃. Fe₃ is a viable candidate for Fe_{ASR}. For the first two experiments, an average of 25% of the total Fe was solubilized from mitochondrial extracts. Of this, Fe_H represented 67% of total mitochondrial Fe in one experiment and 17% in the other. The Fe₃ intensity corresponded to 19% of total Fe in the first experiment and 74% in the second. Despite the large variations, these two peaks collectively dominated the Fe chromatograms of soluble WT mitochondrial extracts. The intermediate-molecular-mass (IMM) Fe species corresponded to *ca.* 10% of the soluble Fe – representing 2.5% of the total Fe. With a total Fe concentration of $880 \mu\text{M}$ in WT mitochondria, these IMM Fe species were present at *ca.* $20 \mu\text{M}$ collectively, with Fe₁₀₀ corresponding to a concentration of *ca.* $9 \mu\text{M}$.

An average of 7% of total Fe was solubilized in the corresponding *mtm1* mitochondria samples. Since the Fe concentration in these mitochondria was $3500 \mu\text{M}$, this corresponded to $245 \mu\text{M}$ Fe injected onto the column. Fe_H and Fe₃ again dominated the chromatograms. Fe₁₀₀ represented 2% of the overall chromatogram intensity, corresponding to a concentration of $5 \mu\text{M}$. Thus, upon deleting *mtm1*, Fe₁₀₀ declined nearly 2-fold.

Under high Mn nutrient conditions, 14% of WT mitochondrial Fe was solubilized; this corresponded to $87 \mu\text{M}$ Fe ($620 \mu\text{M} \times 0.14$) in the soluble sample. Fe₁₀₀ represented 3% of that Fe (*ca.* $3 \mu\text{M}$). For *mtm1* mitochondria, 8% of mitochondrial Fe was solubilized. At $5200 \mu\text{M}$ Fe, this corresponded to $416 \mu\text{M}$ soluble Fe loaded on the column. Fe₁₀₀ represented 0.4%, corresponding to $2 \mu\text{M}$. Thus, deleting *MTM1* under high Mn caused a

slight decline of Fe₁₀₀. This was the same qualitative effect as seen under normal Mn growth conditions.

Fe₁₀₀ approximately coeluted with Mn₁₀₀ raising the possibility that it corresponded to FeSod2p. The absolute concentration of Fe₁₀₀ in mitochondria and the percentage of Fe that it represented would indeed make it impossible to detect by either Mössbauer or XAS spectroscopies. If the percentage of Fe-misincorporated Sod2p is defined as $= 100 \cdot [Fe_{100}] / ([Fe_{100}] + [Mn_{100}])$, then the percentage of FeSod2p was $75 \pm 4\%$ and $79 \pm 3\%$ for WT and *mtm1* mitochondria (ave. of 3 experiments). Assuming that Fe₁₀₀ corresponds to FeSod2p, these percentages show that *there was no significant difference in the extent of Fe misincorporation into apo-Sod2p between WT and mtm1 mitochondria*. Although these results cannot exclude the possibility that Fe₁₀₀ corresponds to FeSod2p, the results of other LC experiments (e.g. see Fig. 9) discount this possibility. Thus, we suggest that only minor portions of Sod2p in our WT and *mtm1* preparations may be misincorporated with Fe.

We performed SOD2 activity gels and Western blots against Sod2p using pooled Sod2p-containing fractions and as-isolated mitochondria. In all three experiments, pooled fractions of SOD2-active *mtm1* extracts revealed specific activities corresponding to $100 \pm 14\%$ of WT levels. This again indicated that the extent of Fe-misincorporation in *mtm1* and WT cells is similar.

The situation was different using isolated mitochondria. The SOD2 specific activity of *mtm1* mitochondrial extracts, prepared from cells grown without Mn supplementation, was *ca.* 20% of the SOD2 specific activity of equivalent WT mitochondrial extracts (Fig. S4). *This suggested that a large percentage of Sod2p in mtm1 mitochondria is inactive but is not associated with the MnSod2p in the Mn₁₀₀-containing fractions.* The inactive Sod2p probably migrates differently through the column or remains in the pellet during solubilization. We performed Western blots on various fractions that eluted from the column, but could not identify any that contained Sod2p, suggesting that the inactive form is in the pellet. This form could be Fe-misincorporated, misfolded or modified in some manner.

There was some recovery in SOD2 specific activity in mitochondria isolated from cells grown on Mn supplemented medium, similar to that reported previously.¹ In this case, the SOD2 specific activity of *mtm1* mitochondria was *ca.* 70% relative to WT mitochondria. This might be caused, not by an increase of MnSod2p, but a decrease in Sod2p expression such that only ~ 30% of Sod2p in *mtm1* mitochondria is inactive. Further studies are required to examine this possibility.

LC-ICP-MS of LMM species

We initially collected the Mn₂₋₃ and Fe₃ fractions from the first group of experiments, and subjected them to the LMM size-exclusion column to determine their molecular masses to greater accuracy. The results indicated multiple overlapping species, with poor reproducibility from batch to batch. More consistent (and simpler) results were obtained by disrupting isolated mitochondria by adding detergent (deoxycholate) rather than by sonication. Supernatant fractions were passed through the 10 kDa cut-off membrane and the

flow-through was injected onto the column. Mn_{2-3} and Fe_3 dominated (Fig. 6c, Experiment 4), and they eluted in accordance with molecular masses between 2 – 3 kDa (explaining the subscripts used). Taken together, these results suggest that the major LMM Mn and Fe species in yeast mitochondria have similar ligand environments (at least in terms of the collective masses of the ligands).

Anion exchange chromatography

We repeated previous LC experiments⁴ using the same column (Mono Q) and run conditions (except that we performed our LC under anaerobic conditions). Due to the high salt concentration involved at the end of the gradient, individual fractions were analyzed (after dilution) for metals using the ICP-MS off-line. Soluble mitochondrial extracts from sonicated WT and *mtm1* mitochondria afforded an intense Mn peak at ~ 14 mL, another less intense peak (or group of incompletely resolved peaks centered) at ~ 19 mL, and unassigned peaks at 2, 36, and 62 mL (Fig. 8, Experiment 5). We tentatively assign the dominant peak to Mn_{2-3} , based on results of the LMM size-exclusion study in which MnSod2p and Mn_{2-3} peaks dominated. SOD2 activity assays and Western blots against Sod2p demonstrated that that Sod2p co-eluted with the second peak(s) (Fig. 8, *insets i, ii*). The specific SOD2 activity in the five fractions containing most Sod2p was 2-fold lower in *mtm1* than in WT fractions, suggesting that one or more inactive forms of Sod2p co-eluted with the active form. The Fe concentrations in these fractions were also determined. One major Fe peak was observed in both *mtm1* and WT chromatograms, which we tentatively assign to Fe_3 , with similar justification. There were low intensity features in the vicinity of the SOD2-active Mn peaks (Fig. 8, *insets iii, iv*), but nothing that comigrated exactly with the SOD2-active fractions or with an intensity that would suggest massive Femisincorporation.

The experiment was repeated but using a shallower salt gradient and with the ICP-MS on-line. Sod2p and SOD activity co-eluted with Mn around 30 mL in both of WT and *mtm1* chromatograms (Fig. 9, Experiment 6) whereas the nearest Fe species eluted at a somewhat greater elution volume. These results again indicate that the extent of Fe mis-incorporation is not significant in *mtm1* cells, and is no greater than in WT cells. SOD2 activity is diminished in *mtm1* mitochondria, but not because of excessive Fe-misincorporation.

The Sod2p in fractions collected from the Mono Q column migrated in four different positions in native gels (see Fig. 9b, *insets*). Sod2p is active as a homotetramer,¹ suggesting that subunits may have different charge, with each combination migrating slightly differently on the column. The situation differs for Sod2p in fractions from size-exclusion columns, because the molecular masses of the different tetrameric forms are so similar (Fig. 6a). Sod2p tetramers in earlier Mono Q fractions are less negatively charged, and more Sod2p in *mtm1* mitochondria eluted earlier compared with WT mitochondria. Although most Sod2p eluted around 30 mL, an Sod2p band was observable down to 20.6 mL (0.14 M NaCl) in the *mtm1* chromatogram, whereas Sod2p was visible only down to 24.7 mL (0.17 M NaCl) in the WT chromatogram. This suggests that Sod2p might require a charge-altering post-translation modification such as phosphorylation to be active, and that the process is

somehow diminished in *mtm1* mitochondria. Consistent with this, threonine (T147 and T149) residues in Sod2p can be phosphorylated.²⁸

DISCUSSION

Disconnect between Fe Accumulation and Sod2 inactivation

The *mtm1/Sod2* mismetallation hypothesis implies that Fe accumulation and Sod2 inactivation in *mtm1* mitochondria are *directly connected* in terms of a reaction network or mechanism. Our characterization of *mtm1* cells grown under anaerobic conditions does not support such a connection. When grown under anaerobic conditions, this strain and the two others that have been studied under anaerobic conditions, namely Yah1p-depleted and Atm1p-depleted cells, do not exhibit the Fe-accumulation phenotype.^{12,14} Yet one of these strains (Yah1p-depleted) exhibits strong SOD2 activity while the other two do not. This demonstrates that Fe accumulation is not tied to diminished SOD2 activity. A previous study⁴ found that Fe/S cluster assembly was not affected by an Mtm1p-deficiency, suggesting a unique Fe accumulation phenotype. In contrast, our results indicate that the Fe accumulation phenotype in *mtm1* mitochondria has all of the standard characteristics as seen in mitochondria deficient in Yfh1p,¹³ Atm1p,¹² and Yah1p.¹⁴ This includes the accumulation of Fe^{III} (phosphate and/or polyphosphate) oxyhydroxide nanoparticles, a defect in Fe/S cluster and heme levels, and an increase in ROS-associated damage. This commonality suggests that these phenomena, for all of these protein-deficiencies, are secondary effects that do not directly provide insight into the function of those proteins, including the function of Mtm1p. Thus, we should discount the Fe accumulation phenotype as we attempt to determine primary functions of Mtm1p and these other proteins.

The Manganese-ome of Yeast Cells

Yeast cells grown in standard medium contain 20 – 40 μM Mn.^{24,29} Although much remains to learn about the distribution of these Mn ions in the cell, we were impressed by the simplicity of the mitochondrial Mn LC traces. Our results suggest that the soluble fraction of yeast mitochondrial extracts contain a single Mn-protein, namely MnSod2p, and what appears to be a single LMM Mn species, namely Mn₂₋₃. (We cannot exclude the possibility that the Mn₂₋₃ peak includes multiple species that are unresolved). A small amount of HMM Mn (Mn_H) is also present. The majority of Mn in yeast cells (grown on minimal media without Mn supplementation) is EPR-active and yields a Mn^{II} hyperfine-split signal at $g = 2.0$.²⁴ As probed by ENDOR spectroscopy, multiple Mn^{II} complexes with phosphate and/or polyphosphate ligands contribute to this signal.²⁹ The location of these EPR-active species is unknown, but they do not appear to be located in mitochondria, as these organelles do not exhibit Mn^{II} EPR signals (Our early mitochondria preparations²⁶ exhibited Mn^{II} EPR, but this has not been observed subsequently.). Isolated vacuoles from cells grown in unsupplemented medium contained only 1.7 μM Mn and exhibited no EPR signals from Mn^{II} ions.²⁷ We propose that the LMM Mn₂₋₃ species in the mitochondria inserts into apo-Sod2 during maturation. The reaction, taking place in the mitochondrial matrix, is proposed to be

The concentration of Mn_{2-3} increased when the growth medium was supplemented with $MnCl_2$, suggesting that it might also function to store Mn or sequester it to avoid deleterious side-reactions.

apo-Sod2p-Reactive Fe

Fe_{ASR} should be a mononuclear NHHS FeII species located in the mitochondrial matrix of *mtm1* mitochondria – the Fe^{II} equivalent of Mn_{2-3} . We initiated our studies assuming that the majority of Fe that accumulated in *mtm1* mitochondria would be this type of species. Our biophysical study of *mtm1* mitochondria immediately indicated that the bulk of the Fe in these organelles was Fe^{III} oxyhydroxide (phosphate or polyphosphate) nanoparticles, as the Mössbauer spectroscopic pattern was indistinguishable from that exhibited by such nanoparticles in Yah1 and Atm1-depleted strains. This is consistent with previous XAS results which suggested that Fe_{ASR} constitutes an insignificant portion of the Fe in *mtm1* mitochondria.¹⁰ The XAS results indicated approximately *equal* proportions of Fe^{II} and Fe^{III} in *mtm1* mitochondria whereas Mössbauer spectra indicate that all but a few percent of the Fe is in the Fe^{III} oxidation state.

The dominance of nanoparticles rendered the problem of identifying Fe_{ASR} more difficult because it meant that we were searching for a tiny amount of a mononuclear HS Fe^{II} complex in an vast excess of inert Fe. We first considered the NHHS Fe^{II} species which afforded a 2% quadrupole doublet in the *mtm1* mitochondrial Mössbauer spectrum. However, similar doublets are also present in spectra of mitochondria from strains that accumulate nanoparticles but do not misincorporate Fe into apo-Sod2p - e.g. those deficient in Yah1p.¹⁴ Fe_{ASR} might *contribute* to the broad NHHS doublet, but if so, it would represent only a small proportion of it.

This suggested that Fe_{ASR} should be present at *very* low concentrations with *no* chance of being characterized or even detected by Mössbauer spectroscopy. Thus, we turned our attention to LC-ICP-MS with the goal of identifying Fe_{ASR} and the Fe-mismetallated FeSod2p. We regard Fe_3 as a viable candidate for Fe_{ASR} , because it is a LMM species with a molecular mass similar to that of Mn_{2-3} . In this case, the mismetallation reaction would be

The most likely candidate for FeSod2p was Fe_{100} , based on molecular mass. However, the fraction of mismetallated Sod2p present in *mtm1* mitochondria, assuming Fe_{100} to be this species, was no different than in WT mitochondria. This was unexpected, and it casts doubt on this assumption.

We were also unable to reconcile our size-exclusion LC-ICP results with a previous study.⁴ The previous Mono Q chromatograms of WT mitochondrial extracts displayed only one Mn

peak (MnSod2) which was absent in chromatograms of *mtm1* mitochondria. In contrast, we observed the MnSod2 peak in chromatograms from both WT and *mtm1* mitochondria. Moreover, our chromatograms exhibited a second major Mn species that has not been observed previously.

In the earlier study, no Fe peaks were observed in the WT chromatogram while two Fe peaks were observed in the *mtm1* chromatogram (one that comigrated with Sod2p and one that eluted in the void volume). We observed a major Fe species in both WT and *mtm1* chromatograms that did not comigrate with Sod2p, as well as minor Fe peaks that comigrated in the vicinity of MnSod2p. We did not observe an Fe species that comigrates *precisely* with MnSod2p. We cannot explain these differences. In the previous study, the bulk of Fe in *mtm1* mitochondria was concluded to be associated with Sod2p (as FeSod2p). Based on our results, we conclude that only a tiny amount of mitochondrial Fe could be associated with Sod2p, and that the level of FeSod2p in *mtm1* cells is similar to that in WT cells.

Observed differences may arise from differences in the yeast strain (W303 vs. By4741), growth medium (minimal vs. YPD), growth conditions or preparation methods, but we have no supporting evidence for any of these possibilities. It is also possible that additional FeSod2p formed in *mtm1* mitochondria but that it was less stable than MnSod2p and that it localized primarily in the pellet of our samples. However, yeast FeSod2p is known to be sufficiently stable and soluble for crystallization studies.³⁰

Physiological Role of Mtm1p

Determining the function of Mtm1p – defined by the species transported by this IM mitochondrial carrier – is a continuing challenge. Our results suggest that the lower SOD2 activity observed in *mtm1 S. cerevisiae* arises, not because of excessive mismetallation, but because a significant portion of Sod2p is misfolded or degraded, and/or because the expression-level of SOD2 is diminished. One possibility is that Mtm1p imports a Maturation Factor (MF) required to help mature apo-Sod2p (Fig. 10). Other proteins that exhibit this phenotype (Ssq1p, Grx5p and Atm1p) might also be involved in apo-Sod2p maturation, which includes installing Mn and folding the protein. In the absence of Mtm1p (or these other proteins), the rate of maturation might slow such that apo-Sod2p misfolds or does not become metallated at as fast a rate as is observed in WT cells. The slow rate of maturation might cause the Mn species that is used for metallation (e.g. Mn₂₋₃) to accumulate. Under Mn- supplemented conditions, this Mn species would be imported at a greater rate.

Another possibility, suggested by the results of Fig. 9, is that Mtm1p helps regulate MnSod2 activity and/or stability by promoting a reaction that affects the *charge* on the protein. Phosphorylation reactions are used to regulate numerous mitochondrial enzymes,³¹⁻³² and are involved in the H₂O₂ stress response.³³ Our results are consistent with the possibility that in the absence of Mtm1p, the degree of phosphorylation declines. However, further studies are required to interpret these results more definitively.

In WT cells, Sod2p is *mainly* metallated with Mn. Fridovich purified Sod2p from *S. cerevisiae*, and found sufficient Fe in the purified Mn-protein solution for no more than 6%

of it to be Fe-bound.¹⁸ Approximately 80% of the Sod2p isolated by Whittaker *et al.*³⁴ from the same organism contained Mn, with ~ 20% containing Fe. Jeong *et al.*³⁵ purified the Sod2p from the closely-related *Schizosaccharomyces pombe* and was unable to detect any Fe (or Cu, Zn, or Ni) in the purified enzyme; the only metal detected was Mn. On the other hand, Kang *et al.*³⁰ determined the X-ray diffraction crystal structure of the Fe-metallated *S. cerevisiae* Sod2p to 1.79 Å resolution. In cell grown under normal conditions, the extent of mismetallation seems to vary from 0% to 20%. The mismetallated protein used for crystallography was obtained by overexpressing yeast apo-Sod2p in *E. coli* grown in medium supplemented with 100 µM Fe, which may have increased the level of mismetallation. Using Mössbauer spectroscopy, Fleisch-hacker *et al.*³⁶ found that 40% of the Fe in whole *E. coli* cells (grown on medium supplemented with just 10 µM ⁵⁷Fe) was mononuclear NHHS Fe^{II}. Higher levels of such Fe might be present in cells grown with 100 µM Fe in the medium. Such Fe, consisting of one or more octahedral complexes with a coordination environment dominated by O and/or N ligands, is the type of Fe expected for mismetallation.

There is no doubt that apo-Sod2p can and does mismetallate with Fe, but our results do not support the view that there is an *increased* level of Fe mismetallation in *mtm1* cells. Rather, they suggest that in *mtm1* cells there is a decline in the degree to which apo-Sod2p is activated, folded, metallated or matured. Establishing the molecular details of this will be a future challenge, but should provide further insight into the physiological role of this fascinating protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation

apo-Sod2p	Sod2 protein prior to metal installation
ASR	apo-Sod2p reactive
CD	central doublet
CIA	cytosolic iron sulfur cluster assembly
CVs	column-volumes
DNP	2,4-dinitrophenylhydrazine
DTPA	diethylenetriamine pentaacetate
ENDOR	electron nuclear double resonance

EPR	electron paramagnetic resonance
Fe_{ASR}	sought-after Fe species that misincorporates into apo-Sod2p
FeSod2p	inactive form of Sod2p, with Fe bound
FPLC	fast protein liquid chromatography
HPLC	high-performance liquid chromatography
HS	high spin
ICP-MS	inductively coupled plasma mass spectrometry
IMM	intermediate molecular mass
INT	iodonitrotetrazolium
ISC	iron sulfur cluster
δ	isomer shift
LC	liquid chromatography
LMM	low molecular mass
MF	apo-Sod2p maturation factor
Mn_{ASR}	Mn species that incorporates into apo-Sod2p
MnSod2p	active form of Sod2p, with Mn bound
Mtm1p	<u>M</u> anganese <u>T</u> rafficking factor for <u>M</u> itochondrial SOD2, YGR257C
Mol. Wt 40	763 Da
NHHS	nonheme high spin
OD₆₀₀	optical density at 600 nm
PMSF	phenylmethanesulfonylfluoride
E_Q	quadrupole splitting
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SOD2	superoxide dismutase 2
WT	wild type
XAS	X-ray absorption spectroscopy
YPAD	yeast extracts, peptone, adenine hemisulfate, and dextrose

Notes and references

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Fig. 1.

5 K Mössbauer spectra of isolated mitochondria from W303 and *mtm1* cells. A, W303/O₂; B, *mtm1*/O₂; C, *mtm1*/O₂/dithionite-reduced; D, same as in B but at 8 T; E, *mtm1*/Ar. Spectra other than D were collected with 0.05 T applied field. Red solid lines are simulations (using components and percentages given in Table S1). Blue solid lines in A, B and E simulate the contribution of NHHS Fe^{II} species. The dashed line in C is a simulation that indicates the contribution of the central doublet.

Fig. 2.

X-band EPR spectra of isolated packed mitochondria from WT and *mtm1* cells. Top panel: A, *mtm1*/O₂ at 10 K; B, same as A, but at 80 K. Microwave power was 20 mW. Middle panel (low-field region): A, W303/O₂; B, *mtm1*/O₂. Temperature and microwave power were 10 K and 0.2 mW, respectively. Bottom panel ($g = 2$ region): A, W303/O₂; B, *mtm1*/O₂; C, W303/Ar; D, *mtm1*/Ar. Temperature and power were 10 K and 0.2 mW. Microwave frequency of all spectra was 9.46 GHz.

Fig. 3. Electronic absorption spectra of mitochondrial suspensions from WT and *mtm1* cells. A, W303/O₂; B, *mtm1*/O₂; C, W303/Ar; D, *mtm1*/Ar; E, *mtm1*/Ar mitochondria isolated from *mtm1*/Ar cells that had been exposed to microaerobic environment before harvesting. Indicated absorbance change corresponds to that expected had spectra been collected in a 1 cm pathlength cuvette.

Fig. 4.

OxyBlot analysis, SOD activity assays and Western blotting against Sod2p. (a) OxyBlot of mitochondria isolated from aerobically grown W303 and $\Delta mtm1$ cells; (b) SOD activity assays and Western blotting against Sod2p for whole cell lysates from aerobically (O_2) and anaerobically (Ar) grown W303, $\Delta mtm1$ and Gal-Yah1 cells. All cells were grown on glucose.

Fig. 5.

5 K, 0.05 T Mössbauer spectra of W303 and *mtm1* cells grown under aerobic and anaerobic conditions. A, WT/O₂; B, *mtm1*/O₂; C, WT/Ar; D, *mtm1*/Ar. Red solid lines are simulations based on percentages in Table S1. Blue solid lines simulate the contribution of NHHS Fe^{II} species in the samples.

Fig. 6.

Mn and Fe traces from SEC-ICP-MS chromatography of soluble fractions of W303 and *mtm1* mitochondria. (a) HMM chromatograms of W303 mitochondria; (b) HMM chromatograms of *mtm1* mitochondria, Experiment 2. Red solid lines simulate Mn and Fe species that co-eluted with Sod2p (Table S4). The inset shows images of SOD2 activity gel, Western blot membrane against Sod2p for each fraction labeled with fraction volume, and SOD2 unit activities determined spectrophotometrically. Metal peaks were labeled according to their approximate molecular weight. Molecular mass was determined using a calibration plot for the Hi-Load Superdex 200 column. (c) LMM chromatograms of W303 (top) and *mtm1* (bottom) mitochondria, Experiment 4. Chromatographic intensities were adjusted proportionally to mitochondrial LMM Mn and Fe concentrations, and then WT-Mn and *mtm1*-Mn were amplified 20-fold for easier comparison.

Fig. 7. Left side, concentrations of MnSod2p (circles) and Mn₂₋₃ (stars) in intact mitochondria from WT and *mtm1* cells grown on media with ~ 2 and 200 μM Mn. Right side, the Mn content of corresponding mitochondrial extracts after sonication and centrifugation. Each circle and star represents 1 μM Mn concentration.

Fig. 8.

Off-line ICP-MS anion-exchange chromatograms of soluble W303 and *mtm1* mitochondria, Experiment 5. A, dotted line, salt gradient; B, W303, Mn concentrations in eluted fractions; C, *mtm1*, Mn; D, W303, Fe; E, *mtm1*, Fe. *Insets*, (i) W303, SOD2 activity gel and Western blot membrane against Sod2p from five adjacent fractions containing most Mn (*top*), SOD2 unit activity from corresponding fractions (*bottom*); (ii) *mtm1*, same as (i); (iii) W303, close-up view of Mn and Fe profiles in 16 – 23 mL region; (iv) *mtm1*, same as (iii).

Fig. 9.

On-line anion-exchange chromatograms of soluble (a) W303 and (b) *mtm1* mitochondria in Experiment 6. Salt gradient is shown in dotted line. Mn detector responses are multiplied by 10. *Insets*, SOD2 unit activity of 15 fractions around 30 mL (*above Fe trace*); images of SOD2 activity gel and Western blot membrane against Sod2p (*below Mn trace*), from every other fraction in 16 – 28 mL (*left*), from five adjacent fractions (28 – 31 mL) containing most Mn (*right*). Differently-migrating MnSod2p bands in native gel are indicated by arrows above the lane exhibiting a differently charged form (reading from right to left). Contrast for the Western blots of the 16 – 28 mL fractions is greater than for the 28 – 31 mL blots.

Fig. 10.

Mtm1p as an importer of a factor (presumed to be a small molecule) that helps mature apo-Sod2p, perhaps involving Mn insertion or protein folding. In the absence of Mtm1p, the rate of maturation slows which causes a build-up of Mn_{ASR} and immature Sod2p that eventually misfolds. The lack of functional MnSod2p increases the concentration of ROS in the matrix, which triggers the Fe accumulation phenotype.

Table 1

Properties of mitochondria and whole cells used in this study. All strains were grown on glucose-containing media.

	W303/O ₂	mtm1/O ₂	Gal-Yah1/O ₂	W303/Ar	mtm1/Ar
[protein] _{initio} (mg/ml)	79 ± 14 ^a (3) ^b	57 ± 9 (3)	-	70 ± 5 (2)	59 ± 11 (3)
[Fe] _{initio} (μM)	880 ± 250 (3) 620 ^c	3500 ± 400 (3) 5200 ^c	-	400 ± 20 (2)	440 ± 70 (3)
[Mn] _{initio} (μM)	13 ± 1 (3) 35 ^c	26 ± 9 (3) 80 ^c	-	8 ± 0 (2)	19 ± 3 (3)
Aconitase ^d (units/mg protein)	1500 ± 45 (2)	350 ± 69 (3)	-	1500 ± 350 (2)	1100 ± 130 (3)
SDH ^d (units/mg protein)	23 ± 4 (2)	2 ± 1 (3)	-	7 ± 1 (2)	8 ± 3 (3)
[Fe] _{cell} (μM)	640 ± 98 (3) 1000 ^c	4900 ± 120 (2) 2400 ^c	-	640 ± 13 (2)	660 ± 190 (2)
[Mn] _{cell} (μM)	35 ± 8 (3) 110 ^c	41 ± 1 (2) 75 ^c	-	39 ± 1 (2)	37 ± 7 (2)
Leu1p ^e (units/mg protein)	48 ± 8 (3)	30 ± 13 (3)	-	100 ± 13 (3)	41 ± 10 (3)
Relative SOD2 activity [*]	100	26 ± 6 (3)	89 ± 17 (3)	66 ± 7 (3)	14 ± 3 (3)
Relative Sod2p expression level [*]	100	71 ± 12 (3)	108 ± 28 (3)	77 ± 19 (3)	36 ± 5 (3)
Relative Specific SOD2 activity ^{**}	100	42 ± 16 (3)	87 ± 11 (3)	102 ± 34 (3)	42 ± 12 (3)

^a Standard error;

^b Number of batches;

^c 200 μM Mn-supplemented medium;

^d mitochondrial suspensions;

^e cell lysates;

^{*} Activities and protein expression level were quantified from activity gels and western blotting membranes;

^{**} Specific activity = relative SOD2 activity divide by the relative Sod2p expression level.