Role of YHM1, encoding a mitochondrial carrier protein, in iron distribution of yeast

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Mitochondrial carrier proteins are a large protein family, consisting of 35 members in Saccharomyces cerevisiae. Members of this protein family have been shown to transport varied substrates from cytoplasm to mitochondria or mitochondria to cytoplasm, although many family members do not have assigned substrates. We speculated whether one or more of these transporters will play a role in iron metabolism. Haploid yeast strains each deleted for a single mitochondrial carrier protein were analysed for alterations in iron homoeostasis. The strain deleted for YHM1 was characterized by increased and misregulated surface ferric reductase and high-affinity ferrous transport activities. Siderophore uptake from different sources was also increased, and these effects were dependent on the AFT1 iron sensor regulator. Mutants of YHM1 converted into rho−, consistent with secondary mitochondrial DNA damage from mitochondrial iron accumulation. In fact, in the ∆yhm1 mutant, iron was found to accumulate in mitochondria. The accumulated iron showed decreased availability for haem synthesis, measured in isolated mitochondria using endogenously available metals and added porphyrins. The phenotypes of ∆yhm1 mutants indicate a role for this mitochondrial transporter in cellular iron homoeostasis.

Key words: carrier protein, haem, iron, mitochondria, Saccharomyces, yeast.

INTRODUCTION

Iron in cells exists primarily in complex with sulphur (Fe–S clusters) or in complex with porphyrin (haem). These critical cofactors mediate many of the myriad functions of iron proteins, including oxidation–reduction, electron transfer and oxygen binding [1]. Iron entering eukaryotic cells from the environment must travel to mitochondria, where haem [2,3] and Fe–S–clusters [4] are made. Iron cofactors must reach proteins in various cellular compartments and, thus, the traffic of iron and iron cofactors into and out of mitochondria are critically important processes. However, molecular mediators for these processes are largely unidentified [4].

In an effort to identify genes that might be involved, we examined a collection of yeast mutants, each deleted for a single member of the mitochondrial carrier family [5]. The mutant collection was screened for abnormal regulation of cellular iron uptake. The rationale for this approach was derived from previous experience demonstrating effects of Fe–S cluster assembly mutants on cellular iron uptake. Mutants of Nfs1p [6,7], Ssq1p [8], Jac1p [9] and other components of the Fe–S cluster assembly machinery [4,10–12], all with principal mitochondrial localizations, were detected by associated increases in cellular ferric reductase and ferrous transport. These activities, involved in iron acquisition and usually repressed during growth in iron adequate media, were inappropriately expressed in Fe–S cluster assembly mutants. The misregulated activities were dependent on the Aft1p iron sensor regulator [6].

MCPs (mitochondrial carrier proteins) constitute a large protein family with 35 members encoded by the yeast nuclear genome [13]. Features of the protein family are integral membrane association and conserved sequence elements. Within cells, most of these proteins are found in the mitochondrial inner membrane. However, one family member is localized to the peroxisomal membrane [14]. Homologues of the yeast ATP/ADP translocators, which belong to this protein family, are found in hydrogenosomes (trichomonas) [15] and amyloplasts (maize) [16]. The topology of family members expressed in mitochondria is shared with the N- and C-termini of the nuclear-encoded polypeptides orientated towards the intermembrane space. The sequences include a tripartite structure consisting of three segments of 100 amino acids, each one containing two membrane-spanning domains and an intervening loop sequence. The proteins form homodimers and they function as exchangers or co-transporters at the mitochondrial inner membrane. A recent low-resolution crystal structure of the ATP/ADP carrier showed homodimerization and 3-fold symmetry in agreement with the 3-fold sequence repeats [17]. The substrates transported by carrier proteins of this family are extremely varied and include adenine nucleotides, phosphate, dicarboxylic acids, tricarboxylic acids, flavins, CoA, arginine-ornithine and pyruvate. The prototype protein for which most data exist is the ATP/ADP carrier. The substrates for particular MCPs were often identified by hints from the phenotypes of the corresponding yeast mutants. In some cases, the hints were straightforward, as in arginine auxotrophy caused by a defect in the arginine-ornithine transporter ARG11 [18], and in other cases the hints were less straightforward, as for leucine auxotrophy associated with the CoA transporter LEU5 [19]. A number of transporters have been studied by incorporation of purified proteins into liposome vesicles, which then reconstituted specific transport activities (e.g. Arg11p for ornithine). Recently, MCPs have been implicated in metal transport, although none has been studied yet by reconstitution. A carrier protein has been shown to mediate transport of iron into mitochondria [20,21] and delivery of iron to the iron sensor regulator AFT1 [22].

Abbreviations used: BPS, bathophenanthroline disulphonic acid; DAPI, 4,6-diamidino-2-phenylindole; ENB, enterobactin; FCH, ferrichrome; FOB, ferrioxamine B; MCP, mitochondrial carrier protein; mtDNA, mitochondrial DNA; PP, protoporphyrin; TAF, triacetylfusarinine C; Zn-PP, zinc PP.

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of manganese to Sod2p in the mitochondrial matrix [22]. To date, more than half of the family members do not have substrates identified [13].

In the present study, we evaluated the regulated expression of ferric reductase and ferrous transport in a collection of yeast mutants, each deleted for a single MCP. We noted major effects of the deletion of YHM1. We observed further a rapid and preferential accumulation of iron in mitochondria of this mutant, suggesting that the carrier plays a role in cellular iron metabolism.

**EXPERIMENTAL**

**Growth media**

Rich media consisted of 1% (w/v) yeast extract, 2% (w/v) peptone, 100 µg/ml adenine and various carbon sources. In most experiments, 2% (w/v) glucose was used as the carbon source (YPAD). In some experiments, cells were precultivated in 2% (w/v) raffinose (YPAR) to induce mitochondrial biogenesis. For assessment of respiratory function, cells were grown on agar plates containing 3% (v/v) ethanol as a non-fermentable carbon source (YE). The deletion strains were grown in YPAD with 200 µg/ml G418 added to the medium to select for the KAN cassette used in knockout construction [23]. For ferrous iron uptake and ferric reductase experiments, 50 µM copper sulphate was added to YPAD.

**Yeasts strains and crosses**

The parental strains BY4741 and BY4742 were exposed to ethidium bromide to ablate mtDNA (mitochondrial DNA), creating BY4741 rho− and BY4742 rho− respectively [24]. The haploid MCP mutants in the BY4741 background were obtained from the EUROSCARF (European Saccharomyces cerevisiae Archive for Functional Analysis, Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany) collection or from Invitrogen (Carlsbad, CA, U.S.A.). Assignment to the MCP family was as described by Belenkiy et al. [13]. The Δleu5 strain was not available and was omitted from the screening. RIM2 and PET9 were listed as essential genes, and we therefore obtained the diploids and sporulated them. The Δpet9 spore clones were non-viable, but Δrim2 spore clones grew into small colonies and several clones were included in the screening. An nfs1-14 strain was created by gene transplacement of a 5 kb BamHI–EcoRI fragment containing the mutant allele and flanking LEU2 gene into strain YPH499. Crosses of haploid strains were performed by micromanipulation of zygotes. Sporulation and tetrad dissection were performed according to published methods [25]. For creating the double Δyhm1 Δaft1 strain, a URA3 cassette inserted into a unique HindIII site in the genome of strain CM3260Aft1 [9] was amplified with flanking sequences and used to transform strain BY4741Δyhm1 and control strains. The correctness of the integration was verified with PCR primers from outside the insertion site.

**Biochemical assays**

Ferric reductase and ferrous iron transport assays were performed as a combined microtitre plate assay. The controls and mutants were inoculated in triplicate in a 96-well plate in YPAD containing 50 µM copper sulphate. After growth for 16 h at 30 °C, the contents of each well were diluted 10-fold into fresh medium of the same composition, and the cells were allowed to reach exponential growth (4 h). The microtitre plates were cooled by floating in an ice-water bath, and the cells were washed free of medium by centrifuging and resuspending in ice-cold citrate buffer (50 mM sodium citrate, pH 6.5/5% glucose). After several washes, the cells were resuspended in 100 µl of citrate buffer for measurement of turbidity (absorbance A at 720 nm). While setting up the assays, samples were maintained on ice. For ferric reductase, 90 µl of the cells were incubated with 1 mM ferric ammonium sulphate and 1 mM BPS (bathophenanthroline disulphonic acid) for 1 h at 30 °C, and the reaction was stopped by the addition of 50% trichloroacetic acid. Cells were removed by centrifugation and the A515 of the ferrous iron–BPS complex was measured. For ferrous iron uptake, 10 µl of cells were added to the 90 µl of iron labelling solution consisting of 1 µM 55Fe (50 Ci/g; Amersham Biosciences, Piscataway, NJ, U.S.A.), 1 mM sodium ascorbate and 50 mM sodium citrate. The uptake was allowed to proceed for 1 h at 30 °C and was terminated by harvesting the cells on 96-well glass-fibre filters, using a Wallac cell harvester. The cells retained on the filter were washed free of unincorporated iron with water, dried and soaked in a liquid-scintillation fluid (Betalplate Scint; Wallac, PerkinElmer Life Sciences, Boston, MA, U.S.A.). The radioactivity was measured by scintillation counting (Wallac 1450 Microbeta). Results are reported as c.p.m. · (106 cells)−1 · h−1. A similar assay was performed for measuring cellular iron uptake from radiolabelled siderophores; in this experiment, radioactivity retained on filters was measured in the presence of a solid sheet of scintillant (MeltiLex, Wallac). The m-syrtine derivative of FOB (ferrioxamine B; Desferal, Novartis) was radiolabelled, and FCH (ferrichrome), TAF (triacetyl fusarinine C) and ENB (enterobactin) were purchased from Biophore Research Products (Tubingen, Germany) and radiolabelled [26]. Aconitase was assayed by measuring the formation of cis-aconitate at 240 nm as described in [27]. Succinate dehydrogenase was measured as described in [28]. Cells for the isopropylamate isomerase (Leu1p) activity assay were grown in the defined medium and lysates were made by vortex-mixing of cells in a microcentrifuge tube to which glass beads were added. Protein concentration was determined by measurement with bicinchoninic acid (Pierce, Rockford, IL, U.S.A.). The substrate citraconic acid was purchased from Aldrich and pH was adjusted to 7.0 with sodium hydroxide. Substrate was added at 4 mM and the rate of decline of A235 was observed. Activity reflecting disappearance of citraconate substrate was expressed in terms of µmol · min−1 · (mg of protein)−1 or units. Details of the assay have been described by Kohlhaw [29].

**Antibodies**

The open reading frame for Isu1p was cloned into pET21b and expressed in Escherichia coli BL21(DE3) codon plus. Protein was purified using a His6 tag, and rabbits were injected for the generation of polyclonal antibodies directed against Isu1p. Antibodies to Aco1p [12], Ssq1p [9], Yfh1p [30] and Nfs1p [6] have been described previously. Rabbit polyclonal antibody against Ccp1p was a gift from Dr Debkumar Pain (Department of Pharmacology and Physiology, UMDNJ-New Jersey Medical School, Newark, NJ, U.S.A.). Mouse monoclonal antibodies against porin, Porlp (mitochondria), Pgp1p (cytoplasm), Dpm1p (endoplasmic reticulum) and Cpy1p (vacuole) were purchased from Molecular Probes (Eugene, OR, U.S.A.).

**Radiolabelling of yeast cells with 55Fe and cell fractionation**

Cells were grown in rich media (YPAD) to A600 1.0. At this time, 55Fe (200 nM final, 50 Ci/g) was added directly to the medium containing growing cells. Cultures were returned to the incubator during the 1 h of labelling. The cultures were then cooled in an ice-water bath, harvested and washed several
times with sodium citrate buffer (50 mM, pH 6.5) to remove any unincorporated counts. The wash buffer was tested and contained negligible radioactivity. Before cell fractionation, an aliquot of washed cells was removed for scintillation counting in a Beckman LS-6500 with ScintiSafe Econo 1 LSC Cocktail (Fisher Scientific, Pittsburgh, PA, U.S.A.). Cell-wall digestion was performed for 20 min with Zymolyase 100T. A crude mitochondrial fraction was isolated as described in [31] after douncing with 15 strokes while on ice. The post-mitochondrial fraction was centrifuged at 100 000 g for 20 min. The supernatant following centrifugation was analysed as the cytoplasmic fraction. Mitochondria were purified further as follows. A portion of mitochondria, equivalent to 5 mg of protein, was layered on top of a step gradient consisting of 2 ml of 40 % (v/v) Percoll in 50 mM Tris/HCl (pH 7.5), 0.6 M sorbitol and 8 ml of 20 % Percoll in the same buffer. Gradients were centrifuged at 105 000 g in a Beckman Sw41Ti swinging bucket rotor for 30 min at 4 °C. The mitochondria were collected from the 20–40 % interface with a Pasteur pipette and washed three times in 15 ml of mitochondrial isolation buffer to remove the Percoll. A portion of the mitochondria and cytoplasm fractions were suspended in 50 mM Tris/HCl (pH 7.5), 0.6 % SDS and analysed for iron content by scintillation counting and for protein content by bichinchoninic acid assay (Pierce). The purified mitochondrial fractions were reactive with antibodies to mitochondrial proteins, but non-reactive with antibodies to vacuolar (anti-Cpy1p), cytoplasmic (anti-Pgk1p) or endoplasmic reticulum (anti-Dpm1p) marker proteins.

Iron release from mitochondrial lysate
Isolated mitochondria were lysed in 0.6 M sorbitol, 10 mM Tris/HCl (pH 7.4) and 0.1 % Tween 80. After lysis, 5 mM sodium ascorbate and 200 µM BPS were added. A
_{435}
 was measured at 25 °C over time and the concentration of the ferrous iron–BPS complex was calculated.

Haem synthesis in intact isolated mitochondria
Isolated mitochondria (0.2 mg of protein/ml) in 0.6 M sorbitol with 50 mM Tris/HCl (pH 7.4), with or without 10 mM EDTA, and no added metals were examined. The reaction was initiated by adding 2 µM protoporphyrinogen. PP (protoporphyrin IX, excitation at 410 nm and emission at 632 nm) or Zn-PP (zinc-PPIX, excitation at 420 nm and emission at 587 nm) formation was studied continuously by their fluorescent emissions. Haem formation was calculated as PPIX formed in the presence of EDTA minus PPIX and Zn-PPIX formed in the absence of EDTA [32]. Protoporphyrinogen was prepared from PP by reduction with a sodium amalgam [33].

Microscopy
Strains were grown for 6 days on YPAR agar plates. Cells were fixed in 70 % ethanol, washed and resuspended in PBS. DNA was stained by adding 0.2 µg/ml DAPI (4,6-diamidino-2-phenylindole) and the cells were attached to polylysine-treated slides and mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, U.S.A.). Cells were viewed with a Nikon Eclipse E800 microscope with a × 60 objective and × 2.5 magnifier tube. Images were recorded with Hamatsu C4742 digital camera and Image-Pro Plus software.

Other methods
SDS/PAGE, immunoblotting and signal development using ECL® (Amersham Biosciences) were performed according to standard methods.

RESULTS
Haploid yeast strains, each deleted for a single MCP, were obtained from the yeast genome deletion collection [5]. Of the 35 predicted MCPs encoded in the yeast genome, we were able to obtain haploid deletion strains for 33. The Δleu5 knockout was unavailable and the Δpet9 knockout was non-viable. RIM2 was listed as an essential gene. However, when the diploid knockout for RIM2 was sporulated, tetrad clones carrying the deletion were found to be viable albeit slow growing, and these were included in the screening assays. The parental strains, BY4741 and BY4742, were included as controls, as were congenic rho° strains made by ethidium bromide treatment. Also, a strain with the nfs1-14 allele transplanted into the genome was included as a control. NFS1 encodes a cysteine desulphurase with a primarily mitochondrial localization, and the nfs1-14 mutation is associated with increased cellular ferric reductase and ferrous iron uptake activities [6].

We previously found that mutants with altered iron distribution between mitochondria and cytoplasm exhibit abnormal increases in these activities [8]. Therefore the yeast MCP mutants and control yeast strains were analysed for cell-surface ferric reductase and high-affinity ferrous transport. These are independent measurements of regulated activities, which in the normal cell are homoeostatically regulated in response to iron availability. In the screening assays (Figure 1), wild-type strains of both mating types and rho° isolates showed similar low levels of ferric reductase and ferrous transport. Marked increases in ferric reductase and ferrous transport were observed in the Δxphm1 (YDL198C) mutant, and these activities were similar to levels observed in the nfs1-14

Figure 1 Screen of MCP mutants for iron regulatory phenotypes
Yeast strains (column no. in parentheses) BY4741 (1), BY4741 rho° (2), BY4742 (3), BY4742 rho° (4), nfs1-14/LEU2 (5), YBR077C Δmir11 (6), YMR056C Δaac1 (7), YBR085W Δaae3 (8), YOR130C Δarg11 (9), YDR100C Δadc1 Δaad1 (10), YBR291C Δpct1 (11), YBR086W Δacr1 (12), YKL120W Δacr1 Δpmrf1 (13), YUR34B Δacr1 (14), YLL134W Δfrr1 (15), YMR241W (16), YPR011C (17), YNL038W Δpmrf2 (18), YGR190W Δfrr2 (19), YOR222W (20), YPL134C Δacr1 (21), YPR058W Δpmrf1 (22), YBR104W Δpmrf2 (23), YLL133W Δmsr3 (24), YKR052C Δmsr4 (25), YNL003C Δpet8 (26), YEL006W (27), YL006W (28), YBR102W Δrim2 (29), YCR128C (30), YER035C (31), YFR045W (32), YPR210C (33), YMR166C (34), YGR257C (35), YDL119C (36), YDL198C Δxphm1 (37) and YDR470C Δa01p (38) were grown at 30 °C in YPAD with 50 µM copper sulphate before measuring ferric reductase (A) and ferrous iron uptake (B). Strains 1–5 represent controls. Strains 6–38 are MCP mutants in the order presented in [13].

Δmsr3 (24) is shown with a (●) and Δxphm1 (37) is shown with a (*) above the corresponding bar graphs. Results are medians for triplicate measurements. For reductase measurements, S.D. < 15 % with the exception of Δrim2 (29), which showed a 42 % S.D. For ferrous uptake measurements, S.D. < 15 % with the exception of Δrim2 (29), which again showed a 42 % S.D., YER053C (31), showed a 19 % S.D. and Δa01p (38), showed a 17 % S.D.
mutant (Figure 1). The Δmrs3 (YJL133W) deletion was also clearly abnormal. Ferrous uptake activity was increased compared with the parental strains, and ferric reductase was also increased. Similar effects were not observed in the mutant deleted for the homologous gene Δmrs4 (YKR052C) (Figure 1). MRS3 and MRS4 have previously been linked to iron metabolism [20,21]. YHM1 was previously identified as a high copy suppressor of mtDNA instability associated with an ABF2 deletion, but YHM1 has not been previously associated with iron metabolism [34].

The markedly increased ferric reductase and ferrous transport activities associated with the Δyhm1 mutation were evaluated further. The Δyhm1 strain was crossed with the parental strain of opposite mating type and the diploid was sporulated. The heterozygote had no detectable phenotype, indicating that the iron regulatory phenotype was recessive. Products of four individual meioses were dissected and analysed. The clones carrying the Δyhm1::KAN knockout were identified by their G418 resistance. The segregants exhibited increased ferric reductase and ferrous transport activities in 2ΔAf1+Δyhm1;ΔAf1−yhm1−rhoΔ strains compared with the rhoΔ control. However, ΔAf1−yhm1 strains were transformed with a cassette to interrupt AFT1. The double mutant showed ferric reductase and ferrous transport activities that were no longer increased compared with the wild-type control strain (Figure 2A). Thus the effect of the Δyhm1 deletion on cellular iron uptake was entirely dependent on the presence of an intact copy of AFT1.

Ferric reductase and ferrous transport activities vary in response to iron levels through control of AFT1 and its parologue AFT2 [36,37]. Iron uptake from siderophores occurs by a separate pathway, also under AFT1/AFT2 control, mediated by four distinct transporters [38–40]. This non-reductive pathway was induced in the Δyhm1 mutant as reflected by increased iron uptake from different siderophores. Iron uptake in the mutant was increased compared with the wild-type control strain (Figure 2B). Therefore a double-mutant strain was constructed lacking both YHM1 and AFT1. The Δyhm1 deletion on cellular iron uptake was entirely dependent on the presence of an intact copy of AFT1.
strains. Thus the \( \Delta yhm1 \) mutant was in fact \( \text{rho}^{\circ} \). Furthermore, the fact that the phenotype recurred in the mutant tetrads following a backcross showed that the effect was a consequence of the nuclear mutation. Iron accumulation in mitochondria has been shown to mediate mtDNA damage in some mutants lacking critical components for Fe–S cluster synthesis. Therefore we considered that the \( \Delta yhm1 \) mutant might be accumulating mitochondrial iron.

Cellular iron distribution was tested in wild-type, \( \text{rho}^{\circ} \) and \( \Delta yhm1 \) strains following a pulse of radioactive iron. A \( \text{rho}^{\circ} \) control was included because \( \Delta yhm1 \) mutants were \( \text{rho}^{\circ} \). Strains were grown to exponential phase in YPAD, and radioactive iron was added directly to the growing culture as a tracer for 1 h. After washing to remove unincorporated counts, radioactivity was assessed in whole cells. Subsequently, the cells were separated into cytoplasmatic and pure mitochondrial subcellular fractions for measurement of radioactive iron and protein concentration. As observed previously, whole cell radioactive iron was increased in the \( \Delta yhm1 \) mutant compared with the controls (Figure 4A). Cytoplasmic iron in the \( \Delta yhm1 \) mutant was only slightly increased (1.3-fold) compared with the \( \text{rho}^{\circ} \) strain (Figure 4B). In contrast, mitochondrial iron in the \( \Delta yhm1 \) mutant was increased 26-fold (Figure 4C). The increase in mitochondria was out of proportion to the increase in total cellular iron (Figure 4D).

The maldistribution of cellular iron observed following a short pulse of radioactive iron was also observed at steady state (24.5 nmol iron/mg of mitochondrial protein in the mutant compared with 2.1 nmol/mg in the wild-type). The characteristics of mitochondrial iron accumulating in the mutant were examined. When mitochondrial lysates from wild-type and mutant were exposed directly to the ferrous iron chelator BPS, no coloured ferrous complex appeared indicating that ferrous iron was not present or was not accessible to the chelator. After addition of ascorbic acid as a reductant, the coloured ferrous iron–BPS complex appeared over time (Figure 5), indicating progressive reduction and mobilization of mitochondrial ferric iron species. The signal was markedly increased in the mutant, consistent with the increased amount of iron in the mutant mitochondria. A \( \text{rho}^{\circ} \) control was indistinguishable from the \( \Delta yhm1 \) mutant (results not shown).

In some respects, the \( \Delta yhm1 \) mutant appeared to phenocopy mutants involved in Fe–S cluster synthesis, exhibiting increased iron uptake and iron accumulation in mitochondria. Therefore we tested the status of cellular Fe–S cluster proteins in the \( \Delta yhm1 \) mutant. The results did not support a generalized or a severe compartmental defect in Fe–S cluster proteins. The mutant had a marked decrease in aconitate, and activities were 98, 178 and 30 nmol of \( cis \)-aconitate formed \( \cdot \) (mg of mitochondrial protein)\(^{-1}\) min\(^{-1}\) for wild-type, \( \text{rho}^{\circ} \) and \( \Delta yhm1 \) respectively. In contrast, succinate dehydrogenase, an Fe–S and haem-containing complex of the mitochondrial inner membrane, was not decreased in the mutant. Levels were 2.6, 0.78 and 1.7 nmol of reduced \( p \)-iodonitrotetrazolium violet substrate formed \( \cdot \) (mg of mitochondrial protein)\(^{-1}\) min\(^{-1}\) for wild-type, \( \text{rho}^{\circ} \) and \( \Delta yhm1 \) respectively. Isopropylmalate isomerase or Leu1p activity in a cytoplasmic lysate was decreased to 45% compared with the \( \text{rho}^{\circ} \) control. The measured activities were 0.21, 0.076 and 0.035 \( \mu \)mol \( \cdot \) min\(^{-1}\) \( \cdot \) (mg of protein)\(^{-1}\) for wild-type, \( \text{rho}^{\circ} \) and \( \Delta yhm1 \) respectively [29].

We wanted to test the nature of iron accumulating in the mutant mitochondria, and to do this haem synthesis from endogenous metals was measured in isolated wild-type and mutant mitochondria. In this experiment (Figure 6), isolated mitochondria were exposed to protoporphyrinogen, allowing for in situ formation of the fluorescent PPIX intermediate. In the presence of metal chelator, the final step of haem synthesis (PPIX to

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**Figure 3** Nuclear mutation of YHM1 (\( \Delta yhm1 \)) associated with loss of mtDNA

(A) Genetic evidence of mtDNA damage. A backcross of the \( \Delta yhm1 \) mutant with a parental strain of opposite mating type, BY4742, was sporulated and \( \Delta yhm1 \) clones were identified by G418 resistance conferred by the knockout cassette. Each of four tetrad clones from a single meiosis was crossed with rho\(^{\circ} \) testers of opposite mating type. Three zygotes from each cross were manipulated and analysed for growth on YE (ethanol) or YPAD (glucose). Failure of the zygotes to grow on ethanol plates is diagnostic of mtDNA inactivation (see tetrads c and d, zygotes 1, 2 and 3). (B) Microscopic evidence of mtDNA loss. Wild-type (squares 1 and 4), \( \Delta yhm1 \) (squares 2 and 5) and rho\(^{\circ} \) (squares 3 and 6) were grown for 6 days on YPAR agar plates. Cells were stained with DAPI and viewed under UV light to visualize DNA (squares 1–3) and differential interference contrast to show cell morphology (squares 4–6).

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The experiment performed with the rho° strain showed no difference from the wild-type control (results not shown). We considered that deficiency in haem synthetic activity in the Δyhm1 mutant might be owing to deficiency of ferrochelatase. However, this was ruled out, because ferrochelatase activity measured with added ferrous ascorbate, protoporphyrinogen and mitochondrial lysate was indistinguishable from wild-type activity. Thus the decrease in haem synthesis indicated that the large amount of accumulated iron in the Δyhm1 mutant was unavailable for haem synthesis.

Protein levels in wild-type, rho° and Δyhm1 mutant mitochondria were examined by immunoblotting of isolated mitochondria with selected antibodies. As a control, we performed blotting with antibody to the outer-membrane protein porin, and porin levels were indistinguishable in the three strains (Figure 7). Ccp1p, cytochrome c peroxidase, a haem protein of the intermembrane space [41], was decreased in the Δyhm1 mutant. Aco1p and aconitase were also decreased consistent with lower aconitase activity of the mutant. In contrast, aconitase activity and Aco1p protein were increased in the rho° control compared with the wild-type. Proteins involved in Fe–S cluster assembly [4] were tested for their abundance by immunoblotting. Isu1p, a scaffold protein involved in assembling Fe–S cluster intermediates [10], was increased in Δyhm1 when compared with the wild-type, although a similar increase was observed for the rho° control. Yfh1p, the yeast frataxin homologue, was decreased when compared with wild-type and rho° controls. Levels for Nfs1p (cysteine desulphurase) and Ssq1p (chaperone protein), were unchanged in the Δyhm1 strain. Low-temperature spectra of whole cells showed deficiencies of b and c type cytochromes in the Δyhm1 mutant and rho° strain that were similar in degree (Figure 8).

DISCUSSION

Iron uptake systems have been characterized, which function at the cell surface [42]. Fe–S cluster forming [43] and haem synthesis [3] activities have been characterized in mitochondria. However, iron transport and transfer processes, which link these two areas of the cell have not been defined. In the present study, we screened MCP mutants for effects on iron homoeostasis.

MCPs are a large family of proteins including 35 members in S. cerevisiae, which are localized in yeast. They have been found in the mitochondrial inner membrane although one exceptional family member was found in peroxisomal membranes [13].
Figure 6  Haem synthesis from endogenous metals is decreased in isolated mitochondria of Δyhm1 cells

Mitochondria were isolated from wild-type and Δyhm1 mutants grown in glucose medium. Isolated mitochondria (0.2 mg of protein/ml) were suspended in buffer consisting of 0.6 M sorbitol with 50 mM Tris/HCl (pH 7.4), with (A) or without (B–D) 10 mM EDTA. No metals were added. The reactions were initiated by adding 2 µM protoporphyrinogen to the suspended mitochondria. Fluorescence from the formation of PPIX, excitation at 410 nm and emission at 632 nm (A, B) or Zn–PPIX, excitation at 420 nm and emission at 587 nm (C) were followed continuously. Haem formation was calculated as PPIX formed in the presence of EDTA minus PPIX + Zn-PPIX formed in the absence of EDTA (D). ■, Δyhm1; ○, WT. The data are from a single experiment.

Family members have been implicated in the transport of varied substrates into and out of mitochondria [44] and, therefore, we reasoned that such transporters of this family with undefined substrates might influence iron homeostasis. We found that the Δyhm1 mutant of yeast, lacking one of these carriers, exhibited abnormally high iron uptake activities and misregulation of the high-affinity iron uptake systems. Iron taken up into the mutant cells rapidly accumulated in mitochondria, leaving the cytoplasm relatively iron-depleted.

We cannot say with certainty if these effects are direct or indirect. In the general scheme of cellular iron metabolism, iron must enter eukaryotic cells via specific permeases and then traverse cytoplasm, and external and internal mitochondrial membranes. Within mitochondria, critical transformations occur, creating haem and Fe–S clusters, and other biologically important forms of iron may also be made here. Subsequently, iron in various forms must exit mitochondria to reach structural and regulatory apoproteins in different extra-mitochondrial locations. Yhm1p in the mitochondrial inner membrane might directly transport iron from mitochondria to cytoplasm as haem, Fe–S or other form. Alternatively, Yhm1p might influence iron uptake and distribution indirectly, by transport of molecules that regulate these functions.

Many features of the Δyhm1 mutant resemble features of Fe–S cluster assembly mutants. These mutants are deficient in mitochondrial proteins implicated in Fe–S cluster assembly. A large body of work has led to identification of more than 12 complementation groups of yeast mutants [43]. These mutants exhibit inappropriate activation of ferric reductase and ferrous transport [6], similar to Δyhm1 mutants. The effects are mediated by AFT1, the sensor regulator responsible for transducing iron signals into regulated gene expression. Similarly, the misregulated high-affinity iron uptake in Δyhm1 mutants was AFT1 dependent and abrogated by AFT1 deletion. Within the cells of Fe–S
cluster assembly mutants, iron distribution shows abnormal accumulations in mitochondria and relative depletion in cytoplasm, also similar to Δyhm1 mutants. Finally, iron accumulation in Fe–S cluster assembly mutants has been linked to mtDNA damage and loss. The proposed mechanism involves generation of oxygen intermediates such as hydrogen peroxide, which in turn produce reactive radicals in the presence of iron (Fenton chemistry) that damage DNA [45,46]. Such a mechanism might also be operative here, with iron accumulation in Δyhm1 mitochondria producing toxic-free radicals, DNA damage and finally complete loss of mtDNA. A prior characterization of the Δyhm1 phenotype did not observe mtDNA instability, although this difference may derive from genetic background effects [34].

The Δyhm1 mutant did not phenocopy the Fe–S cluster assembly mutants entirely. As distinguished from mutants involved in Fe–S cluster assembly, Fe–S cluster protein activities were inconsistently affected in Δyhm1 mutants. In Δyhm1 mutants, decreases in aconitase but increases in succinate dehydrogenase activity, dependent on the formation of an Fe–S cluster on the cytoplasmic protein Leu1p, was moderately decreased in the mutant compared with the rho° control. A virtual total deficiency of this enzyme was used to demonstrate a role for the Atm1p transporter in mediating export of a key component for cytoplasmic Fe–S cluster synthesis [7]. Disparate effects of the Δyhm1 mutation on proteins mediating Fe–S cluster assembly were noted. Isu1p was increased, whereas Yth1p was decreased. Nfs1p and Ssq1p were unchanged.

Effects on haem in the Δyhm1 strain was also observed. Haem synthesis in isolated mitochondria measured from endogenous metals and exogenous porphyrins was decreased, despite the marked increase in iron within the organelle. Thus iron accumulating in the Δyhm1 mutant was not fully available for use in haem synthesis. The defect here was much less severe when compared with that in frataxin mutants, in which haem synthesis from endogenous iron was virtually undetectable owing to a block in delivery of iron to ferrochelatase in the mitochondrial matrix [32]. The rho° strain showed marked deficiencies in cytochromes, making the specific effects of Δyhm1 difficult to extraplate. In Δyhm1 mutants, cyochrome c peroxidase, a haem protein of the mitochondrial intermembrane space, was decreased further compared with the rho°.

Recent work has implicated the MCPs, Mrs3p and Mrs4p, in transport of iron into mitochondria. The double mutant in MRS3 and MRS4 (Δmrs3/4) abrogated mitochondrial iron accumulation of the Δyfh1 mutant [20]. Studies using isolated mitochondria demonstrated a direct role for the corresponding proteins in iron transport and delivery for haem and Fe–S cluster formation in mitochondria [47]. Future work on Yhm1p will seek to address whether it is an iron transporter or transporter of a substrate that indirectly influences iron metabolism.

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REFERENCES

Mitochondrial carriers and iron


