Adrenodoxin Reductase Homolog (Arh1p) of Yeast Mitochondria Required for Iron Homeostasis*

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Arh1p is an essential mitochondrial protein of yeast with reductase activity. Here we show that this protein is involved in iron metabolism. A yeast strain was constructed in which the open reading frame was placed under the control of a galactose-regulated promoter. Protein expression was induced by galactose and repressed to undetectable levels in the absence of galactose, although cells grew quite well in the absence of inducer. Under noninducing conditions, cellular iron uptake was dysregulated, exhibiting a failure to repress in response to medium iron. Iron trafficking within the cell was also disturbed. Exposure of Arh1p-depleted cells to increasing iron concentrations during growth led to drastic increases in mitochondrial iron, indicating a loss of homeostatic control. Activity of aconitase, a prototype Fe-S protein, was deficient at all concentrations of mitochondrial iron, although the protein level was unaltered. Heme protein deficiencies were exacerbated in the iron-loaded mitochondria, suggesting a toxic side effect of accumulated iron. Finally, a time course correlated the cellular depletion of Arh1p with the coordinated appearance of various mutant phenotypes including dysregulated cellular iron uptake, deficiency of Fe-S protein activities in mitochondria and cytoplasm, and deficiency of hemoproteins. Thus, Arh1p is required for control of cellular and mitochondrial iron levels and for the activities of Fe-S cluster proteins.

Iron is required as a cofactor for critical proteins that mediate diverse processes such as cellular respiration and synthesis of metabolic intermediates (1). However, iron is also extremely toxic, capable of generating free radicals that damage proteins, lipids, and DNA (2). Thus, eukaryotic cells such as yeast regulate iron uptake into cells in homeostatic fashion, inducing uptake in response to iron starvation, and repressing uptake in response to iron overload (3). Several mutant strains of yeast have been identified that exhibit dysregulation of cellular iron uptake, deficiency of Fe-S protein activities in mitochondria and cytoplasm, and deficiency of hemoproteins. Thus, Arh1p is required for control of cellular and mitochondrial iron levels and for the activities of Fe-S cluster proteins.

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§ The abbreviations used are: Fe-S, iron-sulfur cluster; HA, hemagglutinin antigen epitope; PCR, polymerase chain reaction.

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Arh1p in Iron Homeostasis

drial iron-sulfur protein activities (20). Atm1p depletion results in iron trafficking abnormalities, deficient cytoplasmic Fe-S proteins but normal mitochondrial Fe-S proteins (7).

Arh1p is an essential yeast protein with reductase activity localized to the inner mitochondrial membrane (21, 22). Arh1p lacks a bacterial homolog on the ice operon. In this work, we demonstrate a role for Arh1p in cellular and mitochondrial iron homeostasis.

**EXPERIMENTAL PROCEDURES**

*Growth Media*—Rich medium consisted of 1% yeast extract, 2% peptone, and various carbon sources. To induce the GAL1 promoter, 2% raffinose and 0.5% galactose served as the carbon source. Expression from the GAL1 promoter was turned off by shifting the cultures to identical medium without galactose. In some experiments, 2% glucose or 3% ethanol was used as carbon source. For experiments with different concentrations of iron, standard defined medium with 2% raffinose or 2% glucose was modified by addition of ferric ammonium sulfate.

*Plasmids and DNA Constructions*—The open reading frame of Arh1p was amplified by PCR using Pfu polymerase and genomic DNA as a template. The primers added XhoI and NdeI sites on the 5’ end and BglII and Xhol sites on the 3’ end. The PCR product was cloned into the XhoI and Xhol sites of pBluescript II, and the resulting plasmid pBS-Arh1 was linearized by BglII and dephosphorylated with calf intestinal phosphatase (Roche Molecular Biochemicals). The BamHI-BglII fragment containing three tandemized copies of the hemagglutinin epitope (HA3) (23) was subcloned into the dephosphorylated BglII site, creating plasmid pBS-Arh1HA3. The correct orientation of the tag was confirmed by sequencing. The XhoI-Xhol fragment containing the open reading frame and tag (Arh1p-HA3) was subcloned into the corresponding sites of the integrating expression vector pEMBlyex4i to create pGal-Arh1HA3. In this vector, a GAL1 promoter, CYC1 transcription start site and terminator, and URA3 marker for selection of yeast transformants. A unique StuI site in the URA3 gene of the plasmid is present for direct integration into the ura3-52 locus. For knock out or deletion of *ARH1*, an interruption/deletion cassette was constructed. A 1.2-kilobase DNA fragment containing the HIS3 gene with BamHI ends was amplified by PCR using pRS413 as template, digested with BamHI to generate cohesive ends, and ligated into the BamHI and BclI sites of the ARH1 open reading frame of pBS-Arh1. The interruption/deletion cassette was released by digestion with XhoI and Xhol.

*Strains*—The parental diploid strain YPH501 (24) was transformed with the fragment of the ARH1 knockout cassette and transformants were selected for histidine prototrophy. Correct integration was confirmed by genomic PCR. Plasmid pGal-Arh1HA3 was linearized at the unique StuI site and integrated into the ura3-52 site of the heterozygous Arh1p/Δarh1 knock out strain. The transformants were sporulated and tetrads were germinated on plates containing galactose as a sole carbon source. A fragment clone that was His+ (marker for pARH1) and Ura+ (marker for pGal-Arh1HA3) was identified and referred to as the Gal-Arh1 strain.

*Bacterial Expression and Antibodies*—For expression in bacteria, the AC01 and YAH1 open reading frames were amplified by PCR from yeast genomic DNA and inserted into the NdeI and Xhol sites of pet22b (Novagen). The overexpressed proteins with C-terminal His6 tags were found to be sequestered in inclusion bodies. These proteins were solubilized in 8 M urea, purified on Ni-NTA columns (Qiagen), and eluted with 0.4 M imidazole. The eluates were further purified by preparative SDS-polyacrylamide gel electrophoresis and used to immunize rabbits for generation of polyclonal antibodies. Antibodies to Cyc1p were generated by immunizing rabbits with yeast holocytochrome c purchased from Sigma. Anti-HA (Roche Molecular Biochemicals) and anti-Cox3p (Molecular Probes) are commercially available mouse monoclonal antibodies.

*Fractionation of Mitochondria*—Mitochondria were isolated as described (25). Intact mitochondria (equivalent to 200 μg of protein) were resuspended in 20 mM Tris-HCl, pH 7.5, 0.6 M sorbitol. For releasing the intermembrane space contents, mitochondria were subjected to hypotonic shock by diluting to 0.1 M sorbitol and incubating at 0 °C for 10 min. The mitoplasts were recovered by centrifuging at 12,000 × g and then solubilized in 0.5% Triton X-100. The lysate was centrifuged for 10 min at 15,000 × g and supernatant and pellet fractions were separated (26). For quantitation of the 55Fe label, fractions were exposed to 2% SDS in 200 μl for 15 min at room temperature and suspended in 1 ml of scintillation mixture for counting in a Beckman scintillation counter.

**Assays**—High-affinity cellular iron uptake was measured as described (27). Aconitase was assayed by measuring the formation of cis-aconitate at 240 nm. The reaction mixture of 1 ml contained of 20 mM iso-citrate, 90 mM Tris-HCl, pH 8.0, 25 μl (100 μg) of purified mitochondria solubilized in 1.5% n-octyl-β-D-glucopyranoside containing 1 mM sodium citrate. The ΔA550 was measured for 2 min at room temperature, and the activity was expressed as nanomoles of cis-aconitate formed per mg of mitochondria protein per min (28). Succinate dehydrogenase was assayed on mitochondrial lysate in 0.5% Triton X-100 by measuring the reduction of p-iodonitrotetrazolium violet to the p-iodonitrotetrazolium-formazan as described (29). Leu1p (3-isopropylmalate dehydratase) activity was measured in concentrated cytoplasmic lysates (~10 mg/ml). The assay mixture of 500 μl consisted of 100 mM potassium phosphate buffer, pH 7.0, 2 mM citraconate, and 20 μl (200 μg) of cell extract. The mixture was transferred to a 2 mm quartz cuvette and the decrease in A555 was recorded over 3 min at room temperature. Activity was expressed as ΔA550/min/mg of protein (30).

For detection of heme, mitochondrial proteins (100 μg) separated by polyacrylamide gel and blotted to nitrocellulose were incubated with peroxide developer and chemiluminescent substrates (Blaze, Pierce) for 5 min prior to exposing the blot to film (31).

**RESULTS**

Gal-Arh1 Strain with Regulated Arh1p Expression—To characterize the function of Arh1p, we replaced a portion of the open reading frame with a HIS3 marker gene in the diploid strain YPH501. Sporulation of this diploid strain resulted in 2+·2− segregation for viable colonies with all the viabies showing auxotrophy for histidine (not shown). This result is consistent with the previously reported essential nature of the ARH1 gene (21, 22). To further study cellular effects of Arh1p, we generated a strain in which the only functional ARH1 gene was replaced with a gene fusion. In this construct, the open reading frame was fused at the C terminus to 3 copies of the HA epitope and placed under control of the GAL1 galactose-inducible promoter. Growth of the strain in galactose medium, inducing for the promoter, resulted in high level expression of the protein as monitored by monoclonal anti-HA antibodies. In the absence of inducer, as expected, Arh1p was undetectable (Fig. 1, panel A).

**FIG. 1.** Panel A, regulated expression of Arh1p. Mitochondria were isolated from a Gal-Arh1 strain grown under inducing conditions (+Gal) or noninducing conditions (−Gal). Mitochondrial proteins were separated by polyacrylamide gel, transferred to nitrocellulose, and probed with antibody to the HA epitope. In the Gal-Arh1 strain, the endogenous ARH1 gene was deleted and replaced with a construct containing the GAL1 promoter driving the open reading frame followed by three tandemized HA epitopes (Arh1p-HA3, 66 kDa). Panel B, growth properties of Gal-Arh1 strain. YPH499 (wild-type parent) or Gal-Arh1 cells were grown on YPAD plates. Cells were suspended in agar plates with different carbon sources. Galactose plate contains 2% raffinose and 0.5% galactose. Glucose plate contains 2% glucose. Ethanol plate contains 3% ethanol. Plates were photographed at 48 h (Galactose and Glucose) or 72 h (Ethanol).
added to the cultures as ferric ammonium sulfate at the indicated concentrations to the iron chelator bathophenanthrolene disulfonate. Iron was contained 2% glucose, which is repressing for the defined media with different concentrations of iron. The defined media containing Arh1p expression. Bathophenanthrolene disulfonate (BPS) refers to the iron chelator bathophenanthrolene disulfonate. Iron was added to the cultures as ferric ammonium sulfate at the indicated concentrations, or bathophenanthrolene disulfonate was added at 10 μM. Cultures were grown for 14 h and then diluted 10-fold into media of the same composition and allowed to reach logarithmic growth. Cellular iron uptake was measured over 1 h (pmol/10^6 cells/h) by incubation with ^55Fe and collection of the cells on glass fiber filters.

The growth properties of this strain were then evaluated. The growth of the Gal-Arh1 strain on galactose containing media was indistinguishable from the wild-type parent YPH499 (Fig. 1, panel B, top). However, surprisingly, when incubated in the absence of inducer and in the presence of glucose, which acts as a strong repressor for the GAL1 promoter, growth and colony formation were minimally affected. Colonies were only slightly smaller than wild-type controls after 2 days (Fig. 1, panel B, middle) but then caught up after another day incubation (not shown). The implication of this observation is that a small amount of “leaky” Arh1p protein expression, undetectable by immunoblotting, was sufficient to support growth. Cells with repressed Arh1p expression were not normal, however. Plating efficiency of the Gal-Arh1 cells on ethanol agar plates was decreased by more than 2 logarithms, and colony size was markedly diminished compared with the wild-type control cells (Fig. 1, panel B, bottom). The ethanol carbon source is noninducing for the GAL1 promoter and furthermore, is nonfermentable. Failure to utilize nonfermentable carbon sources may reflect mitochondrial dysfunction resulting from Arh1p depletion. Such a phenotype would be consistent with a nonredundant mitochondrial function for this protein.

Role for Arh1p in Regulating Cellular Iron Uptake—We next examined cellular iron uptake in the Gal-Arh1 strain and found that it was dysregulated. In the wild-type YPH499 strain grown in the presence of iron chelator (bathophenanthrolene disulfonate) the rate of high-affinity iron uptake was induced, whereas the same strain exposed to iron during growth showed repressed iron uptake. Mutations of several mitochondrial genes implicated in mitochondrial iron homeostasis exhibit altered responses of cellular iron uptake to iron exposures. In the MA14 mutant, carrying a mutation in the NFS1 gene, cellular iron uptake was induced and incompletely repressed by iron exposures in the medium (Fig. 2). Similarly, the Gal-Arh1 mutant grown in the absence of inducer showed a lack of response of cellular uptake to medium iron. At most media iron concentrations, cellular iron uptake was nonrepressed in the Gal-Arh1 strain compared with wild-type levels. At 10 μM medium iron concentration, uptake was completely repressed to the wild-type level (Fig. 2).

Role for Arh1p in Regulating Mitochondrial Iron Homeostasis—Arh1p is localized to the mitochondrial inner membrane (22) and might affect mitochondrial iron usage and distribution. To directly test this possibility, we exposed the wild-type YPH499 strain or the Gal-Arh1 strain to various iron concentrations in defined growth medium lacking the galactose inducer. Radioactive ^55Fe was added as a tracer. After 16 h of growth during which the labeling of intracellular iron pools reached a steady state, mitochondria were isolated and the iron content and distribution were assayed by analysis of the radionuclide. The Gal-Arh1 mutant showed a striking inability to control mitochondrial iron levels (Fig. 3). At the lowest medium iron concentration (0.1 μM, Fig. 3, panel A), wild-type and Gal-Arh1 mitochondrial iron levels were comparable and less than 1 pmol/μg of protein. A 10-fold increase in medium iron exposure had minimal effect on the mitochondrial iron of the wild-type but resulted in more than a 20-fold increase in mitochondrial iron of the mutant (1.0 μM, Fig. 3, panel B). An additional 5-fold increase in medium iron exposure had small effects on the wild-type but resulted in another more than a 5-fold increase in mitochondrial iron in the mutant (5.0 μM, Fig. 3, panel C). The exposure to still higher levels of medium iron did not result in further increase in mitochondrial iron in the Gal-Arh1 mutant (50 μM, Fig. 3, panel D).

Within mitochondria, the characteristics of iron were also strikingly altered in the Gal-Arh1 strain. At the lowest iron concentration, total mitochondrial iron was comparable in the wild-type and Gal-Arh1 strain, as was iron in the intermembrane space and mitoplast fractions. However, when mitoplasts were sonicated disrupting the inner membrane, a significant portion of the iron label was pelleted by low speed centrifugation (not shown). We considered that this iron pool might represent adherence of iron to membrane lipids or accumulation of aggregated iron proteins. Triton X-100 almost quantitatively solubilizes most mitochondrial proteins, and therefore we evaluated the distribution of iron after Triton X-100 solubilization of mitoplasts. In the Gal-Arh1 mutant a significant fraction of mitoplast iron was pelleted even after Triton X-100 treatment (40% versus 13% in the wild-type) consistent with the possibility that the iron label may be present in aggregated proteins (Fig. 3, panel A, TX-100 pellet). Increases in total mitochondrial iron in the Gal-Arh1 strain were associated with marked increases in the Triton X-100-insoluble fraction (38-, 80-, and 10-fold increased compared with wild-type in Fig. 3, panels B-D). Although not to the same degree, iron was also increased.
Arh1p in Iron Homeostasis

Aconitase activity was assayed in mitochondria isolated from the wild-type parent (YPH499, open bars) or engineered Arh1 strain (Gal-Arh1, filled bars) grown under noninducing conditions and exposed to different iron concentrations in the growth medium (0.1, 1.0, 5.0, and 50 μM). The specific activity is defined as nanomole of cis-aconitate formed per mg of mitochondrial protein per min. The aconitase protein level (Aco1p, 85 kDa) was assayed by immunoblotting with monospecific antibody.

in the intermembrane space and in the Triton X-100 soluble fraction of the mitoplasts under these iron loading conditions. In summary, the Gal-Arh1 strain with repressed Arh1p expression showed a loss of mitochondrial iron homeostasis. A distinct Triton X-100-resistant form of iron appeared in the absence of an increase in the total mitochondrial iron level; in addition, after iron exposure during growth, mitochondrial iron accumulated to a remarkable degree.

Iron-Sulfur Protein Deficiencies in the Gal-Arh1 Strain—In view of the marked alterations of iron distribution to mitochondria, iron proteins of the mitochondria were evaluated. Aconitase (Aco1p), a soluble enzyme of the mitochondrial matrix depends on an intact iron-sulfur cluster for its enzymatic activity. Aconitase activity was equivalent to the wild-type level in the Gal-Arh1 strain under inducing conditions for Arh1p expression (not shown), but activity was markedly decreased in the same strain grown in the absence of inducer (Fig. 4). The deficiency was not due to altered protein expression or turnover, because Aco1p could still be detected in normal abundance (Fig. 4). The discrepancy between normal protein levels and decreased activity indicates that enzymatically inactive protein was present. The residual aconitase activity in the Gal-Arh1 strain varied between 16 and 34% of wild-type levels despite more than 100-fold changes in the mitochondrial iron contents (ranging from 0.4 to 54 pmol/mg of protein). The deficiency was not improved by iron starvation as might be expected if it were a toxic effect of iron overload. Conversely, the Aco1p activity was not recovered in the iron loaded mitochondria.

Heme Protein Deficiencies in the Gal-Arh1 Strain—Mitochondrial heme proteins were examined. By immunoblot analysis, cytochrome oxidase subunit 3 (Cox3p) was found to be virtually absent from the Gal-Arh1 mitochondria with elevated iron content (isolated from 1.0, 5.0, and 50 μM iron containing media). By contrast, the protein was present and undiminished in Gal-Arh1 mitochondria with normal iron content (isolated from 0.1 μM iron containing medium) (Fig. 5). Cox3p is mitochondria encoded, and therefore, mtDNA damage might account for the observed effects on Cox3p. However, staining with the fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride showed that mtDNA was present in Gal-Arh1 mitochondria grown in the absence of inducer (not shown). In addition, a similar trend was observed for cytochrome c protein, which is nuclear encoded. This protein was markedly decreased in the Gal-Arh1 mitochondria, with the exception of the low iron point. Covalently bound c-type heme groups of Cyc1p (cytochrome c) and Cyt1p (cytochrome bc1) can be visualized by their intrinsic peroxidase activities (Fig. 5), and these were found to be markedly decreased in the Gal-Arh1 mutant. In mitochondria isolated from low iron growth conditions, the signals from heme in Cyc1p and Cyt1p were increased compared with iron-loaded mitochondria; however, complete normalization was not achieved. Thus, the mutant phenotype characterizing cells depleted of Arh1p included deficiency of heme proteins. The hemoprotein deficiency was mitigated in the Gal-Arh1 mutant mitochondria isolated from low iron medium. In accord with this last observation, these mitochondria were pink in color as distinguished from the paler color of their counterparts isolated from iron replete media.

Time Course of Arh1p Depletion—We examined the appearance of iron regulatory phenotypes after shifting the Gal-Arh1 strain from galactose (inducing) conditions to raffinose (noninducing) conditions, in rich media containing ~1 μM iron. At the time of the switch, expression of HA-tagged Arh1p could be easily detected with anti-HA monoclonal antibody (Fig. 6, panel A). A more rapidly migrating band reactive with anti-HA antibody on immunoblots was frequently observed (* in Fig. 6, panel A). However, the amount of this species varied from experiment to experiment and most likely represents proteolytic clipping at the N terminus of Arh1p. A similar pattern of proteolysis has been described for the Drosophila homolog of Arh1p (32). Subsequently, after growing under noninducing conditions for 12 h, the protein declined to undetectable levels and remained undetectable for the 24-, 36-, 48-, and 60-h time points. During this 60-h time period, cell growth was normal in the absence of detectable Arh1p. At the end of 60 h, galactose was added back to the medium, and the Arh1p protein expression was turned back on for 10 h (Fig. 6, panel A, +10). Control proteins Put2p, a matrix protein involved in proline utilization, and Por1p, a structural outer membrane protein, were present in equivalent amounts in mitochondria isolated from all time points (Fig. 6, panel A).

Cellular iron uptake was examined during the course of Arh1p depletion and repletion. When assessed at the time of the shift to noninducing conditions and 12 h later, cellular iron uptake was indistinguishable from wild-type (not shown), although Arh1p protein was already undetectable at the 12-h time point. Cellular uptake then increased 10-fold at the 24-h
time point (Fig. 6B). The iron uptake level remained elevated throughout the rest of the experiment, failing to recover with the restoration of the Arh1p expression after galactose readdition (Fig. 6, panel B). The delayed appearance of the mutant phenotype (increased iron uptake) at apparent disappearance of Arh1p was observed for the other mutant phenotypes as well, which appeared in coordinated fashion between 12 and 24 h after removal of the galactose inducer. Aconitase activity, normal at the first two time points, declined to less than half of wild-type levels, remaining at this low level until it recovered with restoration of Arh1p at the last time point (Fig. 6, panel C). As noted previously, deficient aconitase activity was not attributed to lack of protein expression or increased turnover, because immunoblotting with monospecific rabbit antisera demonstrated a uniform abundance of protein at all time points (Aco1p in Fig. 6, panel C). In this experiment, activities of other Fe-S proteins were also assayed. Succinate dehydrogenase, a complex of four subunits of the inner mitochondrial membrane, contains Fe-S, heme, and FAD cofactors. Succinate dehydrogenase activity declined and recovered in parallel with the changes in aconitase activity (Fig. 6, panel D). Leu1p, 3-isopropylmalate dehydratase, is a cytoplasmic protein with strong
sequence homology to the 4Fe-4S protein aconitase. Leu1p has been shown to contain iron (7), and this most likely is in the form of a 4Fe-4S cluster. Leu1p activity also changed in parallel with aconitase activity, indicating that both mitochondrial and cytosolic Fe-S proteins were affected by Arh1p depletion (Fig. 6, panel D).

As regards hemoprotein deficiency, cytochrome c protein and heme were independently examined and found to decline together at the 24-h time point (Fig. 6E). The nadirs of protein and cofactor, however, did not occur until the 36-h time point and thus were slightly delayed with respect to the nadir of aconitase activity. During the recovery time point (+10), cytochrome c heme recovered to 20% of wild-type, whereas cytochrome c protein was present at 50% of wild-type; the results suggest that some apoprotein may be present (Fig. 6, panel E). Significantly, the recovery of cytochrome c, like the recoveries of iron uptake, aconitase, and Leu1p were incomplete and delayed after recovery of Arh1p expression. Perhaps the delayed return of these phenotypes to normal results from toxic effects of accumulated mitochondrial iron which take time to reverse, or other downstream effects of Arh1p activity that require time to manifest themselves.

Arh1p might work via effects on Yah1p, analogous to the way human homologs of these proteins are thought to function (33). Therefore, we evaluated Yah1p protein during the time course of Arh1p depletion. Yah1p detected by immunoblotting with specific antibody was unchanged (Fig. 6, panel F) indicating that Arh1p has no effect on Yah1p protein levels.

**DISCUSSION**

Arh1p is an essential protein of mitochondria with reductase activity (21, 22), and we show here that it is required for iron homeostasis of the cell and mitochondria. A yeast strain depleted of Arh1p exhibited a multifaceted phenotype characterized by inability to repress cellular iron uptake appropriately in response to environmental iron exposure. The iron assimilated by these cells accumulated in mitochondria. On the other hand, iron proteins were deficient, including Fe-S proteins and heme proteins.

Apparently distinct threshold levels of Arh1p expression were required for viability and correct iron regulation. During the time course experiment in which expression of Arh1p from the GAL1 promoter was turned off, the level of protein declined, and the cells became dysregulated in terms of iron metabolism. Even then, growth continued unimpeded. Most likely, the Arh1p protein would need to decrease still further to arrest growth, and this decrease was not achieved using the GAL1 promoter. Different threshold levels of protein needed for viability and for correct iron regulation were also noted for Nfs1p, the cysteine desulfurase homolog of mitochondria (8). The implication is that a very minute amount of Arh1p protein suffices for viability. Furthermore, the essential functions of Arh1p are likely to be directly related to its functions in iron metabolism.

What is the function of Arh1p in iron metabolism? Depletion of Arh1p from cells was correlated with a decline in aconitase activity, without alteration in aconitase protein level. This combination of findings suggests that there might be a problem with synthesis, assembly, or maintenance of the Fe-S cluster, which is required for interaction of aconitase with its substrate. Alternatively, aconitase in the Arh1p-depleted cells might be inactive because of improper folding or lack of critical interactions with other proteins. What is clear is that the effects on aconitase do not result from iron toxicity, because Arh1p-depleted mitochondria with more than 100-fold differences in iron content exhibited invariant deficiency of aconitase activity.

A further hint about the function of Arh1p in iron metabolism may be derived from its sequence. Arh1p is a reductase of the mitochondrial inner membrane which contains NADPH and FAD binding motifs, enabling pairs of electrons to be received and then released one at a time to a substrate (21, 22). A possible substrate of the reductase is Yah1p, another essential mitochondrial protein (34). Biochemical studies on adrenodoxin reductase, the human homolog of Arh1p, suggest that transient interaction with a 2Fe-2S ferredoxin, homologous to Yah1p, occurs via a patch of negatively charged residues on the ferredoxin (33). The ferredoxin might undergo a single electron reduction during such an interaction and the reduced ferredoxin, in turn, might transfer reducing equivalents to downstream electron acceptors. A role for the ferredoxin, Yah1p, as the major proximal substrate for the ferredoxin reductase, Arh1p, is supported by the similar phenotypes resulting from depletion of either protein. The cellular depletion of Yah1p has been thoroughly characterized (9) and exhibits strong similarities with Arh1p depletion phenotypes presented here. However, the ultimate substrates in this mitochondrial electron transport chain are unknown. Possibilities are iron or iron chelates, intermediates of Fe-S cluster assembly, or proteins that require reduction as part of their folding pathway. Iron may need to be reduced prior to incorporation into Fe-S clusters. It is not known what form of iron is delivered to the interior of the mitochondria where Arh1p and/or Yah1p might act on it. The iron might be bound as a ferric chelate analogous to an iron-siderophore complex, which could then be reduced and mobilized by the reductase. In fact, the inner mitochondrial reductase resembles the ferric chelate reductase of the cell surface (Fer1p) in that it also uses FAD and NADPH cofactors (35). Alternatively, the Arh1p reductase might be required for release of an intermediate Fe-S cluster transiently assembled on a protein template. Such a mechanism has been described for release of a cluster formed between NifU dimers (15). Finally, the reductase might be involved in protein folding or unfolding related to cluster insertion. Further work is needed to determine which of these possibilities is correct.

In addition to Aco1p, other Fe-S proteins were affected by the Arh1p depletion. Deficiency of succinate dehydrogenase and isopropylmalate dehydratase activities were also observed. This is important because these proteins are Fe-S proteins that reside in distinct cellular compartments. Succinate dehydrogenase is a multisubunit enzyme of the inner membrane and carries heme, FAD, and three Fe-S prosthetic groups (36, 37). Leu1p is a cytoplasmic protein homologous to aconitase and thought to contain a 4Fe-4S cluster involved in isomerization of isopropylmalate as part of the leucine biosynthetic pathway (7, 30). The fact that both of these proteins were deficient and dependent on mitochondrial Arh1p is consistent with a role of mitochondria in synthesis of clusters for varied mitochondrial and extramitochondrial proteins as has been described (7).

Another facet of the role of Arh1p in iron metabolism relates to iron trafficking. Arh1p-depleted cells failed to repress cellular uptake appropriately in response to iron exposure. The excess iron accumulated within mitochondria. The most straightforward explanation for these findings is that one or more iron-sulfur protein regulators control iron trafficking to mitochondria. Regulation might occur at the level of mitochondrial import, storage, or export. Others have proposed that the proteins Yh1p and Atm1p are involved in mitochondrial iron export, and Arh1p might influence this process (38). However, an iron-sulfur protein regulator of iron trafficking to mitochondria has not yet been identified.

The effect of Arh1p depletion in producing deficiencies of heme proteins was likely a toxic effect resulting from iron overload, because the deficiencies correlated with increased
iron content of mitochondria. Cytochrome oxidase subunit 3 was present in the Arh1p-depleted mitochondria with normal iron content but was virtually absent in the iron overloaded mitochondria. Cytochrome c likewise appeared in iron-depleted mitochondria but not in the iron-loaded mitochondria. This iron toxic effect on heme proteins could be the result of repressed heme synthesis or increased heme turnover. Alternatively, the biosynthesis or turnover of the apoproteins prior to heme insertion or after heme removal might be affected. An effect of Arh1p depletion on hemoproteins was not anticipated and was not observed with similar levels of iron overload in the nfs1–14 mutant (not shown). The reason for the difference is not clear.

Detailed biochemical studies on adrenodoxin reductase, the human homolog of Arh1p, have demonstrated a role in steroid synthesis. In humans, an electron transport chain exists in mitochondria of steroidogenic tissues. Reducing equivalents that travel from NADPH to the reductase and then to ferredoxin termed adrenodoxin. The adrenodoxin in turn donates electrons to P450 cytochromes, which participate in hydroxylation reactions involved in steroid synthesis (39). In yeast, homologs of the reductase and ferredoxin exist (Arh1p and Yah1p, respectively), but cytochrome P450 proteins have not been identified in mitochondria, and a role for this electron transport chain in sterol or steroid synthesis has not been demonstrated (22). Conversely, the adrenodoxin reductase and adrenodoxin are expressed ubiquitously outside of steroidogenic tissues in humans (39). In light of our results, it is therefore possible that these human proteins also function in regulation of iron trafficking and in activation of Fe-S cluster proteins, perhaps through aiding in synthesis or maintenance of the clusters.

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