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The ABC transporter Atm1p is required for mitochondrial iron homeostasis

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Abstract The function of the ABC transporter Atm1p located in the mitochondrial inner membrane is not yet known. To study its cellular role, we analyzed a mutant in which *ATM1* was disrupted. Δatm1 cells are deficient in the holoforms, but not the apoforms of heme-carrying proteins both within and outside mitochondria, yet both synthesis and transport of heme are functional. Δatm1 cells are hypersensitive for growth in the presence of oxidative reagents, and they contain increased levels of the antioxidant glutathione, in particular of its oxidized form. Mitochondria deficient in Atm1p accumulate 30-fold higher levels of free iron as compared to wild-type organelles, i.e. three-fold more than mitochondria deficient in frataxin, the protein mutated in Friedreich's ataxia. The increased mitochondrial iron content may be causative of the oxidative damage of heme-containing proteins in Δatm1 cells. Our data assign an important function to Atm1p in mitochondrial iron homeostasis.

Key words: ABC transporter; Mitochondrial iron homeostasis

1. Introduction

ATP binding cassette (ABC) transporters comprise a large family of proteins which catalyze the active transport of a wide variety of substrates across biological membranes [1–3]. These proteins have initially been identified in bacteria [4], and about 80 members are encoded in the genome of *Escherichia coli*. Some ABC transporters catalyze the uptake of nutritional substrates into the bacterial cell, while others secrete toxins and various substrates. In eukaryotic cells, ABC transporters have been found both in the plasma membrane and in intracellular membranes of organelles such as peroxisomes, the endoplasmic reticulum and vacuoles. In mitochondria, only one ABC transporter (termed Atm1p) has been identified so far [5]. Atm1p belongs to the class of 'half-transporters' [6] and is localized in the inner membrane with the nucleotide binding domain facing the mitochondrial matrix. Deletion mutants in *ATM1* do not grow on non-fermentable carbon sources and show only slow growth on glucose-containing media [5]. However, the cellular function of this protein has remained elusive.

We have isolated the *ATM1* gene during a search for mutants defective in mitochondrial cytochromes by complementing a collection of temperature-sensitive *pet* mutants [7,8] with genomic DNA libraries. Here, we describe studies to elucidate the cellular role of Atm1p. A key feature of mitochondria lacking functional Atm1p is the accumulation of high levels of iron. The content of iron is three-fold higher than that reported for mitochondria deficient in frataxin, the protein mutated in patients with Friedreich's ataxia, a neurodegenerative disease [9–12]. Our results suggest that Atm1p, like frataxin, plays an important role in mitochondrial iron homeostasis.

2. Materials and methods

2.1. Yeast strains and growth of yeast

The following *Saccharomyces cerevisiae* strains were used: strain YPH501 (*MATa/α, ura3-52, lys2-801, ade2-101, trp1-63, his3-200, leu2-1*) was used as wild-type; Δcor1 (*MATa, ade2-1, his3-11,15, leu2-3,112, ura3-1, trp1-1, COR1::HIS3* [13]); Δcox6 (*MATa, ade2-1, his3-11,15, leu2-3,112, ura3-1, trp1-1, COX6::URA3* [14]); Δyta12 [15]; CKY10 ρ°_(*MATa, leu2-3,112, ura3-52*). Cells were grown on 1% yeast extract, 2% Bactopeptone supplemented with 2% glucose (YPD), 3% glycerol (YPG), or 2% galactose (YPGal). For selective growth, yeast cells were cultivated in 0.7% yeast nitrogen base, 2% glucose supplemented with leucine (30 mg/l), adenine, histidine, lysine, tryptophan, and uracil (20 mg/l each) according to the auxotrophic requirements.

2.2. Disruption of ATM1

A *Bst*1286-*Spe*I DNA fragment containing the entire *ATM1* coding sequence was subcloned into the *Sma*I site of the pGEM4 vector. A 977 bp internal *Eco*RV fragment was replaced by the *LEU2* yeast auxotrophic marker creating a deletion of amino acid residues 111–495 of Atm1p. The disrupted *ATM1* piece was liberated from the plasmid by *Sst*I-*Hin*dIII digestion and used to transform the isogenic diploid strain YPH501. Correct integration of this construct was confirmed using PCR with primers complementary to *ATM1*. One transformed clone that contained both the wild-type and the disrupted allele was sporulated, and a tetrad dissection was performed.

2.3. Production of antibody against Atm1p

The *Bam*HI fragment of the pGEM4 vector harboring *ATM1* (see above) was subcloned into the pGEX plasmid (Pharmacia). This results in the fusion of the C-terminal 198 amino acid residues of Atm1p to the C-terminus of glutathione *S*-transferase (GST). The plasmid was transformed into *E. coli*, and used to direct the synthesis of the fusion protein after induction with 1 mM IPTG. After 2 h, the GST-Atm1p fusion protein was exclusively found in inclusion bodies. They were isolated, washed three times with PBS containing 1% Triton X-100, dissolved in sample buffer and subjected to SDS-PAGE. After blotting onto nitrocellulose, the fusion protein was excised, the nitrocellulose was dissolved in dimethylsulfoxide and used for immunization of a rabbit.

2.4. Miscellaneous procedures

Standard methods for the manipulation of DNA and for PCR were used [16]. The following published methods were used: transformation of yeast cells [17]; isolation of plasmids from yeast [16]; isolation of yeast mitochondria [18]; Nycodenz density gradient purification of mitochondria [19]; whole cell lysates by breaking the cells with glass

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beads [20] or by the alkaline lysis method [21]; enzyme activities of citrate synthase [22], malate dehydrogenase [23], aconitase [24], and catalase [25]; detection of covalently bound heme [26]; detection of 'non-heme, non-iron-sulfur' iron after solubilization of the mitochondria in 10 mM MOPS-KOH, pH 6.5, 1% Triton X-100 [27]. Overexpression of peroxisomal catalase A was achieved using a YEp352 plasmid harboring the *CTA1* gene (kind gift of Dr. A. Hartig, Vienna).

3. Results

3.1. Δatm1 cells are defective in the holoforms of heme-containing proteins

To initiate investigation of the cellular role of Atm1p, a mutant carrying a disruption of the *ATM1* gene (termed Δatm1) was created. Disruption was confirmed by PCR (not shown) and by immunostaining using a specific antiserum (Fig. 1). Mitochondria were isolated from Δatm1 cells grown at 30°C on galactose-containing rich medium and analyzed for their cytochrome content. A qualitative defect in cytochromes has been noted earlier [5]. Only 10% of holocytochromes aa_3, *b*, *c* and c_1 were detected in differential spectra of isolated Δatm1 mitochondria as compared to wild-type organelles (Fig. 2A). The lack of holocytochromes *c* and c_1 cannot be explained by a defect in the biosynthesis of the apoproteins, as almost wild-type amounts of cytochromes *c* and c_1 were detected in cell lysates by immunostaining analysis (Fig. 2B). However, only a small fraction of these proteins (10% or less) carried a covalently attached heme group as detected by a heme-staining procedure (Fig. 2C). Thus, Δatm1 cells display a severe defect in the holoforms of *c*-type cytochromes, despite functional expression of the apoproteins.

The deficiency in holocytochromes *c* and c_1 could be due to an impaired heme attachment reaction. However, wild-type amounts of cytochrome *c* heme lyase (CCHL) and cytochrome c_1 heme lyase (CC_1HL) were present in Δatm1 mitochondria (not shown). Further, we noticed the exclusive presence of the mature form of cytochrome c_1 in Δatm1 mitochondria. This is remarkably different from the phenotype of a mutant in CC_1HL or mutants defective in the biosynthesis of heme, as all these mutants accumulate the intermediate form of cytochrome c_1 [28–30]. Processing to the mature form strictly depends on preceding covalent attachment of heme catalyzed by CC_1HL. We therefore conclude that in Δatm1 cells holocytochrome c_1 has been formed tran-

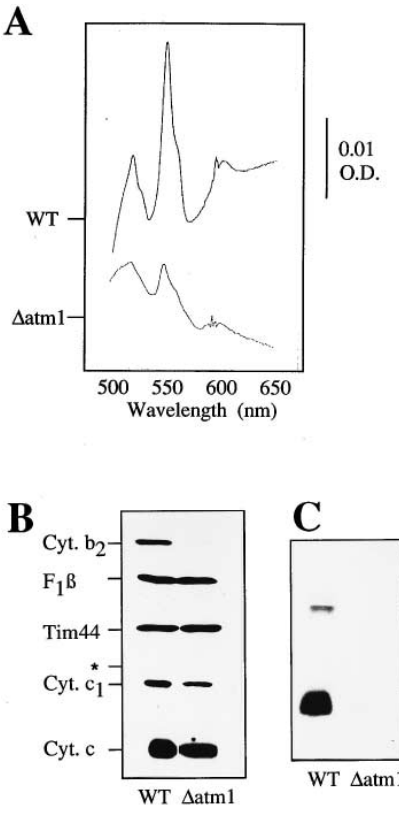

Fig. 2. Δatm1 cells are deficient in the holoforms, but not the apoforms of *c*-type cytochromes. A: Cytochrome spectra were recorded using mitochondria isolated from wild-type (WT) and Δatm1 cells which were grown overnight in YPGal medium at 30°C. Reduced-minus-oxidized difference absorption spectra were recorded at room temperature. The bar on the right represents an absorption difference of 0.01 (OD). B: Proteins from wild-type and Δatm1 mitochondria were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Immunostaining was for cytochrome b_2 (Cyt. b_2), the β-subunit of F_1-ATPase ($F_1β$), Tim44, and cytochromes c_1 (Cyt. c_1) and *c* (Cyt. c). The position of the intermediate form of cytochrome c_1 is indicated by an asterisk. C: Detection of heme covalently bound to *c*-type cytochromes. Mitochondrial proteins were separated by non-reducing SDS-PAGE and blotted onto nitrocellulose membrane. Detection of heme-carrying proteins was performed using the enhanced chemiluminescence method [26]. The asterisk indicates the position of the intermediate form of cytochrome c_1.

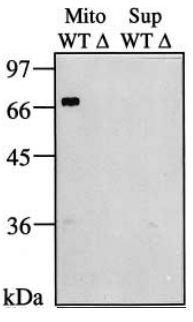

Fig. 1. Localisation of Atm1p. Mitochondria (Mito) and postmitochondrial supernatants (Sup) isolated from wild-type (strain YPH501, WT) and Δatm1 (Δ) cells were subjected to SDS-PAGE, and proteins were blotted onto nitrocellulose membrane. Immunostaining was with antiserum specific for Atm1p.

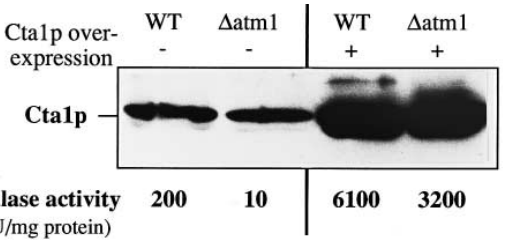

Fig. 3. Δatm1 cells are deficient in peroxisomal catalase activity. Whole cell extracts were prepared by the glass bead method from wild-type (WT) and Δatm1 mutant cells or from corresponding cells overexpressing peroxisomal catalase A (Cta1p). Identical amounts of protein were subjected to SDS-PAGE, and immunostaining was performed using antibodies against Cta1p [31]. The same lysates were used to determine the catalase enzyme activity [25].

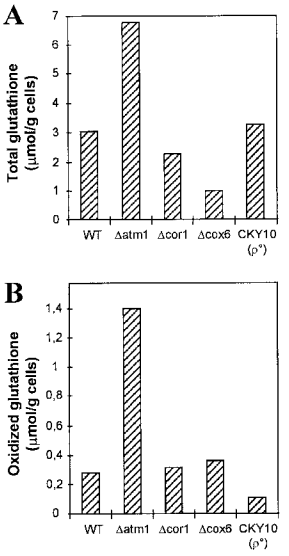

Fig. 4. Δatm1 mutant cells contain increased levels of glutathione. Wild-type cells (YPH501, WT), cells with deletions of ATM1, COR1, and COX6 genes and ρ° cells (CKY10 ρ°) lacking mitochondrial DNA were grown in YPD medium to an optical density of 0.6–0.8, collected by centrifugation and resuspended in buffer (0.1 M sodium phosphate pH 7.4, 5 mM EDTA). The cellular content of total and oxidized glutathione was determined from a perchloric acid extract with the glutathione reductase assay [40]. Data represent the average of three independent determinations with a standard deviation of less than 5%.

siently. How bound heme subsequently became degraded remains unclear (see also below).

We next examined whether heme-containing proteins are affected in other cellular locations of Δatm1 cells. As a marker, we selected catalase A (Cta1p), which represents the majority of catalase enzyme activity in yeast cells and is located within peroxisomes [31]. The cellular level and the activity of this heme-dependent enzyme were measured. Wild-type and Δatm1 cells contained similar amounts of Cta1p as detected by immunostaining (Fig. 3, left). In contrast, the catalase enzyme activity was 20-fold reduced in Δatm1 cells as compared to wild-type cells indicating that the enzyme was lacking its cofactor heme. These data suggest that Δatm1 cells display a deficiency in the holoforms of heme-containing proteins also outside mitochondria.

Surprisingly, only a two-fold difference in the enzyme activities of catalase was detected when Cta1p was overexpressed about 30-fold in both wild-type and Δatm1 mutant cells (Fig. 3, right). Overexpression of Cta1p and the resulting increase in the specific activity of catalase did not alter the growth defect nor the deficiency in mitochondrial cytochromes of Δatm1 cells (not shown). This observation demonstrates that Δatm1 cells are not a priori defective in synthesizing heme and supply it to other parts of the cell such as peroxisomes. Rather, in these mutant cells heme seems to be degraded rapidly.

3.2. *Δatm1 cells display an oxidative stress*

One possibility for the degradation of heme in Δatm1 cells could be an oxidative damage, e.g. by reaction with radicals. We therefore investigated whether Δatm1 cells show typical properties of an oxidative stress. Δatm1 cells were hypersensitive for growth in the presence of oxidizing reagents such as H_2O_2 (not shown). The sensitivity was even more pronounced than that observed for deletion mutants of the yeast homologue of frataxin [10]. Furthermore, the content of glutathione, a major antioxidant protecting cells against free radical damage [32,33] was increased two-fold in Δatm1 cells as compared to wild-type cells (Fig. 4A). Strikingly, the oxidized form of glutathione was elevated five-fold in Δatm1 cells (Fig. 4B). No such increases were detectable in respiratory incompetent *pet* cells (strains Δcor1 and Δcox6) or cells lacking mitochondrial DNA (strain CKY10 ρ°). These results suggest that the deletion of *ATM1* causes an oxidative stress.

3.3. *Δatm1 mitochondria accumulate high levels of iron*

A well-known cause of oxidative stress in cells is an accumulation of iron which at higher concentrations is toxic [34]. We therefore determined the amount of 'free' iron, i.e. iron that is not bound to heme or iron-sulfur (Fe/S) proteins. Wild-type mitochondria contained about 2 nmol free iron per mg mitochondrial protein, as measured by the bathophenanthroline method (Fig. 5A) [27]. This agrees well with published data [9,10,27]. In contrast, mitochondria isolated from Δatm1 cells accumulated 30 times more free iron (Fig. 5A). This increase is three-fold higher than the accumulation of iron in mitochondria lacking yeast frataxin [9,10]. The large content of free iron in Δatm1 mitochondria was confirmed by using atomic absorption spectroscopy (not shown). The post-mitochondrial supernatant of the Δatm1 cells contained only about twice as much free iron as that of wild-type cells (not

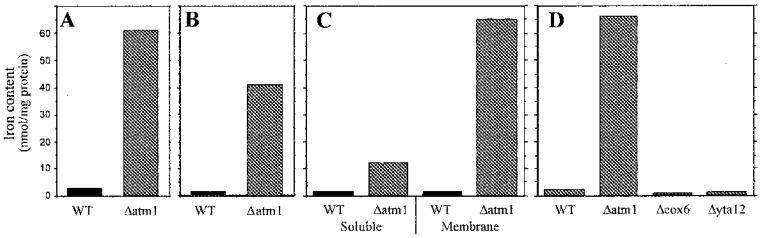

Fig. 5. Δatm1 mitochondria accumulate high amounts of 'non-heme/non-Fe/S' iron. Mitochondria were isolated from wild-type (WT) and Δatm1 mutant cells which were grown in YPGal medium. Organelles obtained with the standard isolation procedure (A) were further purified by Nycodenz density gradient centrifugation (B). The content of 'free' iron (i.e. not associated with heme or iron-sulfur proteins) was determined by the bathophenanthroline method [27]. C: Mitochondria were resuspended in 20 mM HEPES-KOH, pH 7.4 and sonicated on ice using a Branson sonifier (setting 3, 30% duty cycle, 1 min). 0.5 M NaCl was added and the sonication was repeated. Samples were centrifuged for 1 h at 50 000 rpm (Beckman Ti50 rotor). The iron content of the supernatant (Soluble) and the pellet (Membrane) fractions was determined as above. D: Mitochondria were isolated from the indicated cells and free iron was measured as in A.

Table 1
Enzyme activities in Δatm1 mutant mitochondria (U/mg mitochondrial protein)

	WT	Δatm1	Δatm1/WT (%)
Aconitase	0.97 ± 0.12	0.4 ± 0.06	41
Malate dehydrogenase	4.94 ± 0.50	5.45 ± 0.52	110
Citrate synthase	1.42 ± 0.11	1.38 ± 0.12	97

Mitochondria were isolated from wild-type (WT) and Δatm1 cells grown at 30°C in YP medium containing 2% galactose. Organelles were dissolved in lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4 containing 1 mM PMSF), and used for the determination of various enzyme activities. Values represent the average of three independent determinations (standard error is given). One unit is defined as 1 μmol substrate converted per minute.

shown). No increases in mitochondrial iron levels were found for various *pet* cells (Fig. 5D).

Increased free iron concentrations were also found for mitochondria which were highly purified by density gradient centrifugation, a procedure which largely depletes contaminating membranes, e.g. those of the endoplasmic reticulum (Fig. 5B) (cf. [35]). Fractionation of the mitochondria revealed approximately 20% of the free iron to be present in the soluble fraction, while the remaining 80% was pelleted with the membranes (Fig. 5C). Sedimented iron could not be solubilized by treatment with buffers of high ionic strength showing that iron was not peripherally associated with mitochondrial membrane proteins. In conclusion, these results suggest a strong defect in the maintenance of normal iron levels in mitochondria lacking Atm1p.

To investigate whether iron is present in Δatm1 mitochondria in a form which can be incorporated into proteins containing Fe/S clusters, we determined the enzymatic activity of mitochondrial aconitase. As compared to wild-type cells, its activity in Δatm1 cells was decreased about two-fold (Table 1). Citrate synthase and malate dehydrogenase, as control proteins, were virtually identical in mitochondria isolated from the two cells. The amount of the Rieske Fe/S protein of the respiratory chain complex III was decreased 10-fold in Δatm1 mitochondria (not shown). However, a similar effect was found for cells lacking functional cytochrome c_1 or other components of complex III (not shown) [13]. Thus, the reduced amount of Rieske Fe/S protein in Δatm1 cells rather is a pleiotropic consequence of the deficiency in holocytochrome c_1 in these cells. In summary, our results show a drastic accumulation of free iron in mitochondria defective in Atm1p. A fraction of the iron is soluble and can be incorporated into heme and Fe/S proteins.

4. Discussion

We report here several novel findings which are important for the understanding of the cellular role of Atm1p. Cells lacking functional Atm1p display a general deficiency in the holoforms, but not the apoforms of heme-containing proteins both inside and outside mitochondria. This is not due to a general defect in the biosynthesis or transport of heme within the Δatm1 cell. Heme is synthesized in sufficient amounts to generate mature holocytochrome c_1 in the intermembrane space. Moreover, heme can be transported to peroxisomes where it can be incorporated into catalase A. Δatm1 cells are sensitive to oxidative reagents and contain increased levels of glutathione, especially of its oxidized form. Thus, these mutant cells are under oxidative stress. Finally, we observed a drastic increase in the amount of iron within mitochondria of Δatm1 cells.

Based on these observations, we would like to propose a model which explains the phenotype of cells lacking functional *ATM1*. Iron, at elevated concentrations, is known to elicit the formation of free radicals and thus is toxic to cells (see, e.g. [36,37]). The radicals resulting from the increased iron levels could lead to the unspecific oxidative damage of heme-containing proteins observed in Δatm1 cells. This model would readily explain why overexpression of peroxisomal catalase results in at least partial protection of this enzyme against damage in Δatm1 cells (Fig. 3, right). High levels of catalase more effectively remove H_2O_2, a substrate for the iron-dependent formation of hydroxyl radicals by the Fenton reaction, and thus may decrease the oxidative stress within peroxisomes.

A similar, yet less severe increase in free iron was recently observed for mitochondria lacking Yfh1p, the yeast homologue of frataxin [9,10]. This protein is mutated in patients with Friedreich's ataxia. Δyfh1 organelles contain six- to 10-fold increased levels of free iron as compared to mitochondria of wild-type cells, i.e. three-fold less than Δatm1 mitochondria. The differential content of free iron in mitochondria lacking Yfh1p or Atm1p may, at least in part, explain the milder growth phenotype of Δyfh1 cells. In general, an increase in this essential trace element is intriguing as deletion of the major iron-metabolizing protein within mitochondria, ferrochelatase, does not result in significant changes in the free iron concentration within these organelles in yeast [38]. Thus, the content of iron within mitochondria seems to be tightly regulated. Presumably, Atm1p and Yfh1p play a direct role in this regulation [9].

It is clear from our data that Atm1p does not transport iron into the mitochondrial matrix. What role may be envisioned for Atm1p in mitochondrial iron uptake? Atm1p could export from mitochondria a molecule which acts as an iron chelator. The lack of functional Atm1p may lead to an accumulation of the chelator and, in turn, iron inside the mitochondrial matrix. Alternatively, Atm1p could sense the iron concentration in the mitochondrial matrix, and regulate the activity of a yet unknown iron transporter in the inner membrane. Such a proposed regulatory function of an ABC transporter is not unprecedented. An interaction between ABC transporters and channel proteins seems to be a widely observed feature of active transport across cellular membranes [3,39]. Further elucidation of the function of Atm1p will benefit from investigations how iron is imported into mitochondria.

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