Identification of a human mitochondrial ATP binding cassette transporter, the functional orthologue of yeast Atm1p

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Contents

Abbreviations

ABC	ATP binding cassette
Ade	adenine
ALAS	δ-amino-laevulinate synthase
Arg	arginine
Asp	aspartate
Atm1p	ABC transporter protein 1 of the mitochondria
ATP	adenosine triphosphate
CC_1HL	cytochrome c_1 heme lyase
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CCHL	cytochrome c heme lyase
cDNA	complementary deoxyribonucleic acid
CFTR	cystic fibrosis transmembrane regulator
Cta1p	catalase A protein 1
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
DTNB	2-nitro,5,5'-dithiobisbenzoic acid
ECL	enchanced chemiluminescence
EST	expressed sequence tag
Fet3	ferrous transporter 3
FRDA	Friedreich's ataxia
Fre	ferric reductase
Glu	glutamate
GPD	glyceraldehyde-3-phosphate dehydrogenase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-S-transferase

hABC7	human ABC7
His	histidine
HLA	human leucocyte antigen
Ile	isoleucine
IPTG	isopropyl-β-D-thiogalactoside
Leu	leucine
Lys	lysine
Mge1	mitochondrial GrpE-homologue
MTS	mitochondrial targeting signal
$NADPH^+$	nicotineamide adenine dinucleotide phosphate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
ROS	reactive oxygen species
SA	sideroblastic anemia
SDS	sodium-dodecyl-sulphate
Ser	serine
SOD	superoxide dismutase
TCA	trichloroacetic acid
Tim44	translocase of the inner mitochondrial membrane
Trp	tryptophane
Ura	uracil
Val	valine
WT	wild type
Yfh	yeast frataxin homologue

1. Introduction

1.1 Aims

The aim of these studies was to describe the function of the ATP binding cassette transporter protein of the mitochondria (Atm1p) [1], identified in yeast and to characterize its function in the mitochondrial iron metabolism [2]. We also planned to isolate the human homologue of Atm1p to promote the analysis of human disorders caused by the mutation of this gene [3].

1.2 Name and function of ABC transporters

Atm1p belongs to a large family of membrane proteins, to the so-called ABC transporters. These proteins harbor an ATP-binding cassette (ABC) and couple ATP hydrolysis to transport. They catalyze the active transfer of a variety of compounds across numerous biological membranes [4-7]. Each ABC transporter is highly specific for its substrate. ABC transporters have been identified for a wide variety of substrate, including sugars, peptides, inorganic ions, amino acids, oligopeptides, polysaccharides and proteins.

The substrates vary in chemical property and in size. It is not yet possible to predict substrate specificity of an ABC transporter or even the chemical class of substrate, from primary sequence data alone. The majority of these proteins mediate the import of various nutritional substrates (e.g. sugars, amino acids, peptides etc.) by co-operating with specific binding proteins in the [6].

Other transporters are involved in the export of different compounds such

as hemolysin from the bacterial cell [8].

1.3 Structure of ABC transporters

The ABC proteins display a conserved molecular architecture. They are dimers and each monomer of them contains a transmembrane and an ATP binding domain. Therefore, the whole molecule consists of four essential domains, called core domains (Figure 1). Each two hydrophobic transmembrane domains have six membranespanning α helices. The formation of a homodimer yields the typical twelve transmembrane segments in one transporter molecule. These domains are believed to bind the substrate and carry the transport across the lipid bilayer. These domains are supposed to determine the substrate specificity/selectivity of the transporter.

The other two domains of the four core domains are the ATP binding domains, forming the ATP binding cassette (ABC). They are anchored to the cytoplasmic side of the membrane by the transmembrane component. This element, consisting about 200 aminoacids is highly conserved among the species. The sequence identity is 30-50% and independent from the substrate specificity. The ABC motif („Walker motif") in this domain is common in the majority of the nucleotide binding proteins. This short amino acid sequence is responsible for binding and hydrolysis of nucleotides. However, it is important to emphasize that the sequence similarity extends over the complete domain and is fare more extensive than the two short Walker motifs. This fact clearly

distinguishes the ABC transporters from other nucleotide binding proteins: not all proteins with the Walker motif are ABC transporters. ATP binding domains interact specifically, and these induce different conformational changes in the membrane domain, and lead to the active transport. Each ABC transporter has two ATP binding domains and both are required for its function Figure 1). The transport activity is inhibited by the elimination or by a mutation in one of these domains [9].

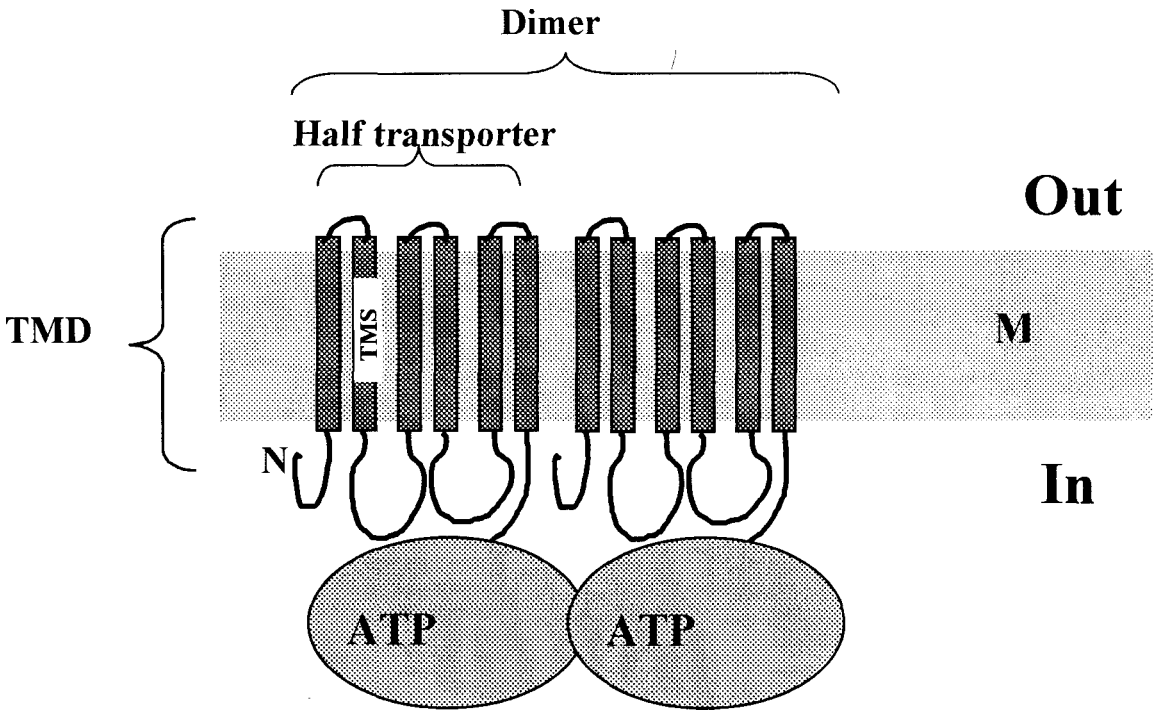

Figure 1.: Membrane arrangement of ATP transporters

Out: Outer side of the membrane
M:Membrane
In:Inner side of the membrane
N: N terminal end of the transporter
ATP: Nucleotide (ATP) binding domain
TMD:Transmembrane domain
TMS:Transmembrane segment

The four core domains can be synthesized as four distinct polipeptides, but they can be fused also into multidomain polipeptides. ABC transporters, where one hydrophobic transmembrane domain is fused to one ATP binding domain are called 'half transporter', and they function as a dimer.

1.4 Phylogenetic occurrence of ABC transporters

Members of ABC transporters are present in every living cell. In the bacterium *Escherichia coli* more than 57 various such transporters are known [10]. The first ABC transporters were characterized in bacterial uptake systems.

A large number of ABC transporters were identified also in eukaryotic organisms. The first yeast gene of these transporters reported in yeast *Saccharomyces cerevisiae* was the STE6 gene product, a plasma membrane pump, responsible for the secretion of the mating pheromone *a* [11].

Many mammalian ABC transporters have been associated with clinically relevant phenotypes. The P-glycoprotein (P-gp) confers the multidrug resistance observed in tumors during therapy to chemotherapeutic drugs [12]. The receptor for sulphonylureas (SUR) used to treat diabetes, is mutated at patients with persistent hyperinsulinemic hypoglycaemia of infancy [13]. ABC transporters are mutated in major genetic inherited syndromes, such as adrenoleukodystrophy [14], Zellweger syndrome [15,16]. A defective ABC transporter is involved also in cystic fibrosis [17,18], Dubin-Johnson syndrome [19], Bechet's syndrome [20].

1.5 Cellular occurrence of ABC transporters, Atm1p

In eukaryotic cells ABC transporters have been localized in a number of different cellular membranes [21,22,23]. Because mitochondria are regarded as bacterial symbiontes, it was expected that they also contain ABC transporters [24]. These could transmit signals to the nucleus from the mitochondria exporting mitochondrially encoded peptides or they could be the mitochondrial inner membrane transporter of metabolites for which no transporter have been identified [25]. Only one ABC transporter has been identified so far in mitochondria, namely the Atm1p [1,2,3].

We have isolated the ATM1 gene during a search for mutants defective in mitochondrial cytochromes, by complementing a collection of temperature-sensitive *pet* [26,27] mutants with genomic DNA libraries. Atm1p belongs to the class of 'half transporters' [28] and is located in the inner membrane with the nucleotide-binding domain facing to the mitochondrial matrix. Deletion of the ATM1 (strain Δatm1) gene results in a drastic growth defect on non-fermentable carbon sources and show only slow growth on glucose containing media [1]. We found that Δatm1 cells display a deficiency of heme-containing proteins both inside and outside of mitochondria. The most striking effect of this mutant phenotype is the accumulation of high levels of iron in the mitochondria. The content of iron is 30-fold higher than that was observed for mitochondria isolated from wild type cells. This suggested a specific role of Atm1p in mitochondrial iron metabolism [2,3].

1.6 Iron, the element with two faces

All the living organisms require iron with some exception [29]. The importance of iron for living organisms is underlined by its role in a large number of proteins, which require its presence in their prosthetic group for their activity (hemoglobins, myoglobins, mono-oxygenases, heme, cytochromes, cytochrome c oxidase, catalase as well as iron-sulphur proteins).

The assimilation of iron poses two problems for the cells: They must overcome its insolubility in order to take up the metal and they must regulate its uptake because iron is potentially toxic. Iron is the fourth most abundant component in the earth's crust. It has two properties at neutral pH, which make it at the same time interesting for biological systems in terms of catalysis, and biologically inaccessible, too. The Fe^{2+} - Fe^{3+} interconversion makes iron an essential cofactor of many oxido-reduction biological reactions [30]. On the other hand Fe^{3+} is practically insoluble, forms stabile hydroxides, and oxyhydroxides at neutral pH. Free Fe^{3+} in aerobic, aqueous environment is limited to an equilibrium concentration of approximately 10^{-17}, a value far below that required for the optimal growth of microbes. (10^{-8}-10^{-6}). Therefore, the first step in microbial iron assimilation is, by necessity, solubilization of free Fe^{3+}. Organisms have two ways to dissolve Fe^{3+} oxides in order to promote its uptake: chelation or reduction [30].

1.7 Iron uptake into pro-, and eukaryotic organisms

Bacteria are predominantly excreting different siderophores for chelation of Fe^{3+}. Plants, fungi excrete siderophores but they have also a surface reductase system. The reduction of Fe^{3+} by membrane bound reductases may be a general feature of eukaryotic iron transport, and the connection between copper and iron transport observed for *Saccharomyces cerevisiae* may have a parallel in mammals.

Saccharomyces cerevisiae does not excrete siderophores, contains only cell surface reductases, and the iron uptake is carried out by a high affinity and a low affinity transporter systems [30]. Both systems recognize Fe^{2+} generated by surface reductases. These reductases are the products of the ferric reductase 1 and 2 (FRE1, FRE2) genes. It was also observed that besides the transporter and the reductase systems another cell surface protein, ferrous transporter protein (Fet3p) is also required for high affinity iron uptake [31]. Mutations in FET3 abolish high affinity iron transport and transcription of the gene is inversely regulated by cellular iron content. Fet3p is presumably not a transmembrane iron transporter, it is a homologue of ceruloplasmin, which is a member of the multicopper oxidase protein family [32]. Because of the homology between Fet3p and ceruloplasmin, it was suggested that Fet3p is also a ferroxidase. Probably it is a part of the heteromultimeric transmembrane transporter. The exact role of Fet3p is unknown, the authors have hypothesized that the transmembrane transport of iron requires that iron have to be transported as Fe^{2+}

and that the catalytic activity of Fet3p is required to oxidize Fe^{2+} to Fe^{3+} and release it from the transporter into the cytoplasm [30].

The microvillar surface of intestinal cells normally takes up iron in a transferrin-free form [33]. Several studies on the uptake of iron by intestinal mucosal cells have demonstrated saturable iron binding to and/or transport across the cellular membrane, suggesting the presence of a carrier-mediated mechanism in the process of iron uptake. Also a ferric reductase activity was localized to the lumenal surface of intestinal epithelial cells in the mouse. This activity was found to correlate with the ability of the cells to assimilate environmental iron [34].

1.8 Iron uptake, storage in the eukaryotic cell

The best-studied cellular iron transport system in higher eukaryotes is the transferrin mediated process [35]. However, cellular uptake of iron does not depend exclusively on transferrin receptor mediated processes. For example, the hepatocyte may also exploit a non-specific (non-saturable) low affinity binding of transferrin to cell membrane followed by endocytotic internalization to an acidifiable compartment where iron is released [36]. This non-specific process is believed to be the principal mechanism operating at a physiological transferrin concentration. The rate of iron uptake by this process studied in cultured rat hepatocytes is quantitatively comparable with the uptake of iron by rat liver in

vivo and on the other hand the iron is largely incorporated into ferritin suggesting that it represents a physiological phenomenon [35].

Recently a *Saccharomyces cerevisiae* type, surface reductase and ceruloplasmin dependent iron transport system was described in fibroblasts [37, 38]. Furthermore, the function of ceruloplasmin in the iron uptake has also been known for a long time in higher level eukaryotes. The copper deficient animals have extremely low levels of ceruloplasmin and, these animals are unable to transport iron [39]. In the human Wilson disease, caused by ceruloplasmin deficiency, severe defects in iron metabolism were found [40].

Once iron became imported, cells have to face the problems inherent with the iron chemistry, i.e., cells have to prevent the precipitation of iron, to keep iron in a bioavailable form, and to prevent the iron toxicity.

This non-toxic, bioavailable storage form of iron in a wide variety of organisms is the ferritin complex [41]. Yeast represents an exception from this point of view because yeast has a low iron content in ferritin, and its iron content of ferritin is essentially independent of intracellular iron concentrations. This fact led us to suggest that another mechanism be implied in transient iron storage. The study of Raguzzi et al [42] showed that vacuole (lysosome) is the major iron storage compartment in the yeast cell. It is the only compartment, which greatly increases its iron concentration when the cells are submitted to a high iron content in their environment. However, it seems that the vacuole is not a passive storage form for excess iron in the yeast cell but its iron can be utilized

by the cell for iron requiring processes. So there are two iron storage pools in yeast cytosol: one associated with a ferritin-like protein and a vacuolar one [42]. In contrast to this, the lysosomal iron pool, the hemosiderin is metabolically inactive in higher eukaryotes.

1.9 Iron in the mitochondria

The mitochondria hold a key position in the cellular handling of iron by metabolizing the majority of the absorbed metal. The immediate iron donor is supposed to be a 'free' non-heme, non-Fe-S iron pool of the mitochondrial matrix. This iron pool was described in isolated rat liver mitochondria, and this is available for heme synthesis [43]. Although the biological nature of this iron pool is not yet known, it is likely that it represents a transit iron pool being the proximate iron donor for the conversion of porphyrin to heme catalyzed by the enzyme ferrochelatase.

Since the immediate iron pool, ferrochelatase and several Fe-S proteins reside in the mitochondrial matrix, the iron must be transported from the cytosol across the mitochondrial inner membrane. The mechanism and its regulation of the iron transport from the cytosol to the matrix are not known. A reduction step liberating iron from the cytoplasmic pools and making it transportable has been suggested. There are some evidences that iron can be reduced by the mitochondrial respiratory chain in yeast and mammals, but the side of the inner mitochondrial membrane on which this occurs is not known, so this result

remains controversial [44]. The mitochondrial iron transporter has not been identified yet, and this fact greatly hampers the analysis of this physiologically important process [45].

1.10 Iron toxicity

A significant feature of the Δatm1 mutant cells besides the mitochondrial iron accumulation is the severe growth defect [1,2,3]. This growth defect is more serious compared to other mutations in mitochondrial energy metabolism [2]. These results suggested, that besides the mitochondrial energy generation, other important cellular processes are also affected in the Δatm1 mutant. One possible explanation for this phenomenon could be the iron toxicity, which appears as a free radical damage to the cells.

Under normal circumstances the absorbed iron will be used for heme or iron-containing enzyme synthesis or will be deposited in storage proteins such as ferritin or in yeast also in lysosome until a later usage. Iron toxicity becomes significant only when an excess of iron, which is not deposited in the relatively non-toxic storage forms appears in the cell, also in various cell compartments.

In the different organisms reactive oxygen species (ROS) such as $O_2^{-}\cdot$, H_2O_2 are continuously produced as natural by-products of cellular metabolism. To protect against free radical mediated damage, cells have the capacity to scavenge or neutralize ROS through the combined action of highly specialized antioxidant enzymes. For example, heme containing catalases specifically attack

H_2O_2, whereas iron manganese or copper containing forms of superoxide dismutase (SOD) are designed to scavenge $O_2^{-}\cdot$[46].

However, in aqueous media $O_2^{-}\cdot$ is relatively unreactive toward most organic compounds, thus its proposed deleterious effects may be the result of its participation in reactions leading to other more reactive species, such as hydroxyl radical (·OH). It attacks all classes of biological macromolecules. It can depolymerize polysaccharides, cause DNA strand breaks, inactivate enzymes, and initiate lipid peroxidation. Because lipid peroxidation is a chain reaction that is geometrically amplified by redox-active iron, it is the action of hydroxyl radical that may have the greatest pathophysiological consequences. This effect has been demonstrated in different human diseases such as e.g. ischaemic heart disease and stroke [46]. Formation of hydroxyl radical requires the presence of transition metal ions such as copper or iron.

ROS or iron toxicity requires the action of other defense mechanisms besides the above-mentioned SOD and catalase. Smaller molecules such as ascorbate, alpha-tokopherol, glutathione (GSH), beta-carotene, uric acid and dihydrolipoamide has been shown to act as an antioxidant, by entrapping free radicals [47,48]. The tripeptide glutathione is the major free thiol in most living cells and plays an important role in the removal of hydrogen peroxide. GSH is also involved in the reduction of alpha-tokopherol, detoxification of xenobiotics, maintenance of sulphhydril status of the cell [47,49]. The oxidized cellular components react with GSH, forming dimers (GSSG). In a reaction catalyzed by

GSH reductase GSSG are reduced back to GSH, utilizing NADPH+H$^+$. The formation of GSSG stops the chain reaction of ROS in a cell exposed to oxidative stress. The determination of GSSG fraction of the total cellular GSH content is a good indication for the formation of the toxic radicals.

The generation of the ROS, and also the protective mechanisms are highly conserved in eukaryotes. The analysis of the iron accumulating mutant yeast cells offers a unique tool to study the cellular reactions to oxidative stress, what represented the second aim of this study besides the investigation of the function of Atm1p.

1.11 Human diseases in point of view of Atm1p

We also decided to identify the functional human orthologue of the yeast Atm1p. Several examples are known that the functional analysis of yeast proteins lead to the discovery of the pathomechanism of human diseases.

The rationale for the use of yeast as a model organism lies on the high conservation of many metabolic and cellular processes between yeast and humans. This is especially true for the mitochondrial processes including metabolism, inheritance and biogenesis [50,51]. We postulated, that a human mitochondrial Atm1p homologue also exists with similar function as the yeast protein. Furthermore, a mutation in the human orthologue would result in the same consequences in humans as ATM1 deletion in yeast and that is iron accumulation in the mitochondria [2,3].

Several human diseases are characterized by increased cellular iron content. The excess iron can be deposited in cytosolic ferritin or in lysosomal hemosiderin as observed for hemosiderosis. A gene encoding a member of the HLA-A3 family (Hfe) was reported to be responsible for this disease [52].

In another type of such disorders are iron deposits in the mitochondria. A recently discovered example for it is Friedreich`s ataxia (FRDA) [53,54]. FRDA is caused by a mutation in a gene, encoding frataxin [55,56,57]. Frataxin is a highly conserved small protein, present in Gram negative bacteria and in eukaryotes, as well. The human frataxin and its yeast homologue, Yfh1p were both localized in the mitochondrial matrix [55]. Disruption of the yeast homologue causes iron accumulation similarly as observed in the case of human mitochondria [53]. A possible function of frataxin in eukaryotic cells has been discussed extensively [53,54,55]. A permanent activation of the mitochondrial iron uptake system triggered by the lack of this protein has been suggested at the first place. Alternatively the decreased iron incorporation in iron-sulphur proteins may similarly lead to the precipitation of the excess iron imported into the mitochondria. Experimentally none of these ideas has been proved yet.

Another example for such a disease is the sideroblastic anaemia [58]. In this hypochromic, microcytic anaemia siderotic granules can be detected using Perl' Prussian-blue iron stain around the nuclei of developing red cells in bone marrow aspirates. The ring shaped perinuclear siderotic granules were later identified as functionally defective iron loaded mitochondria. Lysosomes of

these cells do not contain high amount of iron deposits [58]. Sideroblastic anaemias comprise a heterogeneous group of disorders, they can be either acquired or inherited [59,60]. The mode of transmission may be mitochondrial, autosomal or X-linked. The most common inherited form is X-linked. In some X-linked cases the mutation was found in the δ-amino-laevulinate synthase (ALAS) gene (Xp11,21) [61,62,63], which is expressed in erythropoetic cells. The reaction catalyzed by ALAS is the first and rate-limiting step in heme biosynthesis. Genetic studies, however, clearly demonstrated the involvement of several other genes in this disease.

Based on the knowledge of the phenotype of Δatm1 yeast cells, we thought, that a functional defect of the human homologue of ATM1 would also cause high accumulation of iron in the mitochondria, similar to what was observed in sideroblastic anaemia [3]. These investigations will therefore shed light not only on the still poorly characterized mitochondrial iron metabolism, but also will promote the analysis of pathomechanism of certain type of human iron metabolic disorders.

2. Materials and methods

2.1. Yeast strains and growth of yeast

The following *Saccharomyces cerevisiae* strains were used: Strain YPH500 (MATa, ura3-52, lys2-801, ade2-101, trp1-63, his3-200, leu2-1) and YPH501 (MATa/α, ura3-52, lys2-801, ade2-101, trp1-63, his3-200, leu2-1) served as wild-type cells, strain W303a (MATa, ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3,112) was employed in vitro protein import experiments, Δatm1 cells (MATa, ura3-52, lys2-801, ade2-101, trp1-63, his3-200, leu2-1, atm1::LEU2), Δcor1 (MATa, ade2-1, his3-11,15, leu2-3,112, ura3-1, trp1-1, COR1::HIS3 [64], Δcox6 (MATa, ade2-1, his3-11,15, leu2-3,112, ura3-1, trp1-1, COX6::URA3 [65], Δyta12 [66], CKY10ρ° (MATa, leu2-3,112 ura3-52). Cells were grown on 1% yeast extract, 2% bactopeptone supplemented with 2% glucose (YPD), 3% glycerol (YPG), 2% galactose (YPGal). For selective growth yeast cells were cultivated in 0.7% yeast nitrogen base, 2% glucose supplemented with leucine (30mg/l), adenine, hystidine, lysine, tryptophan, uracil (20mg/l each) according to auxotrophic requirements.

2.2.Disruption of ATM1

A Bst1286-SpeI DNA fragment containing the entire ATM1 coding sequence was subcloned into the SmaI site of the pGEM4 vector. A 977 bp internal EcoRV fragment was replaced by the LEU2 yeast auxotrophic marker

creating a deletion of aminoacid residues 111-495 of Atm1p. The disrupted ATM1 piece was liberated from the plasmid by SstI-HindIII digestion and used to transform the isogenic diploid strain YPH501. Correct integration of this construct was confirmed using polymerase chain reaction (PCR) with primers complementary to ATM1 [1]. 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 BamHI fragment of the pGEM4 vector harboring ATM1 was subcloned into the pGEX plasmid (Pharmacia). This results in the fusion of the C terminal 198 aminoacid residues of Atm1p to the C terminus of glutathione S-transferase (GST). The plasmid was transformed into *Eschericia coli* and the fusion protein was expressed in *Escherichia coli* strain BL21 after induction with 1mM isopropyl-β-D-thiogalactoside (IPTG). After 2h, the GST-Atm1p fusion protein was exclusively found in inclusion bodies. They were isolated, washed three times with phosphate buffered saline (pH 7.4) containing 1% Triton-X-100, dissolved in sample buffer and subjected to sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After blotting onto nitro-cellulose, the fusion protein was excised, the nitro-cellulose was dissolved in dimethylsulfoxide and used for immunization of a rabbit.

2.4.Production of antibody against human ABC7

A PCR fragment corresponding to the C-terminal 321 amino acid residues of hABC7 was subcloned into the BamHI and SalI restriction sites of pGEX-4T-2 plasmid (Pharmacia Biotech). This resulted in the fusion of the ABC domain of hABC7 to the C-terminus of glutathione-S-transferase (GST). The plasmid was transformed into *Escherichia coli*, strain BL21 and used to direct the synthesis of the GST-hABC7 fusion protein after induction by 0.1 mM IPTG. After 3 hours at 30°C, the cells were lysed by sonication on ice and 1% Triton X-100 was added. Cell debris was removed by centrifugation and the GST-hABC7 fusion protein was bound to a glutathione resin (Pharmacia Biotech). The protein was eluted with 10 mM glutathione and used for immunization of a rabbit.

2.5. Preparation of yeast's mitochondria

Cells were harvested by centrifugation (5min at 3000 x *g*) washed once with distilled water, suspended to 0,5g wet weight/ml in 0,1M TRIS-SO_4, pH 9.4 10 mM dithiothreitol, and incubated for 10 min at 30 °C. Cells were then washed once with 1,2 M sorbitol buffer (1.2 M sorbitol, 20 mM KPi, pH 7.4), and resuspended in it to give 0,15 g of cell, wet weight/ml. Zymolase 20T (5mg/g of cell wet weight) was added and the suspension was incubated at 30°C with gentle shaking. Conversion of the cells to spheroplasts was checked as described [67,68]. After 50-60 min, all the cells were usually converted to spheroplasts.

Spheroplast were harvested by centrifugation for 5 min at 3000 x *g* in Sorvall GS3 rotor at 4°C and washed twice with 1.2 M sorbitol buffer. From this point on, all operations were carried out at 0-4°C. For cell breakage spheroplasts were suspended in 0,6 M sorbitol buffer (0,6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 1mM phenylmethylsulfonylfluoride (PMSF)), and were homogenized by 17 strokes in a tight fitting Douncer. All subsequent centrifugation were carried out in Sorvall SS34 rotor at 2°C. The homogenate was diluted with 1 volume of the homogenization buffer and centrifuged for 5 min at 3000 x *g*. The supernatant was saved, and the pellet was rehomogenized as before and recentrifuged at 3000 x *g*. The supernatants were combined and crude mitochondria were sedimented at 10000 x *g* for 10 min. The pellet was carefully resuspended in the homogenization buffer and the suspension was centrifuged for 5 min at 3000 x *g* to remove residual cell debris. The supernatant was saved and centrifuged at 10000 x *g* for 10 min. The mitochondrial pellet was washed twice by resuspension and recentrifugation at 10000 x *g* for 10 min. Mitochondria were resuspended in 0,6 M sorbitol buffer without PMSF to give an approximate final concentration of 10 mg of protein/ml [67].

2.6. Import of preABC7(135)-DHFR into the mitochondria

PreABC78135)-DHFR was synthesized by in vitro transcription and translation in reticulocyte lysate using (35S) methionone as a label. Import of radiolabeled precursor protein was performed in 100 µl of import puffer (0,6 M

sorbitol, 50 mM HEPES-KOH (pH 7,0), 50 mM KCl, 10 mM $MgCl_2$, 2 mM KH_2PO_4, 2,5 mM EDTA, and 1 mg/ml fatty acid free bovine serum albumin) containing 2 mM $NADH_2$ and 50 µg of mitochondrial protein. After incubation for 3 min at 25°C, radiolabeled precursor proteins in reticulocyte lysate (1-5 µl) or 2 µl of urea denaturated precursor proteins (final urea concentration 160mM) were added. Unless stated otherwise incubation was continued for 15 min at 25°C. Samples were chilled on ice and treated with 100 µg/ml proteinase K for 30 min at 0°C. Protease digestion was stopped by the addition of 1 mM PMSF from a freshly prepared 100mM stock solution in ethanol. After 5 min on ice, samples were diluted with SOH buffer (0,6M sorbitol, 20mM HEPES-KOH, pH 7.4 containing 1mM PMSF) to a final volume of 1ml. Mitochondria were reisolated by centrifugation for 12 min at 10000 x g in Beckmann at 2°C. Mitochondria were resuspended in 100µl SOH buffer and proteins were precipitated with trichloroacetic acid (TCA). Following SDS-PAGE and fluorography, radioactive imported proteins were quantitated by densitometry.

Mitoplasts were generated by incubating the mitochondria in 20mM HEPES (pH 7.4) buffer. This treatment results in swelling of the mitochondria and rupture of the outer membrane.

2.7. Determination of total and oxidized glutathione

Cells were grown in liquid YP medium with glucose. Cells were suspended in 0.1M potassium phosphate buffer (pH 7.0) containing 2mM EDTA

and 1/20 volumes of concentrated perchloric acid was added. The mixture was vigorously shaken in the presence of glass beads for 3min. Short pulses of 30s were used, with 30s intervals on ice. The mixture was clarified by centrifugation. Supernatant was neutralized with KOH and was used for GSH determination [69].

The total glutathione content was measured in an enzyme catalytic assay with the help of glutathione reductase and 2-nitro,5,5'-dithiobisbenzoic acid (DTNB) as described by Tietze. [69] The rate of color development was monitored at 412 nm. The concentration was determined by reference to a GSSG standard added to the assay cuvette (internal standard).

Oxidized glutathione was measured in 0,1 M potassium-phosphate buffer, pH 7.2 containing 5mM EDTA by adding $NADPH_2$ till getting optical density (OD) 0,6-0,7E at 340 nm. The reaction was initiated by GSH reductase (GR) (stock concentration 1μmol/ml, SIGMA). The ΔE_{340} of $NADPH_2$ was detected until the slow reoxigenation phase.

2.8. Miscellaneous procedures

Standard methods for the manipulation of DNA and for PCR were used [70]. Expression of hABC7 was achieved by transforming wild type and Δatm1 yeast cells with the multi-copy plasmid pRS424-GPD [71] carrying the hABC7 cDNA under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. The following published methods were used:

transformation of yeast cells [72]; isolation of plasmids from yeast [70]; Nycodenz density gradient purification of mitochondria [73] in vitro transcription and translation and protein import into isolated mitochondria [74,75,76,77]; isolation of mitochondria and post-mitochondrial supernatant from human liver [78]; for preparation of cytosol, the post-mitochondrial supernatant was centrifuged for 1 h at 100000 x g; whole cell lysates by breaking the cells with glass beads [79]; measurement of the enzyme activity of citrate synthase [80]; malate dehydrogenase [81]; aconitase [82]; catalase [83]; detection of "non-heme, non-iron-sulphur,, iron after solubilization of the mitochondria in 10 mM MOPS-KOH, pH 6.5, 1% Triton X-100 [85]. Precipitation of proteins with TCA, alkaline extraction, urea denaturation of radiolabelled precursor proteins and SDS-PAGE, immunoblotting, detected by chemiluminescence using the enchanced chemiluminescence (ECL) system (AMERSHAM) was performed according to the instructions of the suppliers.

Protein concentration was determined by the method using bovine serum albumin as a standard [70].

Overexpression of peroximal catalase A was achieved using a YEP352 plasmid harboring the peroximal catalase protein, catalase A (CTA1) gene (kind gift of Dr. A. Hartig, Vienna).

2.9. Statistics

Calculation and expression of the results are presented as means±SEM.

The mathematical analysis of the results was carried out by the method of Student`s *t* test. The results were taken to be significant if the $P<0.05$.

3. Results

3.1. Localization of Atm1p

The subcellular localization of Atm1p was determined using cell fractionation and immunoblot technics. The antibody raised against Atm1p recognized a single protein in wild-type mitochondria with an approximately molecular weight of 70 kDa. No immune reactivity was detected in cytoplasm of wild type cells as in any subcellular fractions of Δatm1 cells. This result proved that the Atm1p is present only in the mitochondria, further the antibody generated by us is highly specific for this protein (Figure 3/1).

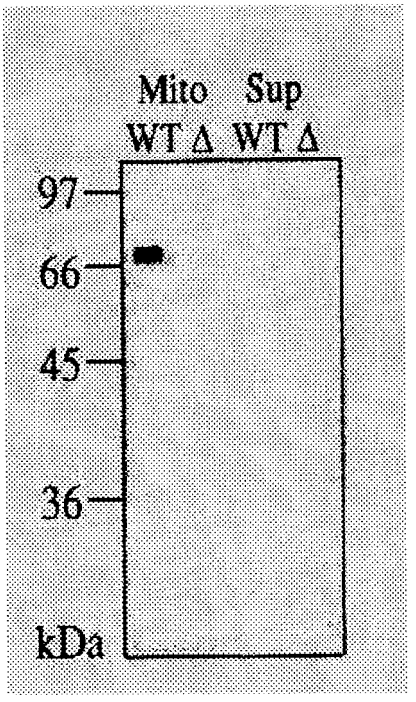

Figure 3.1.

Localization 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 nitro-cellulose membrane. Immunostaining was with antiserum specific for Atm1p.

3.2. *Effect of ATM1 deletion on the mitochondrial cytochromes*

To initiate investigation of the cellular role of Atm1p, a mutant carrying a disruption was created. Confirmation of disruption was done by PCR and by immunostaining using specific antiserum against Atm1p. Mitochondria and postmitochondrial supernatant were isolated from wild type and Δatm1 strains. 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 (Figure 3.2/A). 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 immunostainig analysis (Figure 3.2/B). However, only a small fraction of these proteins (10% or less) carried covalently attached heme group as detected by heme staining procedure (Figure 3.2/C). 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 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 [85,86,87]. 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 transiently. How bound heme subsequently became degraded remains unclear (see also below).

We examined further, 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 peroxisome [88]. 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 (Figure 3.2/D left). In contrast, the catalase enzyme activity was twenty-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 two-fold difference in the enzyme activities of catalase was detected when Cta1p was overexpressed about thirty-fold in both wild type and Δatm1 mutant cells (Figure 3.2/D right). Overexpression of Cta1p and the resulting increase in the specific activity of catalase did not alter the growth defect or 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.

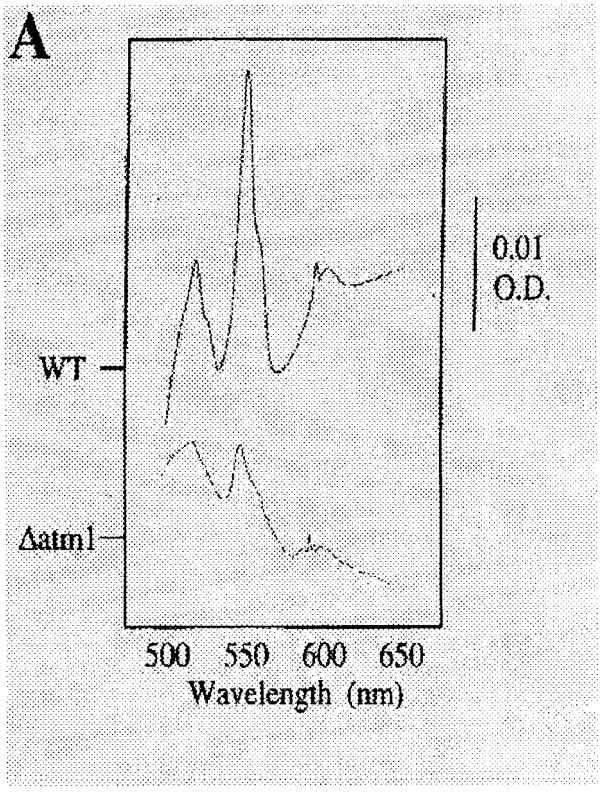

Figure 3.2./A

Δatm1 cells are deficient in the holoforms, but not the apoforms of c-type cytochromes. 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 optical density (O.D.).

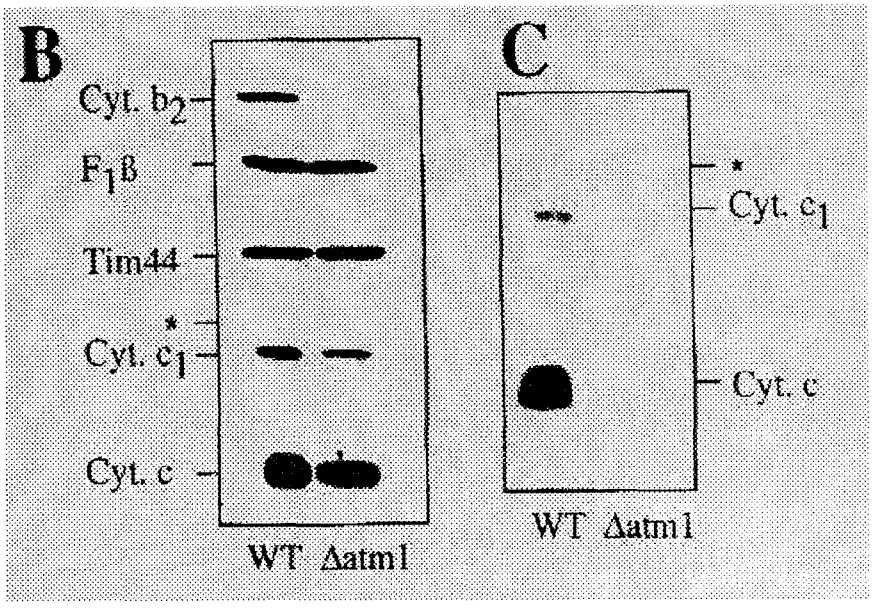

Figure 3.2/B,C

Δatm1 cells are deficient in the holoforms, but not the apoforms of c-type cytochromes. **B**: Proteins from wild type and Δatm1 mitochondria were separated by SDS-PAGE and blotted onto nitro-cellulose membrane. Immunostaining was for cytochrome b_2 (Cyt b_2), the β-subunit of F_1-ATPase ($F_1\beta$), Tim44, and cytochrome 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 nitro-cellulose membrane. Detection of heme-carrying proteins was performed using the enchanced chemiluminescence method [89]. The asterisk indicates the position of the intermediate form of cytochrome c_1.

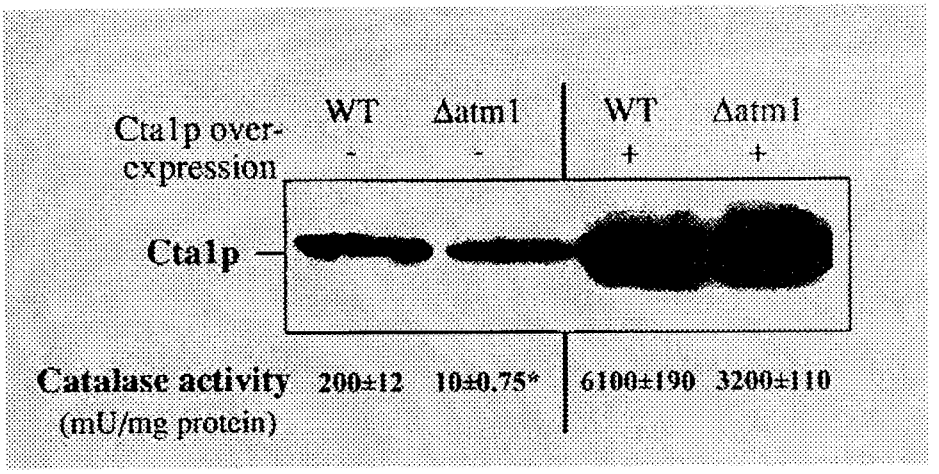

Figure 3.2./D

Δatm1 cells are deficient in peroximal catalase activity. Whole cell extracts were prepared by the glass beads method from wild-type (WT) and Δatm1 mutant cells or from corresponding cells overexpressing peroximal catalase A (Cta1p). Identical amounts of protein were subjected to SDS-PAGE, and immunostaining was performed using antibodies against Cta1p. [88] The same lysates were used to determine the catalase enzyme activity [83] (n=5, *P<0.05, compared to WT).

3.3. *Δatm1 cells display an oxidative stress*

One possibility for 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 [54]. Furthermore the content of glutathione, a major antioxidant protecting cells against free radical damage [90,91] was increased two-fold in Δatm1 cells as compared to wild type cells (Figure 3.3/A). Strikingly the oxidized form of glutathione was elevated five-fold in Δatm1 cells (Figure 3.3/B). No such increases were detectable in respiratory incompetent *pet* cells (strains Δcor1 and Δcox6) or cells lacking mitochondrial DNA (strain CKY 10 ρ°). These results suggest that the deletion of ATM1 causes an oxidative stress.

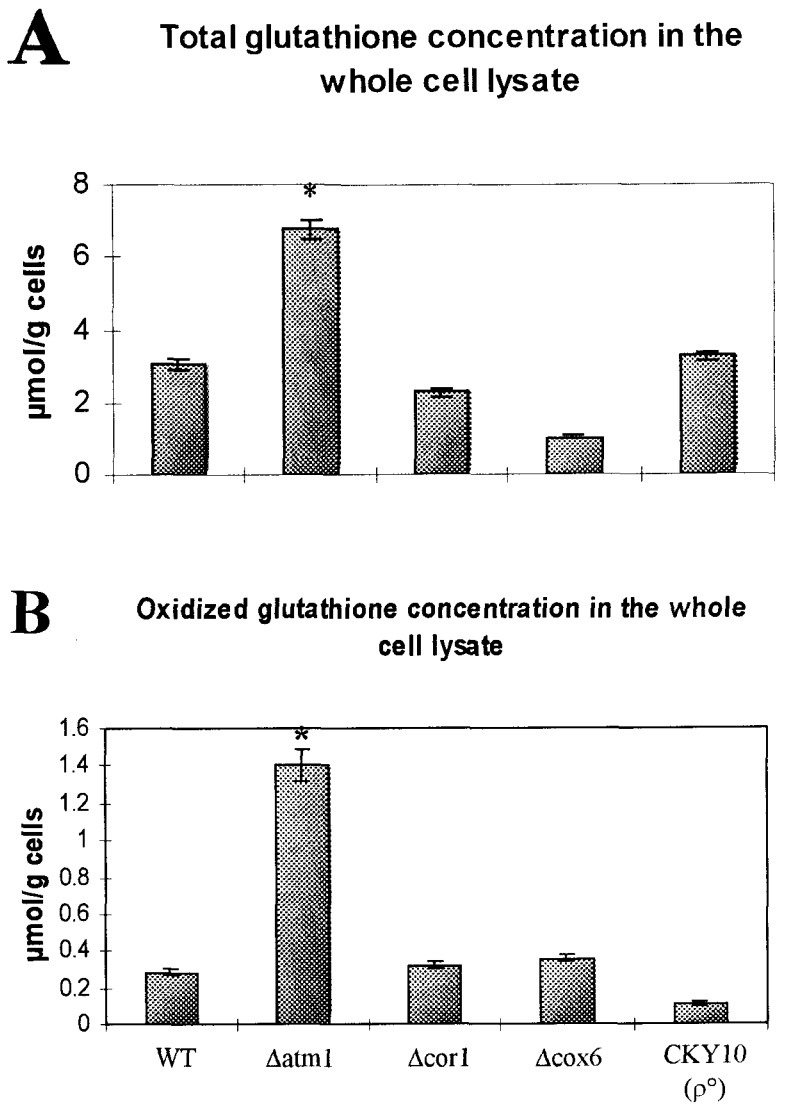

Figure 3.3.

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 (0.1M sodium-phosphate pH 7.4, 5mM EDTA). The cellular content of total and oxidized glutathione was determined from a perchloric acid extract with the glutathione reductase assay [69]. Data represent the average of three independent determinations with a standard deviation of less than 5% (n=3, *$P<0.05$, compared to WT)

3.4. *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 [30]. We therefore determined the amount of „free" iron i.e. iron that is not bound to heme or iron-sulphur (Fe/S) proteins. Wild type mitochondria contained about 2 nmol free iron per mg mitochondrial protein, as measured by the bathophenanthroline method (Figure 3.4/A) [84]. This agrees well with published data [53,54]. In contrast, mitochondria isolated from Δatm1 cells accumulated 30 times more free iron (Figure 3.4/A). This increase is three-fold higher than the accumulation of iron in mitochondria lacking yeast frataxin [53,54]. The large content of free iron in Δatm1 mitochondria was confirmed by using atomic absorption spectroscopy (not shown). The postmitochondrial supernatant of the Δatm1 cells contained only about twice as much free iron as that of wild type cells (not shown). No increases in mitochondrial iron levels were found for various *pet* cells (Figure 3.4/D).

Increased free iron concentration 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 (Figure 3.4/B) [92]. 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 (Figure 3.4/C). 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 3.4/1). 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 cytochromes c_1 or other components of complex III (not shown) [64]. 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.

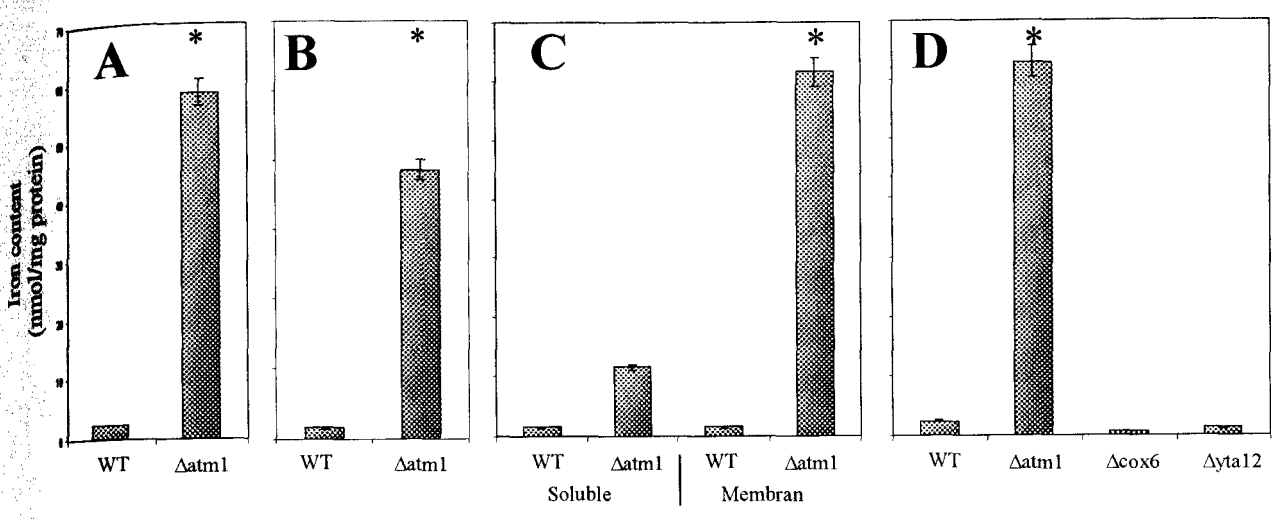

Figure 3.4.

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-sulphur proteins) was determined by the bathophenantroline method [84]. 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 1h at 100000 x *g*. 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. (n=7, *P<0.01, compared to WT)

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

	Wild type	Δatm1	Δatm1/vT(%)
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

Table 3.4./1

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, 50mM 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), *$P<0.05$, compared to WT. One unit is defined as 1 μmol substrate converted per minute.

3.5. *The full-length sequence of human ABC7*

The full-length sequence of human ABC7 (hABC7) was derived from an expressed sequence tag clone (EST No. AA305099) which displayed significant sequence identity to mouse ABC7, encoding an ABC transporter [93]. The entire EST clone was sequenced and the protein sequence of hABC7 protein (752 amino acid residues) was derived (Figure 3.5). During the course of our studies the full-length sequence of hABC7 has been reported also by another group [94]. However, no further information on this protein was provided. The human ABC7 protein shares highest sequence homology with the mouse ABC7 partial sequence (87% identical and 91% chemically similar amino acid residues), with *Saccharomyces cerevisiae* Atm1p (43% and 72%, respectively) and with an open reading frame of *Saccharomyces pombe* (38% and 66%, respectively; Figure 3.5). The sequence identity between these 4 proteins is similar in the C-terminal ABC domain and in the N-terminal membrane-spanning region (about 42% for the human and *Saccharomyces cerevisiae* proteins). Usually, high homology between members of the ABC transporter family is confined to the ABC domains. The significant homology also in the membrane-spanning region raises the possibility that the mammalian and the fungal proteins comprise functional orthologues.

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  hABC7  MALLAMHSWR WAAAAAAFEK RRHSAILIRP LVSVSGSGPQ WRPHQLGALG TARAYQ-IPE SLKSITWQRL GKGNSGQFLD AAKALQVWPL IEKRTCWHGH
  mABC7                                                                  E SLRNTTQQRW GKDNSRQLLD ATKALQTWPL IEKRTCWHGH
ScAtm1p                                MLLLP RCPVIGRIVR SKFRSGLIRN HSRNHSPVIF TVSKLSTQRP LLFNSAVNLW NQAQKD---I THKKSVEQFS
SpAtm1p                    MLE RCPWKLISSP RNIPARSFLN SRGTYLVLRK SNILPLQHIL RFSNFASKQC FPLRNGNNSA SKALWN---- NKSKEKEPLN

  hABC7  AGGGLHTDPK EGLK--DVDT RKIIKAMLSY VWPKDRPDLR ARVAISLGFL GGAKAMNIVV PFMFKYAVDS LNQMSGNMLN LSDAPNTVAT MATAVLIGYG
  mABC7  AGGGLHTDPK EGLK--DVDT RKIIKAMLSY VWPEDRPDLR ARVAISLGFL GGAKAMNIVV PFMFKYAVDS LNQMSGNMLN LSDAPNTVAT MATAVLIGYG
ScAtm1p  SAPKVKTQVK KTSKAPTLSE LKILKDLFRY IWPKGNNKVR IRVLIALGLL ISAKILNVQV PFFFKQTIDS MN------IA WDDPTVALPA AIGLTILCYG
SpAtm1p  TSVKLASDVP DDK---NVTG QMIVKDMLQY IWPKGKTNLK VRVVSALALL VAAKILNVQV PFYFKSIIDT MN------TT LVQEVGALWS TVGAVVLGYG

  hABC7  VSRAGAAFFN EVRNAVFGKV AQNSIRRIAK NVFLHLHNLD LGFHLSRQTG ALSKAIDRGT RGISFVLSAL VFNLLPIMFE VMLVSGVLYY KCGAQFALVT
  mABC7  VSRAGAAFFN EVRNAVFGKV AQNSIRRIAK NVFLHLHNLD LGFHLSRQTG ALSKAIDRGT RGISFVLSAL VFNLLPIVFE MMLVSSVLYY KCGAQFALVT
ScAtm1p  VARFGSVLFG ELRNAVFAKV AQNAIRTVSL QTFQHLMKLD LGWHLSRQTG GLTRAMDRGT KGISQVLTAM VFHIIPISFE ISVVCGILTY QFGASFAAIT
SpAtm1p  FARIFSTVFQ ELRNSVFAIV SQSAIRSVSS NVYQHLLNLD MNFHLSKQTG SITRAMDRGT KGISFILSSM VLHIIPITLE IAMVSGILTY KYGPSFSAIA

  hABC7  LGTLGTYTAF TVAVTRWRTR FRIEMNKADN DAGNAAIDSL LNYETVKYFN NERYEAQRYD GFLKTYETAS LKSTSTLAML NFGQSAIFSV GLTAIMVLAS
  mABC7  LGTLGAYTAF TVAVTRWRTR FRIEMNKADN DAGNAAIDSL LNYETVKYFN NEKYEAQRYD GFLKTYETAS LKSTSTLAML NFGQNAIFSV GLTAIMVLAS
ScAtm1p  FSTMLLYSIF TIKTTAWRTH FRRDANKADN KAASVALDSL INFEAVKYFN NEKYLADKYN GSLMNYRDSQ IKVSQSLAFL NSGQNLIFTT ALTAMMYMGC
SpAtm1p  ATTVALYALF TVRTTSWRTV FRRQANAADS KASAAAIESL INYEAVKTFN NESYEMSRYE KHLSAYEKAN VKVASSLAFL NSGQAIIFST ALTLMMYMGC

  hABC7  QGIVAGTLTV GDLVMVNGLL FQLSLPLNFL GTVYRETRQA LIDMNTLFTL LKVDTQIKDK VMASPLQITP QTA-TVAFDN VHFEYIEGQK VLSGISFEVP
  mABC7  QGIVAGALTV GDLVMVNGLL FQLSLPLNFL GTVYRETRQA LIDMNTLFTL LKVDTRIKDK VMAPPLQITP QTA-TVAFDN VHFEYIEGQK VLNGVSFEVP
ScAtm1p  TGVIGGNLTV GDLVLINQLV FQLSVPLNFL GSVYRDLKQS LIDMETLFKL RKNEVKIKNA --ERPLMLPE NVPYDITFEN VTFGYHPDRK ILKNASFTIP
SpAtm1p  RGIVTSNLTV GDLVMINQLV FQLSIPLNFL GSVYREMRQA FTDMEQLFSL KRINIQVKEA PDARDLVLKG G---SIQFDN VHFSYNPNRP ILNGCSFNIP

  hABC7  AGKKVAIVGG SGSGKSTIVR LLFRFYEPQK GSIYLAGQNI QDVSLVSLRR AVGVVPQDAV LFHNTIYYNL LYGNISASPE EVYAVAKLAG LHDAILRMPH
  mABC7  AGKKVAIVGG SGSGKSTIVR LLFRFYEPQK GSIYLAGQNL QDVSLESLRR AVGVVPQDAV LFHNTIYYNL LYGNINASPE EVYAVAKLAG LHDAILRMPH
ScAtm1p  AGWKTAIVGS SGSGKSTILK LVFRFYDPES GRILINGRDI KEYDIDALRK VIGVVPQDTP LFNDTIWENV KFGRIDATDE EVITVVEKAQ LAPLIKKLPQ
SpAtm1p  AGAKVAFVGA SGCGKSTILR LLFRFYDTDS GKILIDNQRL DQITLNSLRK AIGVVPQDTP LFNDTILYNI GYGNPKASND EIVEAAKKAK IHDIIESFPE

  hABC7  GYDTQVGERG LKLSGGEKQR VAIARAILKD PPVILYDEAT SSLDSITEET ILGAMKD--V VKHRTSIFIA HRLSTVVDAD EIIVLDQGKV AERGTHHGLL
  mABC7  GYDTQVGERG LKLSGGEKQR VAIARAILKN PPVILYDEAT SSLDSITEET ILGAMRD--V VKHRTSIFIA HRLSTVVDAD EIIVLSQGKV AERGTHYGLL
ScAtm1p  GFDTIVGERG LMISGGEKQR LAIARVLLKN ARIMFFDEAT SALDTHTEQA LLRTIRDNFT SGSRTSVYIA HRLRTIADAD KIIVLDNGRV REEGKHLELL
SpAtm1p  GYQTKVGERG LMISGGEKQR LAVSRLLLKN PEILFFDEAT SALDTNTERA LLRNIND

  hABC7  ANPHSIYSEM WHTQSSRVQN HDNPKWEAKK ENISKEEERK KLQEEIVNSV KGCGNCSC
  mABC7  ANSSSIYSEM WHTQSNRVQN QDSLGWDAKK ESLSKEEERK KLQEEIVNSV KGCGNCSC
ScAtm1p  AMPGSLYREL WTIQEDLDHL ENELKDQQEL
SpAtm1p
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Figure 3.5

Figure 3.5.

Sequence alignment of the ABC transporters ABC7 and Atm1p. The figure depicts the primary structures of human ABC7 (hABC7) and mouse ABC7 (mABC7) [93], as well as Atm1p of *Saccharomyces cerevisiae* (ScAtm1p) and an open reading frame of *Saccharomyces pombe* (SpAtm1p). The potential membrane-spanning regions are underlined. The selection of these regions takes into account the distribution of positively and negatively charged residues on both sides of the inner membrane (positive inside rule) [95,96]. The ATP binding motif (P-loop) and the DEAT motif are shown in boldface. The alignment was created using the CLUSTAL multiple sequence alignment program.

3.6. *Human ABC7 is a mitochondrial protein*

The subcellular localization of human ABC7 (hABC7) protein was determined. We tested the ability of the N-terminus of hABC7 protein to serve as a mitochondrial targeting sequence and direct the protein to the organelles in vitro [97,98]. A fusion protein comprised of the first 135 amino acid residues of hABC7 and mouse dihydrofolate reductase (DHFR) was synthesized in reticulocyte lysate. The radiolabelled protein (termed preABC7(135)-DHFR) was incubated with isolated yeast mitochondria to allow import of the preprotein. The reaction mixture was treated with proteinase K to digest the precursor protein that had not been sequestered by the organelles. A substantial fraction of the added preprotein became processed to a shorter form upon incubation with mitochondria (Figure 3.6.1/A, upper panel). The majority of this „mature„ protein was resistant to digestion by proteinase K indicating that it was imported into mitochondria and cleaved by the matrix processing peptidase. When the outer membrane of mitochondria was selectively opened by a swelling procedure (see Figure 3.6.1/A, lower panel), the imported fusion protein was resistant to proteinase K. In contrast, upon lysis of the mitochondria with detergent the fusion protein became sensitive to proteolytic attack demonstrating that it had become imported into the matrix.

The transfer of the presequence across the mitochondrial inner membrane requires a membrane potential, $\Delta\Psi$ [99,100]. We therefore tested the dependence of the import of the fusion protein upon the existence of an

energized inner membrane. The membrane potential was depleted by the addition of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). Under these conditions neither cleavage of preABC7(135)-DHFR to the mature form nor protease resistance of the fusion protein was observed (Figure 3.6.1/B). A small fraction of added preABC7(135)-DHFR remained inaccessible to proteolytic degradation in these experiments which is due to aggregation of the protein (Figure 3.6.1; not shown). Together, these data demonstrate that the N-terminus of hABC7 protein can target an attached passenger protein to mitochondria and direct its import into the organelles in a membrane potential-dependent fashion. This suggests that hABC7 protein represents a constituent of mitochondria.

To test the subcellular localization of hABC7 protein in vivo, we raised an antibody against a fusion protein of hABC7 and glutathione S-transferase. The fusion protein was expressed in *Escherichia coli* and purified by affinity chromatography using a glutathione resin. The antibody could specifically immunoprecipitate hABC7 that was synthesized in vitro (not shown). It recognized a protein of 68 kDa in mitochondria isolated from human liver, but not in a post-mitochondrial supernatant or a cytosolic fraction (Figure 3.6.2). When hABC7 protein was expressed in wild-type yeast cells by transforming with a multi-copy plasmid encoding hABC7, a protein of the same electrophoretic mobility (68 kDa) was recognized by this antiserum (Figure 3.6.2). No immunostaining was observed for the post-mitochondrial supernatant

derived from yeast cells expressing hABC7 protein or for wild-type yeast mitochondria. In summary, our in vitro and in vivo data demonstrate that hABC7 protein is localized in mitochondria.

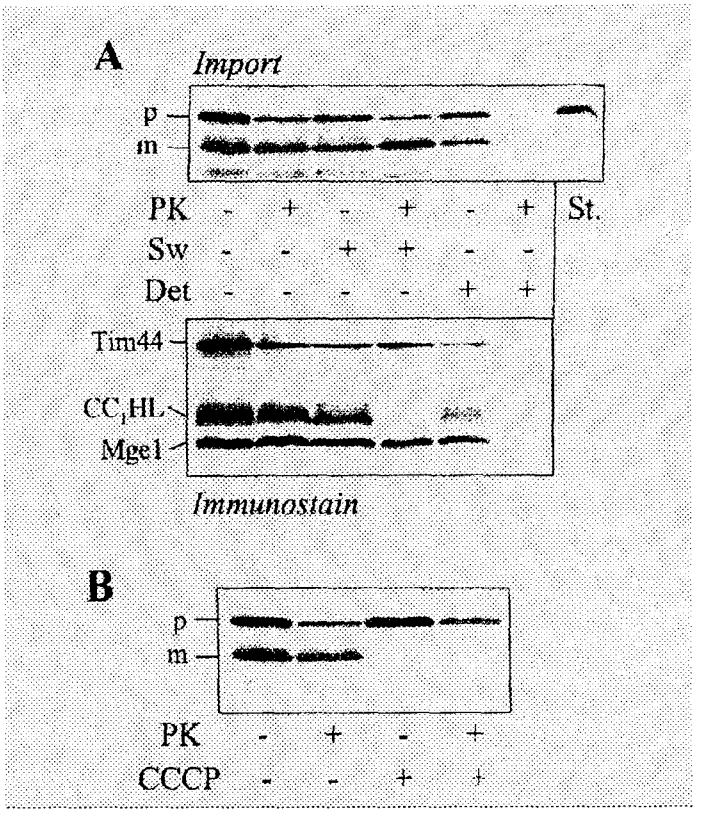

Figure 3.6./1

Human ABC7 is a mitochondrial protein. A) The fusion protein preABC7(135)-DHFR was synthesized in reticulocyte lysate and incubated with isolated yeast mitochondria [77]. After 15 min at 25°C mitochondria were reisolated by centrifugation. Samples were resuspended in SOH buffer (0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.2), subjected to osmotic swelling (Sw), a procedure to selectively rupture the outer membrane [101] or dissolved in 0.5% Triton X-100 detergent (Det). Samples were treated with or without 50 µg/ml proteinase K (PK) on ice for 10 min. Proteins were precipitated with TCA, separated by SDS-PAGE and blotted onto nitro-cellulose membrane. Radiolabelled proteins were visualized by autoradiography of the blot (Import) which then was

immunostained for the indicated marker proteins of the matrix (translocase of the inner mitochondrial membrane (Tim44p) and mitochondrial GrpE-homologue (Mge1p)) and the intermembrane space (CC_1HL). CC_1HL was degraded by protease after rupture of the outer membrane, whereas Tim44p and Mge1p were accessible to proteolysis only after opening of both the outer and inner membranes. p and m, precursor and mature forms of preABC7(135)-DHFR, respectively; St., a standard containing 20% of input preprotein. B) Import of preABC7(135)-DHFR was performed as in A in the presence or absence of 50 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) to deplete the membrane potential. Samples were treated with proteinase K and import was analyzed as described in A.

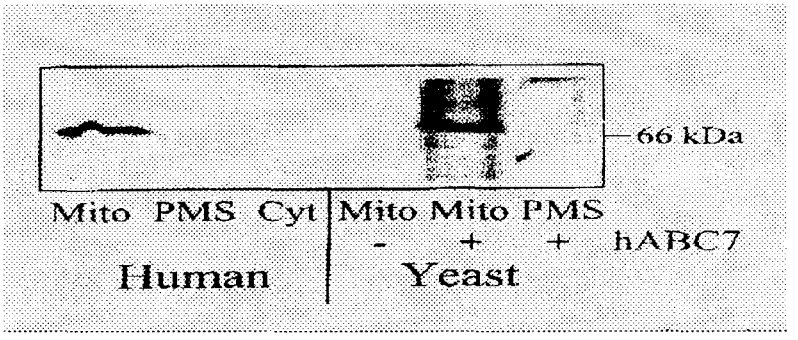

Figure 3.6./2.

Human ABC7 is a mitochondrial protein. A human liver was homogenized and mitochondria (Mito), a post-mitochondrial supernatant (PMS) and a cytosolic fraction (Cyt) were prepared (left part). Mitochondria and post-mitochondrial supernatant were isolated from wild-type yeast cells expressing or not hABC7 protein (right part). Aliquots were subjected to SDS-PAGE and immunostaining was performed using the antiserum raised against the ABC domain of hABC7. The hABC7 protein tends to aggregate after expression in yeast (see staining above hABC7 band).

3.7. *Human ABC7 can functionally complement the deletion of yeast ATM1*

The hABC7 gene was expressed in yeast cells in which the ATM1 gene had been deleted (strain Δatm1) [2]. Synthesis of hABC7 restored almost wild-type growth similar to what was found when the ATM1 gene was reintroduced into these cells (Figure 3.7/A). Thus, hABC7 protein can largely compensate for the growth defects observed upon inactivation of Atm1p.

We asked further whether expression of hABC7 in Δatm1 yeast cells (strain Δatm1/hABC7) [3] can revert the phenotypic consequences observed for Δatm1 cells, namely the high content of iron within mitochondria, the lack of cytochromes and extra-mitochondrial heme-containing proteins as well as the oxidative stress indicated by, e.g., increased levels of cellular glutathione [2]. Mitochondria were purified from Δatm1/hABC7 cells. The isolated organelles contained wild-type levels of cytochromes thus differing markedly from Δatm1 mitochondria, which were largely deficient in all cytochromes (Figure 3.7/B) [3]. A similar result was obtained for the extra-mitochondrial heme-containing protein catalase. The strongly decreased level of this enzyme in Δatm1 cells was restored to almost wild-type activity upon expression of hABC7 (Figure 3.7/C). Furthermore, Δatm1/hABC7 cells contained only twofold increased levels of mitochondrial „non-heme, non-iron/sulphur„ iron as compared to wild-type organelles, whereas a large increase in iron was observed for Δatm1

mitochondria (Figure 3.7/D).

The cellular levels of total and oxidized glutathione were determined using cell lysates. Expression of hABC7 in Δatm1 cells resulted in a substantial, but not complete reduction of the elevated levels of glutathione found in Δatm1 cells (Figure 3.7/E). The reason for this partial complementation is unknown, but may be related to the slightly slower growth of Δatm1/hABC7 relative to wild-type cells (see Figure 3.7/A). In summary, expression of hABC7 can largely or completely revert the known phenotypic consequences of the deletion of yeast ATM1. Our results demonstrate that hABC7 protein represents the functional orthologue of the mitochondrial ABC transporter Atm1p.

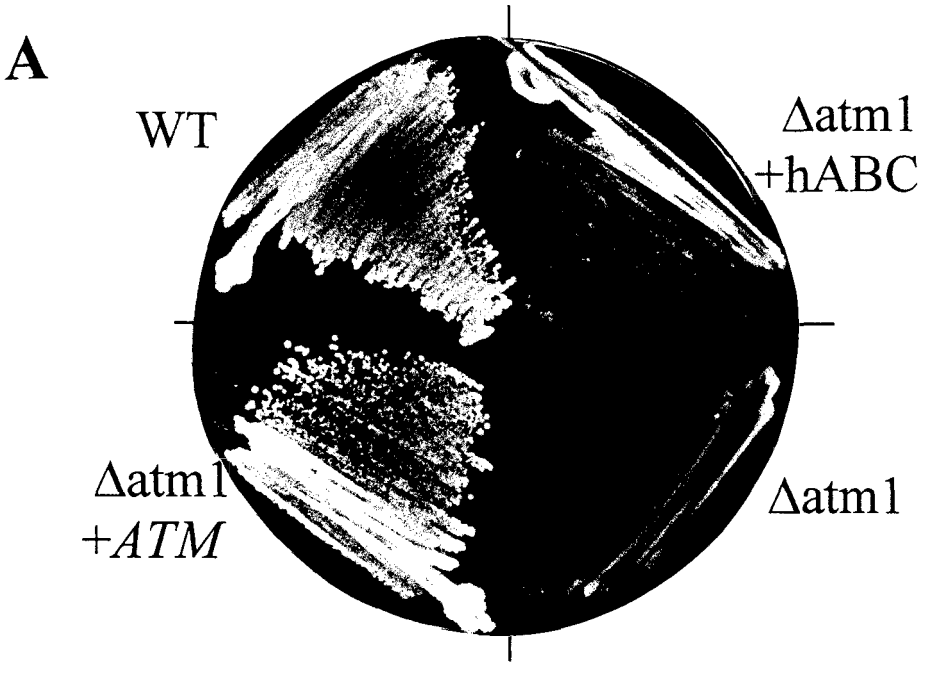

Figure 3.7./A

Human ABC7 can largely restore the phenotypic consequences of the deletion of the yeast ATM1 gene. A) Expression of hABC7 restores growth to Δatm1 cells. Wild-type (WT) and Δatm1 cells harboring either an empty vector (Δatm1), the yeast ATM1 gene or the human ABC7 gene (hABC7) on the multi-copy plasmid pRS424-GPD were grown on agar plates containing YPD medium for 3 days at 30°C.

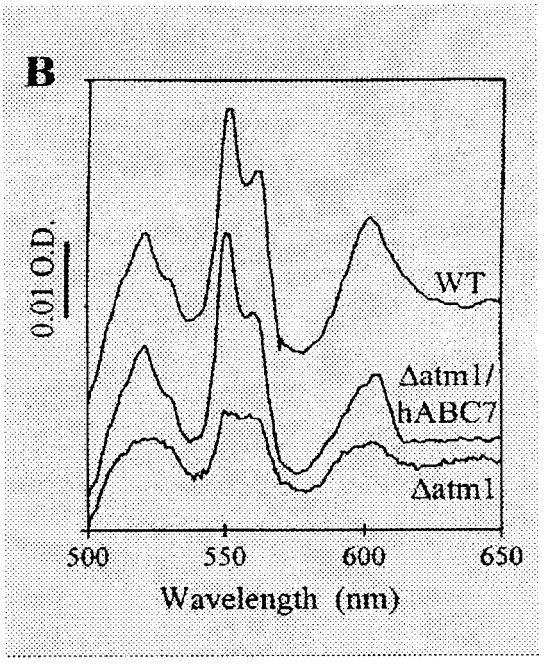

Figure 3.7./B

Human ABC7 can largely restore the phenotypic consequences of the deletion of the yeast ATM1 gene. B) Cytochrome spectra were recorded using mitochondria isolated from wild type, Δatm1 or Δatm1/hABC7 strains. The bar on the left represents an absorption difference of 0.01. O.D.

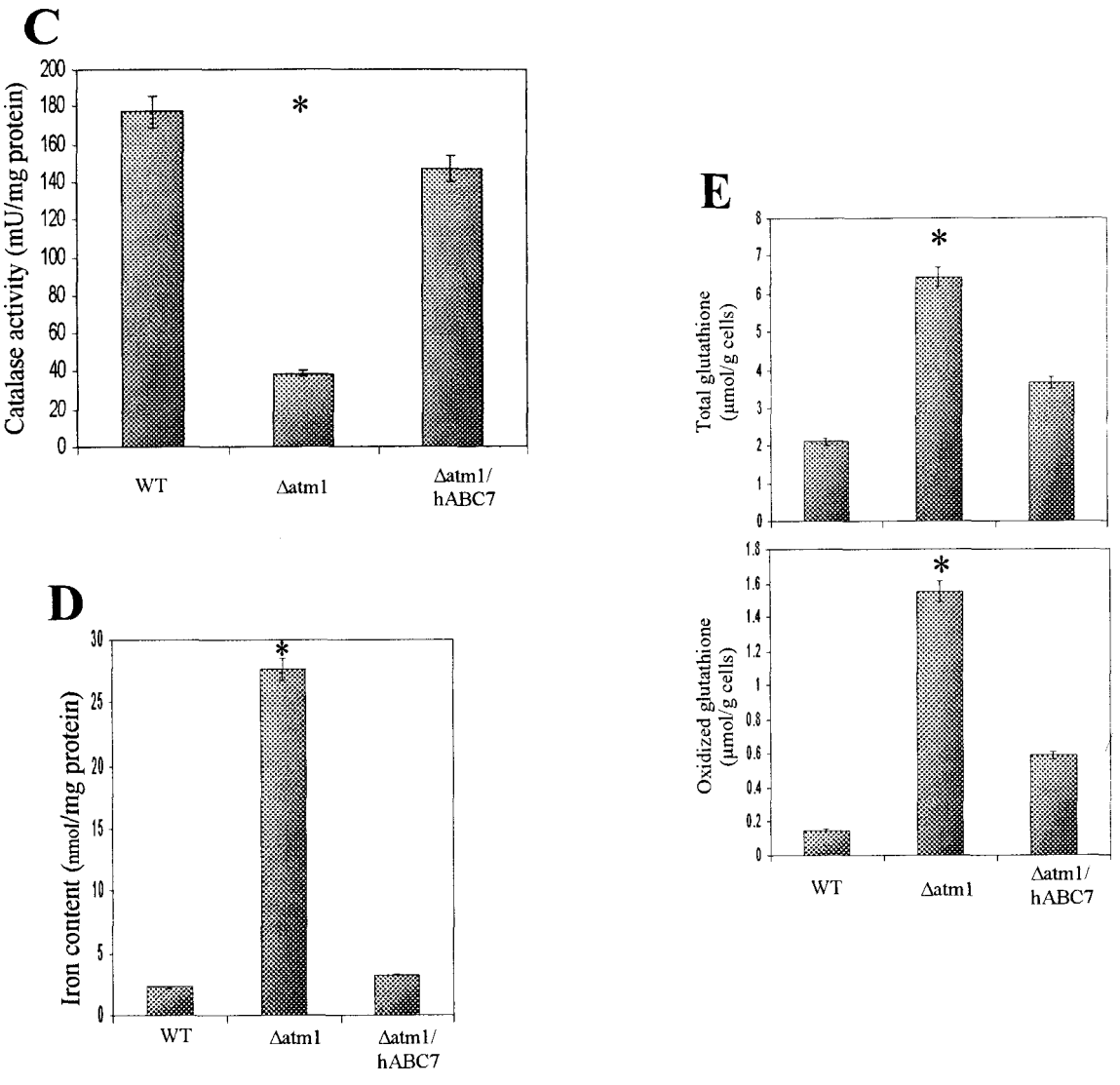

Figure 3.7./C,D,E

Human ABC7 can largely restore the phenotypic consequences of the deletion of the yeast ATM1 gene. C) Catalase activities were measured using extracts of the indicated cells grown in YPD medium [84] (n=3, *P<0.01, compared to WT). D) The iron content of mitochondria isolated from the indicated strains grown on YPGal medium was measured as previously published [85] (n=7, *P<0.01, compared to WT). E) The cellular contents of total and oxidized glutathione were determined for the indicated yeast strains grown on YPD medium as described [63] (n=5, *P<0.01, compared to WT).

4. Discussion

We report here several novel findings that are important for the understanding of the cellular role of Atm1p. This protein is localized in the mitochondria [1,2,3]. Cells lacking functional Atm1p display a general deficiency in the holoforms, but not the apoforms of heme-containing proteins both inside and outside mitochondria [2]. 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 [2,3].

Based on the observations, we propose that the key feature in the phenotype of cells lacking functional ATM1 is this mitochondrial iron deposition [2,3]. Iron, at elevated concentrations, is known to elicit the formation of free radicals and thus is toxic to cells [53,54]. The radicals resulting from the increased iron levels could lead to the unspecific oxidative damage of heme-containing proteins observed in Δatm1 cells. High levels of catalase more effectively remove H_2O_2, a substrate for the iron-dependent

formation of hydroxyl radicals by Fenton reaction, and thus may decrease the oxidative stress within peroxisomes.

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. It is not known whether ABC proteins are channels as in the case of cystic fibrosis transmembrane regulator (CFTR) [102] rather than transporters. However it has emerged that several ABC proteins regulate various channels, and maybe other membrane proteins, besides possessing their own transporter/channel activities [6]. How do ABC transporters regulate the activity of heterologous channels? One mechanism to regulate a channel, that the ABC protein transports a regulatory molecule. Many direct and indirect mechanisms can be envisaged if we assume that the regulation of heterologous proteins by ABC transporters is distinct from their intrinsic channel/transporter activity. An indirect mechanism is vesicle trafficking, in which the ABC protein facilitates insertion of an additional channel protein into the membrane, but of course membrane trafficking does not occur in prokaryotic cells. The direct protein-protein interactions seem the most likely mechanism, but there is a little evidence either

for or against this hypothesis. The ABC protein can form a complex with heterologous channel proteins in the membrane and this could generate specificity of regulation. This variation could occur in both prokaryotic and eukaryotic cells. The possibility that an intermediate protein switches between the ABC protein and channel protein is also imaginable. These regulatory events have in certain clinical cases physiological importance [6]. 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 [6,95]. Further elucidation of the function of Atm1p will benefit from investigations how iron is imported into mitochondria.

To initiate the analysis of human diseases caused by a defect in mitochondrial iron metabolism, we identified the functional human orthologue.

We described the localization of hABC7 within mitochondria and the functional complementation by hABC7 of a yeast mutant lacking the mitochondrial ABC transporter Atm1p. The sequence similarity among various members of the ABC transporter family is limited. Highest sequence conservation is observed in the ABC domain, in particular in the region of nucleotide binding (the P-loop), whereas in the membrane spanning region similarity usually is low. The significant homology of the mammalian ABC7 proteins with the fungal Atm1p in both domains (see Figure 3.5) rendered it likely that these proteins represent functional orthologues.

We provide evidence for the localization of hABC7 protein within mitochondria. An antibody raised against hABC7 recognized a protein of predicted size in human mitochondria and in mitochondria isolated from yeast cells expressing this protein. As demonstrated by our in vitro import studies the targeting information appears to be localized at the N-terminus of hABC7 [3], as this is the case for most mitochondrial preproteins. Mitochondria are probably descended from endosymbiotic bacteria. During evolution, most of the genes of the original endosymbiont were either lost or transferred to the host nucleus. Therefore all mitochondrial proteins encoded in the nucleus are synthesized in the cytoplasm, and must be imported into the organelle [103]. Targeting of proteins to different cellular locations is often mediated by N-terminal topogenic sequences by this respective precursor protein, called mitochondrial targeting signals (MTSs) [96]. In particular this is true for proteins destined for the secretory pathway and for nuclearly encoded mitochondrial and chloroplast proteins. In both groups, translocation across at least one membrane is necessary before the final destination is reached. One possibility for targeting mechanism is used by a group of intermembrane space enzymes that includes cytochrome b_2, cytochrome c_1 [67,101,104]. These proteins are synthesized with transient presequences. The amino terminal portion of each of these presequences resembles a matrix targeting signal. The carboxy terminal portion contains a hydrophobic stretch, and functions as the intermembrane space targeting information is decoded by the mitochondria. In the case of 'stop transfer'

hypothesis the amino terminal portion of the presequence is imported into the matrix, but the inner membrane space targeting domain arrests further translocation [96]. At the way of 'conservative sorting' model the proteins are first imported completely into the matrix and are then translocated back to the inner membrane space [96].

This pathway is thought to be the evolutionary conserved bacterial-type export machinery.

Secretory topogenic sequences (signal peptides) are extremely variable both in length and amino acid sequence, but they do share a common basic design: A positively charged N-terminal region, a central hydrophobic region and a more polar C terminal portion, that defines the cleavage site [95,96]. The mitochondrial targeting sequences are extremely variable, and the only common theme recognized so far is a preponderance of basic and hydroxyl-carrying residues. Mitochondrial targeting sequences are enriched for Arg, Leu, and Ser (but not for Lys) and have few Asp, Glu, Val, and Ile. They can form helices with a higher hydrophobic moment than controls, but the non-polar face does not seem to be strongly selected for high hydrophobicity [105].

Import to each of these compartments appears analogous with the following common features: (a) most polypeptides are initially synthesized as a precursors of higher molecular mass than the corresponding mature form (b) the import process requires an electrochemical gradient across the mitochondrial inner membrane. (c) precursors are processed to the mature polypeptides by a

soluble chelator sensitive protease (located in the mitochondrial matrix) [104].

All inner mitochondrial membrane proteins contain additional sequence determinants, so called topogenic signals, distinct from the MTS-s, which not only ensure their sorting to the inner membrane, but also determine the final orientation in the membrane. These topogenic signals generally comprise hydrophobic cores of varying length, flanked on both sides by charged, hydrophylic amino acids. Mostly this portion forms a transmembrane domain [96]. We have to mention that the translocation into the inner mitochondrial membrane is dependent on a mitochondrial membrane potential also [100].

It should be stressed that the extreme N-terminal region of hABC7 differs from typical mitochondrial presequences [105,106] in that it contains an unusual number of hydrophobic amino acids and a negatively charged residue. Thus, based on sequence information it was impossible to predict a mitochondrial localization of this protein. As estimated from the size difference of the precursor and mature forms of the fusion protein preABC7(135)-DHFR used in our in vitro import studies (about 4-4.5 kDa; see Fig. 3.6.1), the processing site may be localized after residue 43 [3,107,108]. Thus, these first 43 amino acid residues appear to represent the targeting sequence assuring the mitochondrial localization of hABC7 protein.

On the basis of the results reported in this communication, hABC7 protein can be regarded as a functional orthologue of yeast Atm1p [1,2,3]. When expressed in yeast cells lacking Atm1p, the hABC7 protein was localized to

mitochondria and could replace the functions of Atm1p to a great extent. Firstly, it restored almost wild-type growth to Δatm1 cells. Secondly, the Δatm1/hABC7 cells contained wild-type levels of cytochromes and extra-mitochondrial heme-containing proteins such as catalase. Thirdly, mitochondria did not contain the largely elevated iron levels that were reported for Δatm1 organelles. Finally, the cellular content of glutathione was substantially reduced by expression of hABC7 protein in Δatm1 cells. These data suggest that the functions of yeast Atm1p and human ABC7 overlap and that they likely transport the same substrate across the mitochondrial inner membrane.

Based on the orientation in the membrane with the ABC domain facing the matrix space [1], Atm1p/hABC7 functions as an exporter. Which substrate might require active export from mitochondria mediated by Atm1p/hABC7? Clues to answer this important question might come from the functional reconstitution of the ABC transporters and from the analysis of components interacting with these proteins. One protein which has been reported to genetically interact with Atm1p is the mitochondrial branched-chain amino acid transaminase Bat1p of yeast, which upon over-expression can restore growth to the temperature-sensitive strain *atm1-1* at the non-permissive condition [109]. Branched-chain α-keto acids have been described to function as siderophores in certain bacteria. [110] It thus seems possible that α-keto acids may serve a similar function in eukaryotic cells and alleviate the toxic effects of increased iron levels in cells defective in Atm1p. More investigations are needed to solve

these interesting questions.

The human ABC7 gene has been mapped to chromosome Xq13.1-q13.3 [93]. This region has been implicated in hereditary X-linked sideroblastic anaemia, in which cells contain high deposits of non-heme iron within mitochondria which are arranged in ring-like structures around the nucleus (ring sideroblasts [58].

Based on our results obtained during the analysis of ATM1 mutant yeast cell we suggested that a mutation in hABC7 might be connected to this iron storage disease. Shortly after the publication of our study, a report appeared in Human Molecular Genetics, [111] that fully proved our ideas. The authors of this paper showed, that an X-linked sideroblastic anaemia concomitant with ataxia (XLSA/A) [61] is a recessive disorder characterized by an infantile to early childhood onset of non-progressive cerebellar ataxia and mild anaemia with hypochromia and microcytosis. A gene encoding an ATP-binding cassette (ABC) transporter was mapped to Xq13, a region previously shown by linkage analysis to harbor the XLSA/A gene. The authors proved also, that the gene, ABC7, is an orthologue of the yeast ATM1 gene is involved in iron homeostasis. The full-length ABC7 cDNA was cloned and the entire coding region screened for mutations in a kindred in which five male members manifested XLSA/A. The mutation in this variant caused isoleucine 400 - methionine exchange in a predicted transmembrane segment of the ABC7 gene in patients with XLSA/A. The mutation was shown to segregate with the disease

in the family and was not detected in at least 600 chromosomes of general population controls. Introduction of the corresponding mutation into the *Saccharomyces cerevisiae* ATM1 gene resulted in a partial loss of function of the yeast Atm1 protein.

This study represents the first steps in the analysis of the mitochondrial iron metabolism in an approach where the identification of the participants and the functional analysis was the aim. A deletion of the first identified yeast component, Atm1p or a mutation of its human orthologue, hABC7 results in severe consequences in both cell types, what emphasizes the highly conserved nature of the mitochondrial iron metabolism and also the importance of this process in the life of the eukaryotic cells.

5. *References*

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