The EMBO Journal Vol.18 No.14 pp.3981–3989, 1999

The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins

Gyula Kispal[1], Peter Csere, Corinna Prohl and Roland Lill[2]

Institut für Zytobiologie und Zytopathologie der Philipps-Universität Marburg, Robert-Koch-Strasse 5, 35033 Marburg, Germany

[1]Permanent address: Institute of Biochemistry, University Medical School of Pecs, Szigeti 12, 7624 Pecs, Hungary

[2]Corresponding author
e-mail: Lill@mailer.uni-marburg.de

Iron–sulfur (Fe/S) cluster-containing proteins catalyse a number of electron transfer and metabolic reactions. Little is known about the biogenesis of Fe/S clusters in the eukaryotic cell. Here, we demonstrate that mitochondria perform an essential role in the synthesis of both intra- and extra-mitochondrial Fe/S proteins. Nfs1p represents the yeast orthologue of the bacterial cysteine desulfurase NifS that initiates biogenesis by producing elemental sulfur. The matrix-localized protein is required for synthesis of both mitochondrial and cytosolic Fe/S proteins. The ATP-binding cassette (ABC) transporter Atm1p of the mitochondrial inner membrane performs an essential function only in the generation of cytosolic Fe/S proteins by mediating export of Fe/S cluster precursors synthesized by Nfs1p and other mitochondrial proteins. Assembly of cellular Fe/S clusters constitutes an indispensable biosynthetic task of mitochondria with potential relevance for an iron-storage disease and the control of cellular iron uptake.

Keywords: ABC transporter/iron homeostasis/iron–sulfur protein/mitochondria/sideroblastic anemia

Introduction

Iron–sulfur (Fe/S) cluster-containing proteins are involved in numerous metabolic processes such as isomerization and dehydration reactions and they serve as electron carriers in various redox reactions (Cammack, 1992; Johnson, 1998). The structure and function of these clusters and their associated proteins have been intensely studied in the past two decades (Beinert *et al*., 1997). Likewise, the chemical synthesis and properties of the various Fe/S clusters have been well documented. However, our knowledge about the components and mechanisms involved in Fe/S protein biosynthesis inside a living cell is still scarce. The process of Fe/S cluster assembly has been studied almost exclusively in bacteria (see Zheng *et al*., 1998). The genomes of prokaryotes contain operons in which genes involved in Fe/S biogenesis are clustered. The best-characterized operon is the Nif operon required for the synthesis of nitrogenase, a complex enzyme containing several Fe/S clusters (Peters *et al*., 1995). The recently identified Isc operon contains several genes that are homologous to genes of the Nif operon, and the encoded proteins may perform a housekeeping function in the assembly of cellular Fe/S proteins (Zheng *et al*., 1998). Only a few proteins encoded by the Nif, Isc and related operons have been functionally investigated. The pyridoxal phosphate-dependent protein NifS has been shown to produce elemental sulfur for Fe/S cluster synthesis by functioning as a cysteine desulfurase (Zheng *et al*., 1993; Zheng and Dean, 1994). Even though NifS is sufficient for synthesis of an Fe/S cluster *in vitro*, the *in vivo* assembly pathway is much more complex and involves the assistance of additional components encoded by the Nif operon.

In eukaryotes, most known Fe/S proteins are localized within the mitochondria, but an increasing number of Fe/S cluster components have been identified residing outside this organelle. These proteins include the iron regulatory protein 1 involved in the translational control of iron uptake into higher eukaryotes (Hentze and Kuhn, 1996; Rouault and Klausner, 1996), enzymes with biosynthetic functions in amino acid anabolism (Kohlhaw, 1988c), an ATP binding cassette (ABC) protein with proposed function as an inhibitor of RNase L (Bisbal *et al*., 1995), and an endonuclease (Augeri *et al*., 1997). Currently, little is known of how and where the synthesis of cellular Fe/S clusters and their assembly into apoproteins takes place and which proteins catalyse the process. Only recently a mutation in the protein Nfs1p, the eukaryotic homologue of bacterial NifS, has been shown to have some influence on the activity of two mitochondrial Fe/S proteins (Strain *et al*., 1998). Stronger effects were observed for mutants in two mitochondrial heat shock proteins (Jac1p and Ssq1p) providing a first indication that mitochondria may synthesize their own Fe/S clusters.

We became interested in the biogenesis of cellular Fe/S proteins in the course of our functional investigation of the mitochondrial ABC transporter Atm1p (Leighton and Schatz, 1995; Kispal *et al*., 1997). The protein is localized in the inner membrane with its ABC domains facing the matrix space and is thus predicted to function as an exporter. Deletion of the *ATM1* gene of the yeast *Saccharomyces cerevisiae* results in defects in cytochromes, respiration-incompetent mitochondria, increased glutathione levels indicating an oxidative stress, and an accumulation of iron in mitochondria (Leighton and Schatz, 1995; Kispal *et al*., 1997). A mutation in hABC7, the human functional orthologue of Atm1p, has been reported to cause X-linked sideroblastic anemia and ataxia (XLSA/A) in which mitochondria accumulate iron (Csere *et al*., 1998; Allikmets *et al*., 1999). The dramatic influence of defects in Atm1p and hABC7 on mitochondrial iron levels suggests an important role for these ABC transporters in mitochondrial iron metabolism, but their precise

function has remained elusive. Clearly, elucidation of the function of yeast Atm1p would help understanding of the molecular basis of the iron-storage disease.

Here, we present evidence for a function of Atm1p in the mitochondrial export of components required for Fe/S cluster incorporation into apoproteins located in the eukaryotic cytosol. Biosynthesis of the Fe/S clusters is initiated in the mitochondrial matrix by the cysteine desulfurase Nfs1p, which provides elemental sulfur for biogenesis. Nfs1p is essential for the assembly of Fe/S proteins both inside and outside of the mitochondria, while Atm1p is specific for cytosolic Fe/S cluster-containing proteins. Our study suggests that mitochondria are the primary site of Fe/S cluster synthesis in the eukaryotic cell. The findings ascribe a novel biosynthetic function to mitochondria in the assembly of Fe/S proteins outside the organelle.

Results

The ABC transporter Atm1p is required for generation of extra-mitochondrial Fe/S proteins

In our attempts to elucidate the function of the mitochondrial ABC transporter Atm1p, we noted a leucine auxotrophy of mutant yeast cells defective in Atm1p. We therefore tested which of the three specific steps of leucine biosynthesis might be responsible for this defect (Jones and Fink, 1982; Hinnebusch, 1992). The enzymes α-isopropyl malate synthase (Leu4p, Beltzer *et al*., 1988; and Leu9p proteins, W.Pelzer and R.Lill, unpublished) and β-isopropyl malate dehydrogenase (Leu2p) catalysing the first and third steps of leucine biosynthesis displayed wild-type activities in extracts isolated from cells in which the *ATM1* gene was deleted (Δatm1 cells; Kispal *et al*., 1997; see Materials and methods; Figure 1A). On the contrary, extracts of Δatm1 cells contained no detectable activity of the enzyme isopropyl malate isomerase (Leu1p; Kohlhaw, 1988c). This defect was not due to defective synthesis of the Leu1p polypeptide chain, since wild-type levels of Leu1p were detected by immunostaining analysis of extracts derived from Δatm1 cells (Figure 1A, insert).

Leu1p contains an Fe/S cluster and shares sequence homology with the Fe/S proteins aconitase and 'iron regulatory protein'. Our findings made it likely that the defect of Leu1p in Δatm1 cells is due to an impaired formation of the Fe/S cluster in this protein which is localized in the cytosol (Kohlhaw, 1988c; see below). To study directly the assembly of the Fe/S cluster of Leu1p, we employed a novel approach to follow the incorporation of radioactive ^{55}Fe into Leu1p *in vivo*. Yeast cells were radiolabelled with ^{55}Fe for 1 h and cell lysates were prepared. Leu1p was immunoprecipitated under native conditions using specific antibodies, and the Leu1p-associated radioactivity was quantitated by liquid scintillation counting. Using wild-type cells, significant amounts of ^{55}Fe were immunoprecipitated with the Leu1p-specific antiserum, whereas hardly any radioactivity was precipitated employing antibodies derived from preimmune serum (Figure 1B). With Δatm1 cells, no specific labelling of Leu1p with ^{55}Fe was detectable, even though the uptake of ^{55}Fe by Δatm1 cells was comparable to wild-type cells (left panel). The procedure is highly specific for Leu1p, as no significant amounts of radioactive iron were precipitated by anti-Leu1p antibodies using extracts prepared from cells in which the *LEU1* gene was inactivated (leu1 cells; Figure 1C). Hardly any difference in the assembly of the Fe/S cluster of Leu1p was observed in cells defective in other mitochondrial proteins, e.g. in cytochrome c_1 heme lyase (encoded by the *CYT2* gene; Zollner *et al*., 1992; Figure 1C). Together, our *in vivo* and *in vitro* data demonstrate a defect in the assembly of the Fe/S cluster

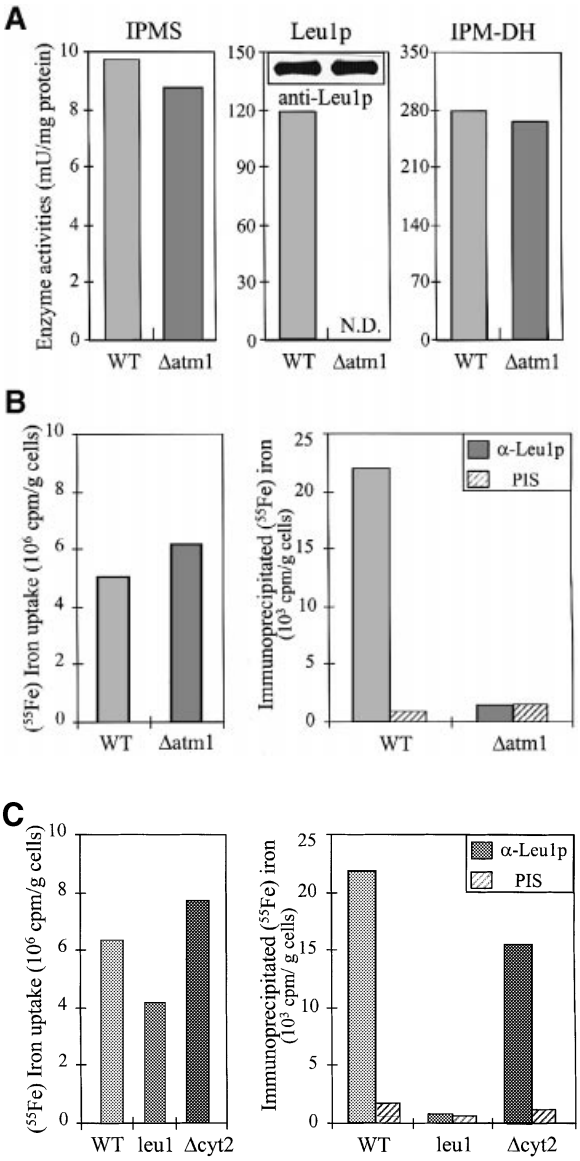

Fig. 1. Deletion of *ATM1* encoding a mitochondrial ABC transporter causes a defect in the assembly of the cytosolic Fe/S protein Leu1p. (**A**) Impairment of isopropyl malate isomerase (Leu1p) activity is responsible for the leucine auxotrophy of Δatm1 cells. The enzyme activities of the three specific steps of leucine biogenesis were measured using cell extracts prepared from wild-type (WT) or Δatm1 cells after growth in glucose-containing minimal media in the presence of leucine. The insert shows an immunostaining of Leu1p present in these extracts. IPMS, α-isopropyl malate synthase; IPM-DH, β-isopropyl malate dehydrogenase. N.D., not detectable, i.e. <25 mU/mg protein. (**B** and **C**) Δatm1 cells display a specific defect in the *in vivo* assembly of the Fe/S cluster of cytosolic Leu1p. Wild-type, Δatm1, leu1 and Δcyt2 cells were radiolabelled with ^{55}Fe, cell lysates were prepared, and ^{55}Fe uptake was quantitated by liquid scintillation counting. Immunoprecipitation using anti-Leu1p (α-Leu1p) antibodies or preimmune serum (PIS) was performed and co-precipitated ^{55}Fe was estimated by liquid scintillation counting.

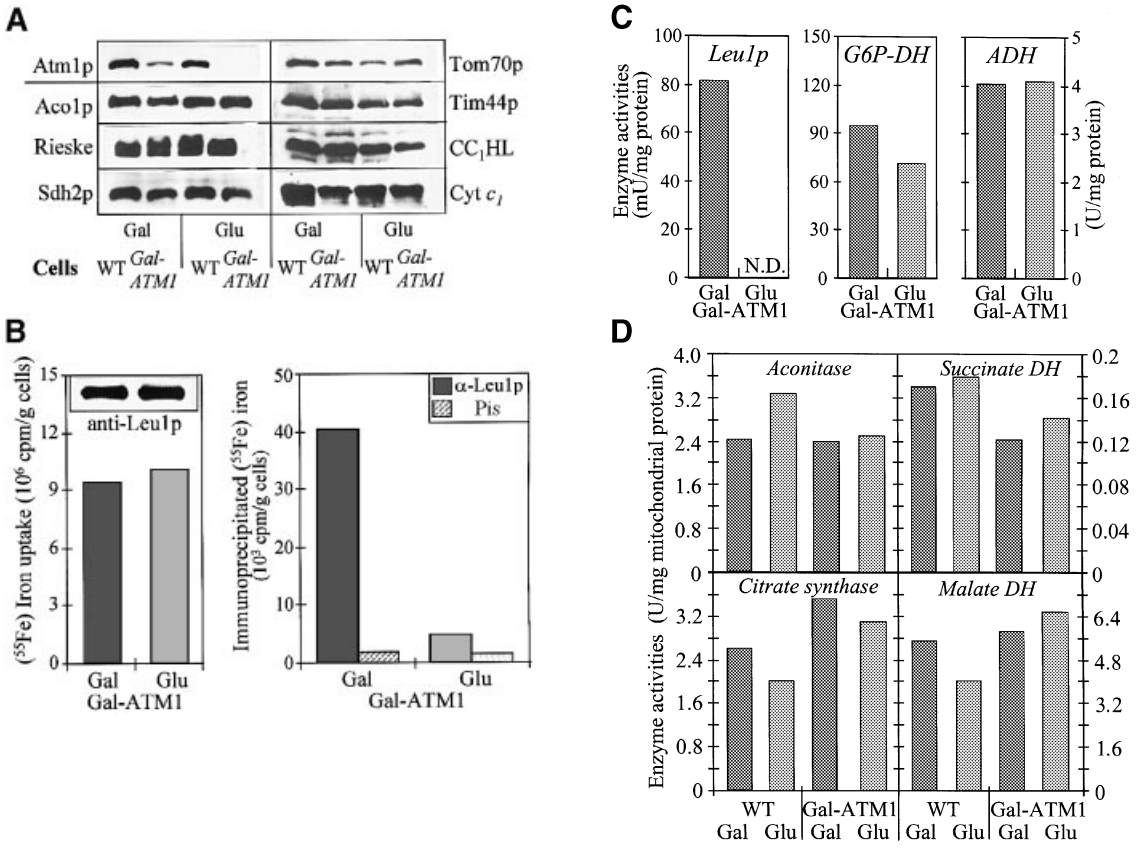

Fig. 2. Depletion of Atm1p impairs the assembly of cytosolic, but not of mitochondrial Fe/S cluster-containing proteins. (**A**) Immunostaining analysis for the indicated proteins using mitochondria isolated from wild-type (WT) or Gal–ATM1 cells containing the *ATM1* gene under control of a galactose-inducible promoter. Cells were grown for 40 h in YP medium containing galactose (Gal) or lactate medium containing 0.1% glucose (Glu). Cyt c_1, cytochrome c_1; Rieske, Rieske Fe/S protein of respiratory complex III. (**B**) Assembly of the Fe/S cluster of Leu1p *in vivo* was estimated as in Figure 1B using Gal–ATM1 cells grown in medium A containing galactose or glucose. (**C**) The enzyme activities of Leu1p, glucose-6-phosphate dehydrogenase (G6P-DH) and alcohol dehydrogenase (ADH) were measured employing cell extracts of Gal–ATM1 cells after growth as in (A). (**D**) Mitochondria were isolated from cells grown as in (A) and the enzyme activities indicated were measured. DH, dehydrogenase.

of cytosolic Leu1p in cells lacking the mitochondrial ABC transporter Atm1p. This suggests a crucial function of Atm1p in the biosynthesis of cytosolic Fe/S cluster-containing proteins.

To exclude the possibility that the defective biosynthesis of the Fe/S cluster of Leu1p in the absence of functional Atm1p is a secondary consequence of the severe phenotype of Δatm1 cells, we constructed a mutant carrying the *ATM1* gene under the control of a galactose-inducible promoter (Gal–ATM1 cells). Atm1p was depleted below the detection limit of our immunostaining analysis after 40 h of growth of Gal–ATM1 cells on glucose-containing media (Figure 2A) without causing a detectable decrease in the growth rate (not shown). After longer periods of cultivation, growth of Gal–ATM1 cells was considerably retarded, but not as strongly as that of Δatm1 cells. No significant effects of Atm1p depletion (for 40 h) were observed on the amounts of other mitochondrial proteins including cytochromes (Figure 2A; data not shown) or of cytosolic Leu1p (Figure 2B, insert). Furthermore, mitochondria isolated from Gal–ATM1 cells did not contain increased levels of iron as had been observed for organelles prepared from Δatm1 cells (data not shown). Thus, Atm1p can be severely depleted in Gal–ATM1 cells without the strong phenotypical consequences arising from disruption of the gene.

The Gal–ATM1 cells were used to measure the *in vivo* assembly of the Fe/S cluster of cytosolic Leu1p. A 2-fold higher incorporation of ^{55}Fe into Leu1p compared with wild-type cells was observed after growth of Gal–ATM1 cells in the presence of galactose (Figure 2B). On the contrary, hardly any ^{55}Fe was precipitated by Leu1p-specific antibodies after depletion of Atm1p by growth of Gal–ATM1 cells in the absence of galactose. Comparable results were obtained for the enzyme activity of isopropyl malate isomerase (Leu1p) in extracts isolated from Atm1p-depleted Gal–ATM1 cells (Figure 2C). Other cytosolic enzyme activities such as glucose-6-phosphate dehydrogenase and alcohol dehydrogenase were not significantly influenced by the lack of Atm1p. When Leu1p was overexpressed in Gal–ATM1 cells, 4-fold higher Leu1p enzyme activities were measured in cells containing Atm1p, but no activity was detectable upon depletion of Atm1p (data not shown). We conclude from these results that the mitochondrial ABC transporter Atm1p is required for efficient formation of Fe/S proteins in the cytosol. The phenotypical differences noted for Δatm1 and Gal–ATM1 cells are likely to be due to leaky expression known to occur from the galactose-regulated promoter in the absence of this sugar. This may allow synthesis of small amounts of Atm1p, sustain some Atm1p-mediated transport and hence preserve residual function of extra-mitochondrial Fe/S proteins.

Is Atm1p also required for the biogenesis of mitochon-

drial Fe/S cluster-containing proteins? The enzyme activities of aconitase and of succinate dehydrogenase (which contains the Fe/S subunit Sdh2p; Lombardo *et al*., 1990) were measured using mitochondria isolated from wild-type or Gal–ATM1 cells grown in the presence of galactose or glucose. The deficiency in functional Atm1p hardly affected the activities (Figure 2D) and the amounts (Figure 2A) of these mitochondrial Fe/S proteins or, as a control, of citrate synthase and malate dehydrogenase. Similarly, no detectable influence of the presence or absence of Atm1p was noted on the level of the Rieske Fe/S protein of the mitochondrial respiratory complex III (Figure 2A). Taken together, Atm1p is not required for biosynthesis of mitochondrial Fe/S proteins, but rather appears to be specifically involved in the generation of cytosolic Fe/S proteins. This strongly suggests that the ABC transporter Atm1p functions in the export from mitochondria of components required for Fe/S cluster assembly in the cytosol. Our findings predict the function of other mitochondrial proteins in the synthesis of cytosolic Fe/S proteins.

Nfs1p is required for the biosynthesis of intra- and extra-mitochondrial Fe/S proteins

In bacteria, biosynthesis of Fe/S clusters is initiated by the protein NifS which functions as a cysteine desulfurase liberating elemental sulfur from cysteine (Zheng *et al*., 1993; Zheng and Dean, 1994). A highly homologous protein termed Nfs1 is present in eukaryotic cells (Land and Rouault, 1998; Nakai *et al*., 1998). In *S.cerevisiae*, the Nfs1p protein was found exclusively in isolated mitochondria, and was not detectable in post-mitochondrial supernatants (Nakai *et al*., 1998; data not shown). Fractionation of mitochondria isolated from wild-type cells revealed that Nfs1p was located in the matrix and behaved as a soluble, protease-sensitive protein (data not shown). Nfs1p was highly enriched in density gradient-purified mitochondria (Schlenstedt *et al*., 1995) excluding localization in other organelles such as the endoplasmic reticulum (data not shown).

Does mitochondrial Nfs1p perform a function as a cysteine desulfurase in the biogenesis of cellular Fe/S clusters? The essential nature of Nfs1p for cell viability made it necessary to construct a mutant in which the *NFS1* gene is under the control of a galactose-inducible promoter. The resulting yeast strain (Gal–NFS1) grew at wild-type rates in the presence of galactose and cells contained 2-fold higher amounts of mitochondrial Nfs1p compared with the wild-type strain (not shown). Upon cultivation of Gal–NFS1 cells in the absence of galactose, Nfs1p was rapidly depleted and was not detectable by immunostaining analysis after growth for 16 h (corresponding to approximately four cell doublings; Figure 3A). After six generations (40 h) Gal–NFS1 cells ceased to duplicate (not shown). Growth of Gal–NFS1 cells in the absence of galactose was restored upon transformation with a plasmid carrying the coding information for a fusion protein consisting of the presequence of subunit 9 of mitochondrial F_0-ATPase and *Escherichia coli* NifS (not shown). Without the presequence, no restoration of growth was noticed. These data show that bacterial NifS can functionally replace yeast Nfs1p, but only when it is imported into mitochondria. Upon depletion of Nfs1p we noted a strong accumulation of 'free' iron (i.e. iron which is not bound to heme or Fe/S clusters) in mitochondria (Figure 3B). A similar iron accumulation within mitochondria has been reported for cells lacking the mitochondrial

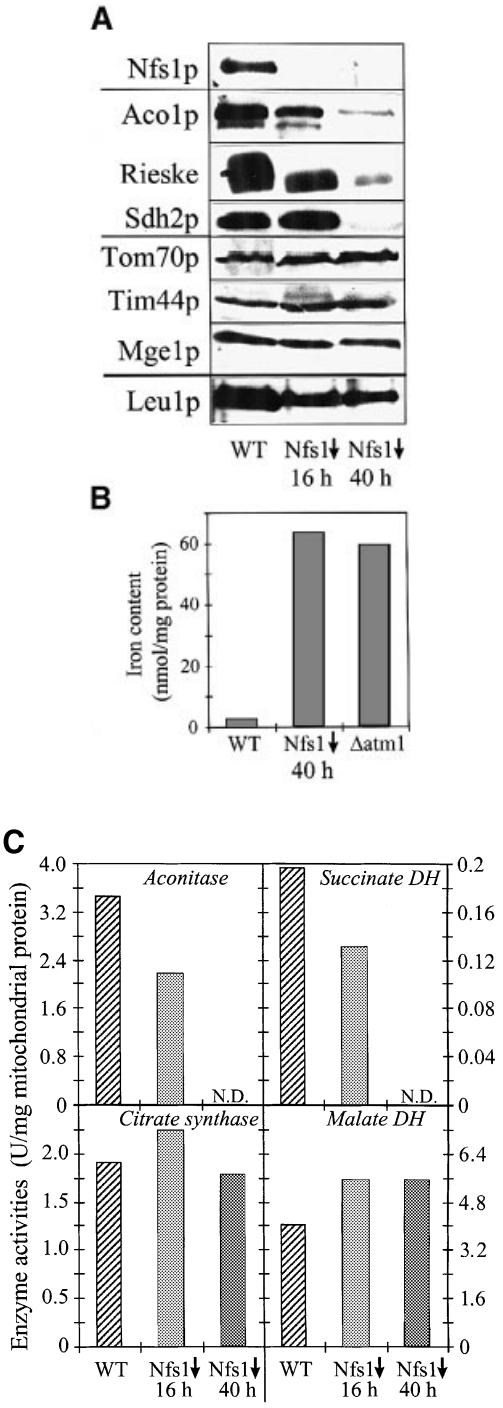

Fig. 3. Depletion of Nfs1p results in a defect of mitochondrial Fe/S proteins. (**A**) Immunostaining of the indicated proteins using mitochondria or cell extracts (for Leu1p) derived from wild-type (WT) or Gal–NFS1 cells grown in the presence of lactate medium for 16 or 40 h (corresponding to four and six cell doublings, respectively). (**B**) Non-heme non-Fe/S ('free') iron associated with isolated mitochondria was measured by the bathophenantroline method (Tangeras *et al*., 1980). (**C**) The enzyme activities indicated were measured using isolated mitochondria. DH, dehydrogenase. N.D., not detectable, i.e. <0.05 (aconitase) or 0.005 (succinate dehydrogenase) U/mg protein.

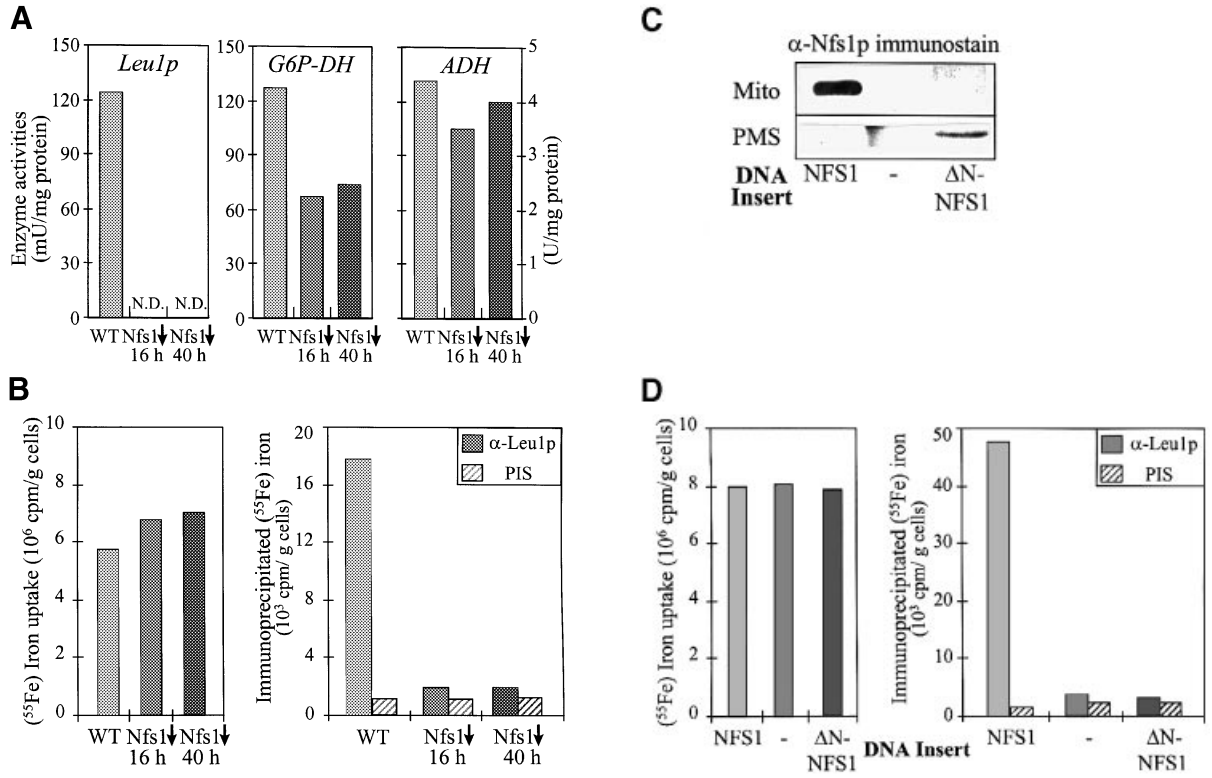

Fig. 4. Mitochondria-localized Nfs1p is required for the generation of the Fe/S cluster of cytosolic Leu1p. (**A**) The enzyme activities of Leu1p, glucose-6-phosphate dehydrogenase (G6P-DH) and alcohol dehydrogenase (ADH) were measured in post-mitochondrial supernatants of wild-type (WT) or Gal-NFS1 cells grown as in Figure 3A. N.D., not detectable. (**B**) The assembly of the Fe/S cluster of Leu1p *in vivo* was determined as in Figure 1B. Nfs1p was depleted by cultivation of Gal–NFS1 cells as described in Figure 2B. (**C**) Immunostaining of Nfs1p using isolated mitochondria (Mito) or post-mitochondrial supernatants (PMS) derived from Gal–NFS1 cells. The cells harbored plasmids with either no DNA insert (–), the full-length (NFS1) or the N-terminally truncated (ΔN-NFS1) *NFS1* gene. Growth was for 16 h in the presence of glucose. (**D**) The assembly of the Fe/S cluster of Leu1p in the cells described in (C) was estimated as in Figure 1B after growth in medium A.

proteins Atm1p (Δatm1 cells; Figure 3B), frataxin and Ssq1p (Babcock *et al*., 1997; Foury and Cazzalini, 1997; Kispal *et al*., 1997; Knight *et al*., 1998).

Using Gal–NFS1 cells we tested the influence of Nfs1p depletion on the function of mitochondrial Fe/S proteins. The activities of aconitase and succinate dehydrogenase were slightly decreased compared with wild-type samples after growth for 16 h in glucose-containing media, and not detectable after growth for 40 h (Figure 3C). In contrast, the function of other mitochondrial enzymes such as citrate synthase or malate dehydrogenase was virtually unchanged. The inactivation of aconitase and succinate dehydrogenase in Nfs1p-depleted cells was accompanied by a strong decrease in the amounts of these proteins (Figure 3A). A similar reduction was observed in the level of the Rieske Fe/S protein, while other mitochondrial proteins did not change significantly in their amounts (Figure 3A). The decrease of the mitochondrial Fe/S proteins was specific for cells depleted in Nfs1p, as no such effect was observed for yeast mutant cells lacking other mitochondrial proteins such as cytochrome c_1 heme lyase (not shown). These findings suggest that Nfs1p plays a crucial role in the biosynthesis and/or maintenance of mitochondrial Fe/S proteins. Presumably, the impaired formation and/or repair of the mitochondrial Fe/S clusters result in apoproteins that are susceptible to proteolytic degradation.

To investigate the potential function of Nfs1p in the *de novo* formation of cytosolic Fe/S cluster-containing proteins the enzyme activity of Leu1p was measured. Cell extracts isolated from Gal–NFS1 cells depleted in Nfs1p contained no detectable Leu1p enzyme activity, while the activities of two other cytosolic enzymes (glucose-6-phosphate dehydrogenase and alcohol dehydrogenase) were hardly affected (Figure 4A). The level of Leu1p was lowered 2- to 3-fold upon depletion of Nfs1p (Figure 3A). Most likely, this slight decrease results from proteolytic degradation of Leu1p in Gal–NFS1 cells (which have stopped duplicating under these conditions) rather than from the impaired synthesis of the Leu1p polypeptide. At any rate, decreased expression of Leu1p can only partially account for the defect in Leu1p enzyme activity. Taken together, these data suggest that Nfs1p is essential for generation of functional Leu1p in the cytosol.

To prove directly the function of Nfs1p in the assembly of the Fe/S cluster in cytosolic Leu1p, the incorporation of radiolabelled ^{55}Fe into this protein was followed *in vivo*. Wild-type cells or Gal–NFS1 cells depleted in Nfs1p by growth on glucose-containing media were labelled with radioactive ^{55}Fe and Leu1p was immunoprecipitated under native conditions. Using wild-type cells (Figure 4B) or Gal–NFS1 cells grown in the presence of galactose (not shown), ^{55}Fe could be efficiently incorporated into Leu1p. No specific labelling of Leu1p with radioactive ^{55}Fe was detectable after depletion of Nfs1p by growth for 16 or 40 h in glucose-containing media. The iron uptake by the yeast cells was not affected by the deficiency of Nfs1p (Figure 4B, left panel). These results indicate that Nfs1p

performs a crucial role in the synthesis of cytosolic Fe/S cluster-containing proteins. In contrast to Atm1p, this protein is also required for generation of mitochondrial Fe/S clusters.

Nfs1p must be localized within mitochondria to perform its essential function in cellular Fe/S cluster biogenesis

The requirement for Nfs1p function in the synthesis of cytosolic Fe/S proteins could be due to minute amounts of this protein present in the cytosol. These amounts may have escaped detection by immunostaining analysis. To examine whether Nfs1p performs its function in the formation of cytosolic Fe/S proteins within mitochondria or the cytosol, we expressed Nfs1p with and without its N-terminal mitochondrial targeting sequence in Gal–NFS1 cells. Plasmids carrying either no DNA insert, the entire *NFS1* gene or a truncated version of *NFS1* lacking the DNA segment encoding the N-terminus of Nfs1p including its presequence (ΔN-NFS1) were transformed into Gal–NFS1 cells. The plasmid containing the entire *NFS1* gene was able to retain viability of the Gal–NFS1 cells in the absence of galactose (not shown). Cells expressing the truncated version of Nfs1p (termed $Nfs1p^{cyt}$) did not grow under these conditions. Growth was restored to wild-type rates when the N-terminally truncated *NFS1* gene was fused to a DNA segment encoding the presequence of subunit 9 of F_o-ATPase mediating mitochondrial import of the fusion protein (not shown). The Nfs1p protein derived from the plasmid-encoded full-length *NFS1* gene was exclusively localized within mitochondria, while the N-terminally truncated $Nfs1p^{cyt}$ was detectable only in the post-mitochondrial supernatant (Figure 4C). This indicated that mitochondrial localization of Nfs1p is crucial for its essential function. These results are consistent with our findings of functional complementation by bacterial NifS (see above).

To examine whether the cytosolic form of Nfs1p can support the synthesis of Fe/S clusters in the cytosol, the incorporation of radioactive ^{55}Fe into Leu1p was measured employing Gal–NFS1 cells transformed with the three plasmids described above. Expression of the nucleus-encoded Nfs1p was inhibited by growth in the presence of glucose. Mitochondrial Nfs1p synthesized from the plasmid-encoded full-length *NFS1* gene allowed efficient synthesis of the Fe/S cluster of Leu1p as evident from the efficient immunoprecipitation of radioactive ^{55}Fe by Leu1p-specific antiserum (Figure 4D). Conversely, no significant formation of ^{55}Fe-labelled Leu1p was detectable upon expression of $Nfs1p^{cyt}$ from the ΔN-NFS DNA insert or without expression of any Nfs1p protein. Cell extracts prepared from the three strains contained similar amounts of total radioactive ^{55}Fe (Figure 4D, left panel), i.e. the cellular uptake of ^{55}Fe was not affected by the different plasmids. These results provide strong evidence that Nfs1p performs its essential function in the synthesis of cytosolic and mitochondrial Fe/S proteins only when it is localized within mitochondria.

A mitochondrial membrane potential is required for biosynthesis of cytosolic Fe/S proteins

The production of precursors of cytosolic Fe/S clusters within mitochondria necessitates the transport of substrates and products of synthesis across the mitochondrial inner membrane. We therefore tested whether the generation of cytosolic Fe/S proteins depends on energized mitochondria. Wild-type cells were treated with the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) during labelling with ^{55}Fe to deplete the mitochondrial membrane potential (Wienhues *et al.*, 1991). Hardly any radiolabelled ^{55}Fe above background could be immunoprecipitated with the Leu1p-specific antiserum using extracts from CCCP-treated cells (Figure 5). This was not due to impaired uptake of radioactive iron upon treatment with CCCP, as even slightly higher amounts of ^{55}Fe were present in the lysate derived from these cells compared with untreated cells (left panel). These findings indicated that energized mitochondria are crucial for incorporation of an Fe/S cluster into a cytosolic protein. This result provides a further criterion for the central role that mitochondria play in the biogenesis of cytosolic Fe/S proteins.

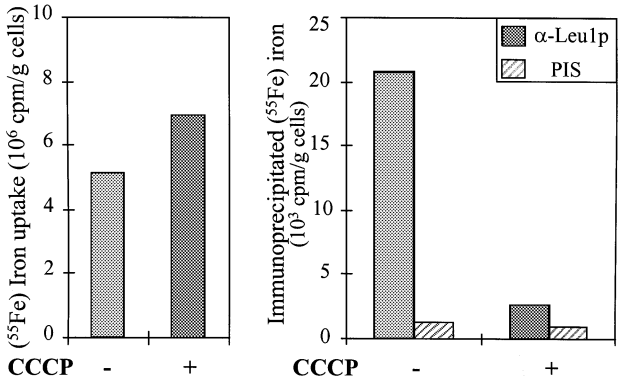

Fig. 5. Incorporation of an Fe/S cluster into a cytosolic protein requires a mitochondrial membrane potential. The assembly of the Fe/S cluster of Leu1p *in vivo* was measured as in Figure 1B using wild-type cells that were treated with 60 μM CCCP during radiolabelling with ^{55}Fe. Control cells (–) received CCCP after radiolabelling.

Discussion

The results presented here demonstrate a central role for mitochondria in the biosynthesis of both intra- and extra-mitochondrial Fe/S cluster-containing proteins. The unexpected requirement for mitochondria in the generation of cytosolic Fe/S proteins was seen either by measuring the enzyme activity of isopropyl malate isomerase (Leu1p) or by the novel approach of directly assessing the incorporation of the Fe/S cluster into Leu1p through co-immunoprecipitation of radioactive iron with Leu1p-specific antibodies. Our study represents the first investigation into the biogenesis of Fe/S proteins in the eukaryotic cytosol and establishes the basis for further molecular analyses of this fundamental biosynthetic process.

Two mitochondrial proteins perform a decisive role in cellular Fe/S cluster formation. The matrix protein Nfs1p, as the functional orthologue of bacterial NifS, initiates the process by liberating elemental sulfur from cysteine (Figure 6). Its activity is required for the synthesis of both mitochondrial and cytosolic Fe/S clusters. Nfs1p executes its crucial function in the assembly of cytosolic Fe/S clusters only when it is localized within mitochondria.

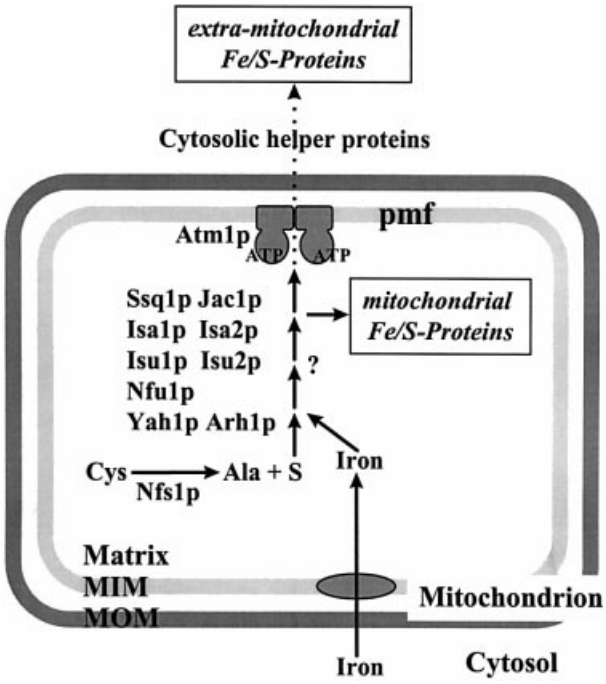

Fig. 6. Working model for the biogenesis of Fe/S proteins in eukaryotic cells. The central role of mitochondria in Fe/S cluster biosynthesis is evident from the involvement of the yeast proteins Nfs1p, Atm1p, Ssq1p and Jac1p in this process. The potential participation of the other components was inferred from their homology to bacterial proteins involved in Fe/S cluster biogenesis. As demonstrated in this report, generation of cytosolic Fe/S proteins requires Nfs1p, Atm1p and a proton-motive force (pmf). The assistance of further components of both mitochondria and the cytosol is likely. See Discussion for further explanations. MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane.

When Nfs1p or bacterial NifS were expressed in the cytosol, the Fe/S cluster did not become incorporated into Leu1p. These observations strongly suggest that the elemental sulfur needed for cytosolic Fe/S clusters is generated inside the organelles and must be exported.

The mitochondrial ABC transporter Atm1p appears to perform such an export function in the biogenesis of cytosolic Fe/S clusters (Figure 6). The protein is specifically involved in the formation of Fe/S clusters in cytosolic proteins. Neither deletion of the *ATM1* gene (Kispal *et al*., 1997) nor depletion of Atm1p in Gal–ATM1 cells (this study) had any significant effect on the function of Fe/S proteins inside the organelle. Thus, Atm1p mediates the export of components required for Fe/S cluster incorporation into extra-mitochondrial proteins. Microanalytical studies will now have to be employed to elucidate the chemical nature of the transported substrate.

In addition to the sulfur moiety, iron may also be exported from mitochondria into the cytosol. In part, this expectation is based on the strongly increased mitochondrial iron levels observed in *atm1* and *nfs1* yeast mutants. In the simplest view, iron might accumulate in the organelles as a result of its decreased consumption due to the impaired production and/or the export of Fe/S clusters or its precursors. Interestingly, export of iron from mitochondria has recently been proposed to occur (Askwith and Kaplan, 1998) and may require the function of the matrix protein frataxin (Radisky *et al*., 1999; see below).

Our findings suggest that mitochondria are the primary site of Fe/S cluster assembly in the eukaryotic cell. In addition to Nfs1p and Atm1p, other mitochondrial components may participate in this biosynthetic pathway (Figure 6). Two such proteins are the heat shock proteins Jac1p and Ssq1p, which have been shown to be required for efficient function of two intra-mitochondrial Fe/S proteins (Strain *et al*., 1998). It will be interesting to investigate whether these proteins also participate in the biogenesis of cytosolic Fe/S clusters. The function of a minimum of six additional proteins in Fe/S cluster biogenesis in the eukaryotic cell may be inferred from their sequence similarity to bacterial proteins encoded by the Nif/Isc operons (Figure 6). In *S.cerevisiae*, most if not all of these homologues are predicted to be localized in mitochondria based on their N-termini resembling mitochondrial targeting sequences. An interesting example is a ferredoxin homologue (Yah1p in Figure 6) which might co-operate with a mitochondria-localized ferredoxin reductase (Arh1p; Lacour *et al*., 1998) in the reduction of sulfur and/or iron during the assembly of Fe/S clusters.

Apparently, the biogenesis of cellular Fe/S proteins is one of the most central biosynthetic tasks of mitochondria. At least four mitochondrial proteins (potentially) involved in this process (Nfs1p, Jac1p, ferredoxin and its reductase; Lacour *et al*., 1998; Strain *et al*., 1998) are essential for viability of yeast cells. Almost all other essential mitochondrial proteins perform a role in the import, processing or folding of nucleus-encoded proteins (reviewed by Lill *et al*., 1996; Schatz and Dobberstein, 1996; Neupert, 1997; Pfanner *et al*., 1997). The frequency of essential components participating in Fe/S cluster biosynthesis emphasizes the importance of Fe/S proteins in the eukaryotic cell. It is therefore surprising that so little is known to date about the mode of production of these bio-organic assemblies that are localized in mitochondria, the cytosol and the nucleus.

The essential role of mitochondria in cytosolic Fe/S cluster assembly and/or repair may have profound implications on the mode of iron uptake into the cell. In higher eukaryotes, key components controlling cellular iron uptake and storage are the iron regulatory proteins 1 and 2 (IRP1 and IRP2; Hentze and Kuhn, 1996; Rouault and Klausner, 1996). IRP1 senses the cellular iron concentration through the dynamic addition and loss of an Fe/S cluster. The apoform of IRP1 binds to the mRNAs of transferrin receptor or of ferritin and thereby regulates their stability or translation, respectively. The predominant or exclusive localization of human and mouse Nfs1 in the mitochondrial matrix (Land and Rouault, 1998; Nakai *et al*., 1998) and the functional overlap of the human ABC transporter hABC7 with yeast Atm1p (Csere *et al*., 1998) suggests that in mammalian cells also, cytosolic Fe/S cluster formation is performed mainly by mitochondria. Thus, the mitochondrial iron concentration may directly affect the efficiency of the assembly and regeneration of the Fe/S cluster in IRP1 and hence play a decisive role in the control of cellular iron uptake. Such a potential cross-talk between mitochondria and the cytosol may serve to couple the mitochondrion's need for iron to the general control of iron uptake into the cell, and guarantee the proper cellular distribution of iron. For yeast cells, a function of Fe/S proteins in the control of cellular iron

uptake has not yet been reported (see Yamaguchi-Iwai *et al*., 1996).

Our investigation is relevant to various medical problems. The ABC transporter hABC7, the human orthologue of Atm1p (Csere *et al*., 1998), is implicated in X-linked sideroblastic anemia and ataxia (Allikmets *et al*., 1999), an iron-storage disease in which affected cells harbor iron-loaded mitochondria. The functional complementation of *ATM1*-deletion mutants (Δatm1 cells) by expression of hABC7 protein (Csere *et al*., 1998) suggests that hABC7 also participates in the biogenesis of cytosolic Fe/S clusters in human cells. It will be interesting to study the impact of mutations in hABC7 on the activity of cytosolic Fe/S proteins. Furthermore, our findings are important for the molecular understanding of the interaction of the intracellular bacterium *Rickettsia prowazekii* with its host cell. At present, this organism is the only known prokaryotic species that encodes a homologue of the ABC transporter Atm1p (Andersson *et al*., 1998). The *Rickettsia* Atm1p may be proposed to influence the assembly and/or repair of Fe/S proteins in the host cytoplasm and consequently may be meaningful for the communication between the pathogen and the eukaryotic cell via the activity of some of these proteins. Strikingly, all of the relevant Nif/Isc operon-encoded proteins (cf. Figure 6) are contained within the genome of this simple organism, indicating that the bacterial pathogen is capable of producing its own (and presumably foreign) Fe/S proteins. Finally, our study raises the interesting question of whether the mitochondrial matrix protein frataxin may also contribute to the biogenesis of cytosolic Fe/S cluster-containing proteins. Mutations in this protein cause the neurodegenerative disorder Friedreich's ataxia, and mitochondria lacking functional frataxin accumulate iron similarly to mutants in Atm1p/hABC7 and Nfs1p (Campuzano *et al*., 1996; Babcock *et al*., 1997; Foury and Cazzalini, 1997). A slight reduction in the activity of mitochondrial Fe/S proteins has been reported (Rotig *et al*., 1997). Notably, frataxin is not contained within the Nif/Isc operons and therefore a function in Fe/S cluster biogenesis was not predicted. Our investigation provides the foundation to address these challenging medical problems.

Materials and methods

Yeast strains and cell growth

The following strains of *S.cerevisiae* were used: W303 (*MATα, ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3 112*) served as wild-type; strains carrying deletions in the genes *ATM1* (Δatm1; Kispal *et al*., 1997), *CYT2* (Δcyt2; cytochrome c_1 heme lyase; Steiner *et al*., 1996) or mutations in *LEU1* (strain 4808–14A; *MAT*a and *gal1 trp5 met2 ade1 ade6 leu1 lys7 pet2858*). Exchange of the promoters of *NFS1* and *ATM1* genes for a galactose-inducible promoter (Gal–NFS1 and Gal–ATM1 strains) was performed as described previously (Sirrenberg *et al*., 1996). In brief, PCR fragments corresponding to the coding region (nucleotides –6 to 1620) and the 5′-upstream region (nucleotides –314 to –7) of *NFS1* were cloned into the *Bam*HI–*Hin*dIII and *Hin*dIII–*Hpa*I restriction sites, respectively, of the Yep51 vector carrying the *GAL10* promoter. The plasmid DNA was linearized by *Hin*dIII and used to transform W303 cells. Selection for integration of the DNA was for the *LEU2* auxotrophic marker present on this DNA fragment. For Gal–ATM1 cells, PCR fragments corresponding to nucleotides –5 to 1100 and –396 to –27, respectively, of *ATM1* were used. Cells were grown as detailed previously using rich (YP) or minimal (SD) media (Sherman, 1991; Kispal *et al*., 1996, 1997) and lactate media (Daum *et al*., 1982) containing the required carbon sources.

Measurement of ^{55}Fe incorporation into Leu1p

Cells were grown overnight in medium A (minimal SD medium lacking added iron chloride) in the presence of the desired carbon source at 30°C. At an optical density of 1–2 OD_{600} cells (0.5 g wet weight) were collected and resuspended in 10 ml medium A, and 0.1 mM ascorbate and 10 μCi ^{55}Fe (Amersham) were added. Radiolabelling was for 1 h at 30°C. After addition of 100 μM iron sulfate and further incubation for 5 min at 30°C, cells were collected and washed with 50 mM citrate pH 7.4, 1 mM EDTA. A cell lysate was prepared by breaking cells with glass beads (Woonter and Jaehning, 1990). Aliquots of the clarified lysate (10 min, 12 000 *g*) were used for determination of ^{55}Fe uptake into cells by liquid scintillation counting, for immunostaining and for immunoprecipitation employing antibodies raised against Leu1p or antibodies derived from preimmune serum. Radioactivity associated with immunoprecipitated material was quantitated by liquid scintillation counting. The standard error for detection of Leu1p-associated ^{55}Fe in a cell lysate was 15%; the cellular uptake of ^{55}Fe varied by 30%.

Miscellaneous methods

The following published methods were used: manipulation of DNA and PCR (Sambrook *et al*., 1989); transformation of yeast cells (Gietz *et al*., 1992); isolation of plasmids from yeast (Sambrook *et al*., 1989); isolation of yeast mitochondria (Daum *et al*., 1982); sub-fractionation of mitochondria (Glick, 1995); whole-cell lysates by breaking cells with glass beads (Woonter and Jaehning, 1990); enzyme activities of citrate synthase, malate dehydrogenase, aconitase (Kispal *et al*., 1997), succinate dehydrogenase (Robinson *et al*., 1991; Robinson and Lemire, 1995), α-isopropyl malate synthase (Kohlhaw, 1988a), isopropyl malate isomerase (Kohlhaw, 1988c) and β-isopropyl malate dehydrogenase (Kohlhaw, 1988b). For measurement of the latter enzyme, wild-type and Δatm1 cells were transformed with the multi-copy plasmid pRS426 carrying the *LEU2* gene. The standard error of the determination of enzyme activities was between 5 and 15%.

Acknowledgements

We thank M.Weidgans for expert technical assistance. We gratefully acknowledge the invaluable help of Professor G.Kohlhaw concerning the biogenesis of leucine. We thank Drs M.Brunner, B.Lemire and R.Stuart for antibodies. Our work was supported by grants of the Sonderforschungsbereich 286 of Deutsche Forschungsgemeinschaft, the Fonds der chemischen Industrie, the Volkswagen-Stiftung, the Kempkes-Stiftung, and the Hungarian Fund OKTA (T6378, T020079, T022581). G.K. acknowledges support by the Alexander-von-Humboldt-Foundation.

References

Allikmets,R., Raskind,W.H., Hutchinson,A., Schueck,N.D., Dean,M. and Koeller,D.M. (1999) Mutation of a putative mitochondrial iron transporter gene (ABC7) in X-linked sideroblastic anemia and ataxia (XLSA/A). *Hum. Mol. Gen*., **8**, 743–749.

Andersson,S.G. *et al.* (1998) The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature*, **396**, 133–140.

Askwith,C. and Kaplan,J. (1998) Iron and copper transport in yeast and its relevance to human disease. *Trends Biochem. Sci*., **23**, 135–138.

Augeri,L., Lee,Y.M., Barton,A.B. and Doetsch,P.W. (1997) Purification, characterization, gene cloning and expression of *Saccharomyces cerevisiae* redoxyendonuclease, a homolog of *Escherichia coli* endonuclease III. *Biochemistry*, **36**, 721–729.

Babcock,M., De Silva,D., Oaks,R., Davis-Kaplan,S., Jiralerspong,S., Montermini,L., Pandolfo,M. and Kaplan,J. (1997) Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. *Science*, **276**, 1709–1712.

Beinert,H., Holm,R.H. and Münck,E. (1997) Iron–sulfur clusters: Nature's modular, multipurpose structures. *Science*, **277**, 653–659.

Beltzer,J.P., Morris,S.R. and Kohlhaw,G.B. (1988) Yeast *LEU4* encodes mitochondrial and nonmitochondrial forms of α-isopropylmalate synthase. *J. Biol. Chem*., **263**, 368–374.

Bisbal,C., Martinand,C., Silhol,M., Lebleu,B. and Salehzada,T. (1995) Cloning and characterization of a RNase L inhibitor. A new component of the interferon-regulated 2–5A pathway. *J. Biol. Chem*., **270**, 13308–13317.

Cammack,R. (1992) Iron–sulfur clusters in enzymes: themes and variations. In Cammack,R. (ed.), *Iron–Sulfur Proteins*. Vol. 38. Academic Press, San Diego, CA, pp. 281–322.

Campuzano,V. *et al*. (1996) Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science*, **271**, 1423–1427.

Csere,P., Lill,R. and Kispal,G. (1998) Identification of a human mitochondrial ABC transporter, the functional orthologue of yeast Atm1p. *FEBS Lett.*, **441**, 266–270.

Daum,G., Böhni,P.C. and Schatz,G. (1982) Import of proteins into mitochondria: cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.*, **257**, 13028–13033.

Foury,F. and Cazzalini,O. (1997) Deletion of the yeast homologue of the human gene associated with Friedreich's ataxia elicits iron accumulation in mitochondria. *FEBS Lett.*, **411**, 373–377.

Gietz,D., St Jean,A., Woods,R.A. and Schiestl,R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, **20**, 1425.

Glick,B.S. (1995) Pathways and energetics of mitochondrial protein import in *Saccharomyces cerevisiae*. *Methods Enzymol.*, **260**, 224–231.

Hentze,M.W. and Kuhn,L.C. (1996) Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. *Proc. Natl Acad. Sci. USA*, **93**, 8175–8182.

Hinnebusch,A.G. (1992) General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*. In Jones,E.W., Pringle,J.R. and Broach,J.R. (eds) *The Molecular and Cellular Biology of the Yeast Saccharomyces. Vol. 2: Gene Expression.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 319–414.

Johnson,M.K. (1998) Iron–sulfur proteins: new roles for old clusters. *Curr. Opin. Chem. Biol.*, **2**, 173–181.

Jones,E.W. and Fink,G.R. (1982) Regulation of amino acid and nucleotide biosynthesis in yeast. In Strathern,J.N., Jones,E.W. and Broach,J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces. Metabolism and Gene Expression.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 181–299.

Kispal,G., Steiner,H., Court,D.A., Rolinski,B. and Lill,R. (1996) Mitochondrial and cytosolic branched-chain amino acid transaminases from yeast, homologs of the *myc* oncogene-regulated Eca39 protein. *J. Biol. Chem.*, **271**, 24458–24464.

Kispal,G., Csere,P., Guiard,B. and Lill,R. (1997) The ABC transporter Atm1p is required for mitochondrial iron homeostasis. *FEBS Lett.*, **418**, 346–350.

Knight,S.A., Sepuri,N.B., Pain,D. and Dancis,A. (1998) Mt-Hsp70 homolog, Ssc2p, required for maturation of yeast frataxin and mitochondrial iron homeostasis. *J. Biol. Chem.*, **273**, 18389–18393.

Kohlhaw,G.B. (1988a) α-isopropylmalate synthase from yeast. *Methods Enzymol.*, **166**, 414–423.

Kohlhaw,G.B. (1988b) β-isopropylmalate dehydrogenase from yeast. *Methods Enzymol.*, **166**, 429–435.

Kohlhaw,G.B. (1988c) Isopropylmalate dehydratase from yeast. *Methods Enzymol.*, **166**, 423–429.

Lacour,T., Achstetter,T. and Dumas,B. (1998) Characterization of recombinant adrenodoxin reductase homologue (Arh1p) from yeast. Implication in *in vitro* cytochrome p45011beta monooxygenase system. *J. Biol. Chem.*, **273**, 23984–23992.

Land,T. and Rouault,T.A. (1998) Targeting of a human iron–sulfur cluster assembly enzyme, nifs, to different subcellular compartments is regulated through alternative AUG utilization. *Mol. Cell*, **2**, 807–815.

Leighton,J. and Schatz,G. (1995) An ABC transporter in the mitochondrial inner membrane is required for normal growth of yeast. *EMBO J.*, **14**, 188–195.

Lill,R., Nargang,F.E. and Neupert,W. (1996) Biogenesis of mitochondrial proteins. *Curr. Opin. Cell Biol.*, **8**, 505–512.

Lombardo,A., Carine,K. and Scheffler,I.E. (1990) Cloning and characterization of the iron–sulfur subunit gene of succinate dehydrogenase from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **265**, 10419–10423.

Nakai,Y., Yoshihara,Y., Hayashi,H. and Kagamiyama,H. (1998) cDNA cloning and characterization of mouse nifS-like protein, m-Nfs1: mitochondrial localization of eukaryotic NifS-like proteins. *FEBS Lett.*, **433**, 143–148.

Neupert,W. (1997) Protein import into mitochondria. *Annu. Rev. Biochem.*, **66**, 861–915.

Peters,J.W., Fisher,K. and Dean,D.R. (1995) Nitrogenase structure and function: a biochemical–genetic perspective. *Annu. Rev. Microbiol.*, **49**, 335–366.

Pfanner,N., Craig,E.A. and Honlinger,A. (1997) Mitochondrial preprotein translocase. *Annu. Rev. Cell Dev. Biol.*, **13**, 25–51.

Radisky,D.C., Babcock,M.C. and Kaplan,J. (1999) The yeast frataxin homologue mediates mitochondrial iron efflux. Evidence for a mitochondrial iron cycle. *J. Biol. Chem.*, **274**, 4497–4499.

Robinson,K.M. and Lemire,B.D. (1995) Flavinylation of succinate: ubiquinone oxidoreductase from *Saccharomyces cerevisiae*. *Methods Enzymol.*, **260**, 34–51.

Robinson,K.M., von Kieckebusch-Guck,A. and Lemire,B.D. (1991) Isolation and characterization of a *Saccharomyces cerevisiae* mutant disrupted for the succinate dehydrogenase flavoprotein subunit. *J. Biol. Chem.*, **266**, 21347–21350.

Rotig,A., de Lonlay,P., Chretien,D., Foury,F., Koenig,M., Sidi,D., Munnich,A. and Rustin,P. (1997) Aconitase and mitochondrial iron–sulfur protein deficiency in Friedreich ataxia. *Nature Genet.*, **17**, 215–217.

Rouault,T.A. and Klausner,R.D. (1996) Iron–sulfur clusters as biosensors of oxidants and iron. *Trends Biochem. Sci.*, **21**, 174–177.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Press, Cold Spring Harbor, NY.

Schatz,G. and Dobberstein,B. (1996) Common principles of protein translocation across membranes. *Science*, **271**, 1519–1526.

Schlenstedt,G., Harris,S., Risse,B., Lill,R. and Silver,P.A. (1995) A yeast DnaJ homolog, Scj1p, can function in the endoplasmic reticulum with BiP/Kar2p via a conserved domain that specifies interactions with Hsp70s. *J. Cell Biol.*, **129**, 979–988.

Sherman,F. (1991) Getting started with yeast. *Methods Enzymol.*, **194**, 3–21.

Sirrenberg,C., Bauer,M.F., Guiard,B., Neupert,W. and Brunner,M. (1996) Import of carrier proteins into the mitochondrial inner membrane mediated by Tim22. *Nature*, **384**, 582–585.

Steiner,H., Kispal,G., Zollner,A., Haid,A., Neupert,W. and Lill,R. (1996) Heme binding to a conserved CysProVal motif is crucial for the catalytic function of mitochondrial heme lyases. *J. Biol. Chem.*, **271**, 32605–32611.

Strain,J., Lorenz,C.R., Bode,J., Garland,S., Smolen,G.A., Ta,D.T., Vickery,L.E. and Culotta,V.C. (1998) Suppressors of superoxide dismutase (SOD1) deficiency in *Saccharomyces cerevisiae*. Identification of proteins predicted to mediate iron–sulfur cluster assembly. *J. Biol. Chem.*, **273**, 31138–31144.

Tangeras,A., Flatmark,T., Bäckström,D. and Ehrenberg,A. (1980) Mitochondrial iron not bound in heme and iron–sulfur centers. Estimation, compartmentation and redox state. *Biochim. Biophys. Acta*, **589**, 162–175.

Wienhues,U., Becker,K., Schleyer,M., Guiard,B., Tropschug,M., Horwich,A.L., Pfanner,N. and Neupert,W. (1991) Protein folding causes an arrest of preprotein translocation in mitochondria *in vivo*. *J. Cell Biol.*, **115**, 1601–1609.

Woonter,M. and Jaehning,J.A. (1990) Accurate initiation by RNA polymerase II in whole cell extracts from *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **265**, 8979–8982.

Yamaguchi-Iwai,Y., Stearman,R., Dancis,A. and Klausner,R.D. (1996) Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. *EMBO J.*, **15**, 3377–3384.

Zheng,L. and Dean,D.R. (1994) Catalytic formation of a nitrogenase iron–sulfur cluster. *J. Biol. Chem.*, **269**, 18723–18726.

Zheng,L., White,R.H., Cash,V.L., Jack,R.F. and Dean,D.R. (1993) Cysteine desulfurase activity indicates a role for NifS in metallocluster biosynthesis. *Proc. Natl Acad. Sci. USA*, **90**, 2754–2758.

Zheng,L., Cash,V.L., Flint,D.H. and Dean,D.R. (1998) Assembly of iron–sulfur clusters. Identification of an *iscSUA–hscBA–fdx* gene cluster from *Azotobacter vinelandii*. *J. Biol. Chem.*, **273**, 13264–13272.

Zollner,A., Rödel,G. and Haid,A. (1992) Molecular cloning and characterization of the *Saccharomyces cerevisiae CYT2* gene encoding cytochrome c_1 heme lyase. *Eur. J. Biochem.*, **207**, 1093–1100.

Received April 21, 1999; revised and accepted May 20, 1999