GTP Is Required for Iron-Sulfur Cluster Biogenesis in Mitochondria*

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Boominathan Amutha †1, Donna M. Gordon †1,2, Yajuan Gu ‡, Elise R. Lyver §, Andrew Dancis ‡, and Debkumar Pain †3,4

From the †Department of Pharmacology and Physiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07101 and the ‡Department of Medicine, Division of Hematology-Oncology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Iron-sulfur (Fe-S) cluster biogenesis in mitochondria is an essential process and is conserved from yeast to humans. Several proteins with Fe-S cluster cofactors reside in mitochondria, including aconitase [4Fe-4S] and ferredoxin [2Fe-2S]. We found that mitochondria isolated from wild-type yeast contain a pool of apoconitase and machinery capable of forming new clusters and inserting them into this endogenous apoprotein pool. These observations allowed us to develop assays to assess the role of nucleotides (GTP and ATP) in cluster biogenesis in mitochondria. We show that Fe-S cluster biogenesis in isolated mitochondria is enhanced by the addition of GTP and ATP. Hydrolysis of both GTP and ATP is necessary, and the addition of ATP cannot circumvent processes that require GTP hydrolysis. Both in vivo and in vitro experiments suggest that GTP must enter into the matrix to exert its effects on cluster biogenesis. Upon import into isolated mitochondria, purified apoferrredoxin can also be used as a substrate by the Fe-S cluster machinery in a GTP-dependent manner. GTP is likely required for a common step involved in the cluster biogenesis of aconitase and ferredoxin. To our knowledge this is the first report demonstrating a role of GTP in mitochondrial Fe-S cluster biogenesis.

GTP is required for numerous cellular functions both inside and outside of mitochondria. Mitochondria have their own genome attached to the organellar inner membrane at the matrix side (1). Processes associated with mitochondrial DNA replication, repair, and transcription require GTP. Likewise, critical steps involved in mitochondrial protein synthesis (initiation, elongation, and termination) utilize GTP (2). Other GTP-requiring processes also likely exist in the mitochondrial matrix, as evidenced by the presence of several GTPases of unknown function in this compartment (3). GTPases outside the mitochondrial matrix and attached to the inner membrane or outer membrane function in fission and fusion of mitochondria, and these are required for morphologic changes of the organelle associated with the metabolic demand of the cell (4).

These GTP-requiring processes in mitochondria are apparently conserved from yeast to humans. However, the compartmentalization of GTP synthesis and transport are quite different between mammalian and yeast mitochondria. In mammalian mitochondria, GTP is synthesized in the matrix by two different enzymes, a GTP-specific isoform of succinyl-CoA ligase and a nucleoside diphosphate kinase. Succinyl-CoA ligase converts succinyl-CoA to succinate with the generation of GTP in the tricarboxylic acid cycle (5). Nucleoside diphosphate kinases catalyze the transfer of γ-phosphate from nucleoside triphosphates (NTPs) to nucleoside diphosphates (6). The high energy phosphate is usually supplied by ATP, and the enzyme regulates the crucial balance between ATP and GTP or other NTPs. The human Nm23/nucleoside diphosphate kinase family consists of eight related genes and widely expressed proteins termed Nm23-H1 to Nm23-H8. One of these isoforms, Nm23-H4, contains a mitochondrial targeting signal and is localized to the mitochondrial matrix (7, 8).

Unlike in the case of mammalian mitochondria, GTP is not made in the matrix of Saccharomyces cerevisiae mitochondria, and therefore, yeast mitochondria are dependent on cytosolic GTP supply (2). In yeast, succinyl-CoA ligase produces ATP instead of GTP (9). Furthermore, yeast nucleoside diphosphate kinase is encoded by a single nuclear gene YNK1 (10), and the corresponding protein (Ynk1p) and activity are present in the cytosol and mitochondrial intermembrane space but not in the matrix (11). How cytosolic GTP is transported across the inner membrane into the matrix of yeast mitochondria remained elusive until recently (see below). For decades, the mitochondrial inner membrane was considered to be impermeable to GTP, and this explains why the possible role of matrix GTP in various metabolic processes has not received much attention.

Mitochondrial inner membrane contains a family of carrier proteins that allow exchange of various substrates across this membrane (12). Palmieri and co-workers (2) identified one such protein in yeast mitochondria as the GTP/GDP carrier and named the protein Ggc1p. They demonstrated that Ggc1p allows exchange of cytosolic GTP for matrix GDP across the inner membrane. Ggc1p-deficient (Δggc1) mitochondria lack this exchange activity, and the matrix contains greatly reduced levels of GTP and increased levels of GDP. Surprisingly, in an

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† These authors contributed equally to this work.

‡ Present address: Dept. of Biological Sciences, Mississippi State University, MS 37962.

§ To whom correspondence should be addressed: Dept. of Pharmacology and Physiology, UMDNJ, NJ Medical School, 185 South Orange Ave., MSB I-669, Newark, NJ 07101-1709. Tel.: 973-972-3439; Fax: 973-972-7950; E-mail: paide@umdnj.edu.
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earlier study the same Δggc1 yeast mutant (previously called Δyhm1) was found to exhibit abnormal iron regulation such as increased surface ferric reductase and high-affinity ferrous transport activities and accumulation of excess iron (~30-fold) in mitochondria (13). How Ggc1p participates in mitochondrial iron homeostasis was not clear from these studies. We recently investigated whether the role of Ggc1p in mitochondrial iron metabolism is mediated by its effects on GTP/GDP levels in the mitochondrial matrix. We showed that in vivo targeting of the human nucleoside diphosphate kinase Nm23-H4 to the mitochondrial matrix of Δggc1 rescues high cellular iron uptake and mitochondrial iron accumulation. The Nm23-H4 enzyme exhibits activity capable of converting GDP to GTP using ATP as the phosphate donor in the mitochondrial matrix, and it is this activity that is responsible for rescuing the iron phenotypic defects of the mutant (8). These results suggest that GTP in the mitochondrial matrix plays an important role in organelar iron homeostasis.

In most eukaryotic cells iron (Fe) primarily exists in complex with porphyrin (heme) or with sulfur (Fe-S clusters). Iron-sulfur clusters are critical cofactors of proteins that participate in numerous important cellular processes. Cluster biogenesis occurs in the mitochondrial matrix; it is a multistep process and requires multiple components (for review, see Ref. 14). Various yeast mutants that lack components involved in Fe-S cluster biogenesis exhibit abnormal cellular iron uptake and mitochondrial iron accumulation. The Nm23-H4 enzyme reversibly catalyzes the conversion of citrate to isocitrate in the tricarboxylic acid cycle and requires a [4Fe-4S] cluster as a cofactor (22). Here we show a requirement for GTP in the mitochondrial matrix plays a role in Fe-S cluster biogenesis.

Several Fe-S cluster-containing proteins, such as aconitase [4Fe-4S] and ferredoxin [2Fe-2S], reside in mitochondria. Aconitase reversibly catalyzes the conversion of citrate to isocitrate in the tricarboxylic acid cycle and requires a [4Fe-4S] cluster for this activity. Ferredoxin itself is known to participate in Fe-S cluster biogenesis (22). Here we show a requirement for nucleotides, particularly GTP, in Fe-S cluster biogenesis of aconitase (Aco1p) and ferredoxin (Yah1p).

MATERIALS AND METHODS

Yeast Strains—No significant difference was noted in cluster biogenesis in mitochondria isolated from D273-10B (ATCC 24657) and BY4741 (Invitrogen) wild-type strains. D273-10B mitochondria were used for experiments in Table 1 and Figs. 2, 4, 5, and 7; these experiments involved the use of only wild-type mitochondria. BY4741 was used as the wild-type strain for experiments in Figs. 1, 3, and 6; these experiments were performed to compare cluster biogenesis in wild-type and various mutant mitochondria. The Δggc1 mutant strain in the BY4741 background was generated by plasmid shuffling. A similar strain was constructed carrying a high copy number plasmid pRS425 with a strong glyceraldehyde phosphate dehydrogenase promoter to express the human nucleoside diphosphate kinase Nm23-H4 targeted to the mitochondrial matrix (8). A congenic BY4741 haploid yeast strain that expresses Aco1p with a C-terminal TAP (tandem affinity purification) tag from the ACO1 promoter in the genome was purchased from Open Biosystems. An aco1 mutant strain, which expresses aconitase with a mutation in a cysteine residue involved in iron binding (C448S), was a generous gift of Dr. Ronald A. Butow (23). Expression of the cysteine desulfurase Nfs1p from the regulated galactose-inducible GAL1 promoter has been described elsewhere (15). For the experiment in Fig. 8, the scaffold protein Isu1p with a C-terminal His6 tag was constitutively overexpressed in a wild-type strain (BY4741) from the plasmid pRS426 with glyceraldehyde phosphate dehydrogenase promoter essentially as described (24).

Insertion of Radiolabeled Clusters Into Apoproteins—Mitochondria were isolated from various strains and purified on Percoll gradients as described (25). Experiments were performed with or without prior depletion of endogenous nucleotides in isolated mitochondria. To deplete endogenous nucleotides, mitochondria were preincubated at 25 °C for 5–10 min (26). Isolated mitochondria contain a stored pool of iron that can be used for the synthesis of Fe-S clusters. Unless otherwise indicated, experiments were performed with no added iron salt. The procedures for iron depletion and supplementation are described in Fig. 4 legend.

Insertion of newly formed and radiolabeled clusters into an endogenous apoprotein in isolated intact mitochondria was examined as follows. The assay mixture (50 μl) contained mitochondria (100–200 μg of proteins) in HSB buffer (20 mM Hepes/KOH, pH 7.5, 0.6 mM sorbitol, 0.1 mg/ml bovine serum albumin, 10 mM Mg(0Ac)2, and 40 mM KOAc) containing 5 mM NADH and 1 mM dithiothreitol. Nucleotides (GTP and/or ATP) and poorly hydrolyzable nucleotide analogs (GTPγS or AMP-PNP) were included at various concentrations as indicated in the legends for Figs. 5, 7, and 8. After the addition of [35S]cysteine (10 μCi, 1075 Ci/mmol), samples were incubated at 30 °C for 15–20 min. Reaction mixtures were diluted 20-fold with ice-cold HSB buffer, and mitochondria were resoitated by centrifugation at 15,000 × g for 5 min at 4 °C. The pellet was resuspended in 35 μl of 50 mM Tris/HCl, pH 8.0, containing 0.5 mM phenylmethylsulfonyl fluoride. Mitochondrial membranes were ruptured by freezing the samples at −80 °C followed by thawing and bath sonication for 30 s at 4 °C. This process was repeated three times. Samples were centrifuged at 15,000 × g for 15 min at 4 °C, and supernatant fractions containing soluble proteins were subjected to native PAGE. The gel was fixed with 20% methanol in 50 mM Tris/HCl, pH 8.0, for 1 h at 4 °C, dried, and exposed to film for autoradiography. Radiolabeled protein bands were quantitated using the software NIH Image.

Insertion of newly formed and radiolabeled clusters into imported apoferrredoxin was examined as follows. The precursor form of ferredoxin with a C-terminal His6 tag was expressed in bacteria and found to be sequestered in inclusion bodies. The protein was solubilized with 8 M urea in 50 mM Tris/HCl, pH 8.0, and centrifuged at 250,000 × g for 20 min at 20 °C to remove insoluble material. The supernatant fraction analyzed by SDS-PAGE followed by Coomassie Blue staining showed a single major protein band and was ~95% pure (27). Import assay was performed in HSB buffer containing 5 mM NADH, 1

4 The abbreviations used are: GTPγS, guanosine 5’-3-O-(thio)triphosphate; AMP-PNP, 5’-adenyl imidodiphosphate; Ni-NTA, nickel-nitrilotriacetic acid.
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mm dithiothreitol, and 1 mM ATP (27–29). Import was initiated by adding urea-denatured ferredoxin precursor (400 ng) to mitochondria (200 μg of proteins) in the assay buffer (total volume 50 μl). The final urea concentration was 0.16 M. After incubation at 30 °C for 10 min, samples were diluted 20-fold with HSB buffer, and mitochondria were reisolated by centrifugation at 15,000 × g for 2 min at 4 °C. Mitochondrial pellet with ferredoxin imported into the matrix was resuspended in HSB buffer (50 μl) containing 5 mM NADH, 1 mM dithiothreitol, 10 μCi of [35S]cysteine (1075 Ci/mmol), and 1 mM ATP with or without 1 mM GTP. Reaction mixtures were incubated at 30 °C for 15 min and diluted with HSB buffer, mitochondria were reisolated, membranes were ruptured, and soluble proteins were analyzed by native PAGE followed by autoradiography as described above.

**Assembly of Cluster Intermediates on the Scaffold Protein Isu1p**—Mitochondria were preincubated at 25 °C for 15 min to promote depletion of endogenous nucleotides just before use. The reaction mixture (100 μl) contained mitochondria (500 μg of proteins with or without overexpressed Isu1p-His6) in HSB buffer supplemented with [35S]cysteine (20 μCi, 1075 Ci/mmol) and ferrous ascorbate (10 μM). Reaction mixtures were incubated at 30 °C for 15 min in the absence or presence of GTP or GTPγS (2 mM). Samples were diluted 12-fold with HSB buffer, and mitochondria were reisolated by centrifugation at 15,000 × g for 5 min at 4 °C. Mitochondrial pellets were resolubilized with 200 μl of buffer A (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) containing 10 mM imidazole and centrifuged at 15,000 × g for 15 min at 4 °C. Supernatant fractions were incubated with Ni-NTA-agarose (30 μl of settled beads) for 1 h at 4 °C. After washing of the agarose beads with buffer A containing 10 mM imidazole, bound proteins were eluted with 100 μl of 0.4 M imidazole in buffer A. Aliquots of eluted fractions (50 μl) were analyzed by scintillation counting of radioactivity and also by SDS-PAGE followed by immunoblotting using anti-Isu1p antibodies.

**Aconitase Activity Measurement**—Aconitase activity was determined using the whole cell extract or isolated mitochondria. For the whole cell extract, cells were treated with zymolase to generate spheroplasts as described in the protocol for the isolation of mitochondria (25). Spheroplasts or isolated mitochondria were incubated with 50 mM Tris/HCl, pH 8.0, containing 50 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM sodium citrate, and 200 units/ml catalase in a total volume of 50 μl for 30 min on ice. Samples were centrifuged at 15,000 × g for 10 min at 4 °C. Supernatant fractions were subjected to native PAGE, and aconitase activity was measured by an in-gel assay as described (30). Bands displaying aconitase activity appeared blue and were quantitated using the software NIH Image. Data from the scanned gels are presented in black and white (Figs. 1B, 3B, and 6A).

The nucleotide-dependent insertion of Fe-S clusters into endogenous apoAco1p was correlated with the activity of holo-Aco1p thus formed as follows. Briefly, mitochondria (500 μg of proteins) in HSB buffer containing 20 μM unlabeled cysteine, 10 μM ferrous ascorbate, 5 mM NADH, and 1 mM dithiothreitol were incubated at 30 °C for 15 min in the absence or presence of added nucleotides (1 mM GTP and 4 mM ATP) in a total volume of 250 μl. Reaction mixtures were diluted 5-fold with ice-cold 50 mM Tris/HCl, pH 7.5, containing 0.6 M sorbitol, and mitochondria were reisolated by centrifugation at 15,000 × g for 5 min at 4 °C. Mitochondrial pellets were treated with 125 μl of solubilization buffer (50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 10% glycerol, 1 mM sodium citrate, 0.5% glucose, 1.5% n-octyl-β-D-glucopyranoside, and 1 mM phenylmethylsulfonyl fluoride) and kept on ice. Aliquots (10–20 μl) corresponding to 40–80 μg of proteins were assayed in triplicate for aconitase activity. The assay follows conversion of isocitrate to cis-aconitate over time measured by absorbance change at 240 nm (27, 31).

**RESULTS**

**Insertion of Newly Formed Fe-35S Clusters into Endogenous Apoconitase in Isolated Intact Mitochondria**—We investigated Fe-S cluster biogenesis in isolated mitochondria using assays that we developed for this purpose (27). In these assays radioactive sulfur was generated from [35S]cysteine and utilized for iron-sulfur cluster synthesis. Newly formed Fe-35S clusters were subsequently incorporated into apoproteins. Samples were analyzed by native PAGE, thereby allowing direct visualization of labeled proteins. The polypeptide backbone of the protein was unlabeled. The clusters were destroyed by denaturants such as SDS, and thus, labeled proteins could only be analyzed by native gels.

Isolated intact mitochondria were incubated with [35S]cysteine in the presence of added nucleotides (ATP and GTP). Mitochondrial membranes were ruptured, and soluble proteins in duplicate were analyzed by a native gel. After electrophoresis, the gel was divided into two parts; one part was processed for autoradiography to detect radiolabeled proteins (Fig. 1A), whereas the other part was processed for an in-gel enzymatic
assay to detect active holo-aconitase (Fig. 1B). In a separate experiment, mitochondrial proteins were analyzed by SDS-PAGE followed by immunoblotting using anti-Aco1p antibodies (Fig. 1C).

In wild-type mitochondria, an endogenous apoprotein was radiolabeled (Fig. 1A, lane 1). This protein was identified as aconitase by three independent findings. 1) The migration pattern of the radiolabeled protein (Fig. 1A, lane 1) was identical to that of the band exhibiting aconitase activity in wild-type mitochondria (Fig. 1B, lane 1). 2) As control, we used mitochondria isolated from an aco1 mutant strain, which expresses aconitase with a mutation in a cysteine residue (C448S) involved in iron binding (23). The mutant protein is unable to coordinate an Fe-S cluster, and in this case, no radiolabeled protein was detected (Fig. 1A, lane 2) even though the mutant protein was present at wild-type levels (Fig. 1C, compare lanes 1 and 2). As expected, the mutant protein did not exhibit aconitase activity (Fig. 1B, lane 2). 3) We also used mitochondria isolated from a strain in which ACO1 was replaced with a tagged version of the gene that encodes Aco1p with a C-terminal TAP tag. The TAP tag adds ~20 kDa to the molecular mass of the wild-type protein, and the difference in migration on gels was clearly detected not only by SDS gels followed by immunoblotting (Fig. 1C) but also by native in-gel activity assay (Fig. 1B).

Nucleotide-dependent Fe-35S Labeling of Endogenous Aco1p—To investigate the requirement of nucleotides for cluster biogenesis, wild-type mitochondria were incubated with [35S]cysteine in the absence or presence of added ATP and/or GTP, and samples were analyzed by native PAGE followed by autoradiography. Endogenous aconitase was found to be radiolabeled in a nucleotide-dependent manner (Fig. 2A). The radiolabeling was observed in the presence of added ATP or GTP, and the process was most effective in the presence of both ATP and GTP, suggesting that ATP and GTP together are required for efficient cluster biogenesis.

Radiolabeling of the endogenous aconitase was not altered in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis (Fig. 2B). These results show that protein synthesis on mitochondrial ribosomes did not contribute to aconitase labeling. Incubation of mitochondria with [35S]cysteine followed by chase with a large excess of unlabeled cysteine did not diminish the intensity of radiolabeled aconitase (Fig. 2C).

Cluster Biogenesis of Endogenous Aco1p Is Dependent on the Cysteine Desulfurase Activity—In yeast cells, the cysteine desulfurase Nfs1p supplies sulfur from cysteine for Fe-S cluster biosynthesis (15, 16). We, therefore, investigated whether Nfs1p is required for nucleotide-dependent cluster biogenesis of endogenous apoAco1p in isolated mitochondria. Because Nfs1p is essential for cell viability, we used a strain in which Nfs1p expression is under control of the GAL1 promoter (15). When switched from raffinose-galactose medium (inducing conditions) to raffinose medium (non-inducing conditions), Nfs1p was no longer synthesized (Fig. 3A). These Nfs1p-deleted cells (nfs1Δ) were characterized by high cellular iron uptake and iron accumulation in mitochondria (15). Aconitase activity in these mutant cells was virtually undetectable (Fig. 3B) even though the Aco1p protein level was only modestly reduced to roughly 50% that of the level in wild-type cells (Fig. 3C). Tom40p is the major component of the protein translocase of the mitochondrial outer membrane (32) and served as the internal loading control (Fig. 3D). These results suggest that in the absence of sufficient Nfs1p, cluster biogenesis of aconitase in vivo is impaired. Such a notion is further supported by our in vitro experiments as follows.

Isolated mitochondria (wild-type or nfs1Δ) were incubated with [35S]cysteine, ATP, and GTP, looking for insertion of newly formed Fe-35S clusters into endogenous apoAco1p. Unlike in the case of wild-type mitochondria, Aco1p labelin...
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A. Nfs1p protein

WT

nfs1

Immunoblot

SDS-PAGE

B. Aco1p activity

Native PAGE

C. Aco1p protein

Immunoblot

SDS-PAGE

D. Tom40p protein

Immunoblot

SDS-PAGE

E. Aco1p (Fe-35S)

Native PAGE

FIGURE 3. Cluster biogenesis of aconitase both in vivo and in isolated mitochondria requires cysteine desulfurase (Nfs1p) activity. The Nfs1p open reading frame was placed under the control of GAL1 promoter (15). The resulting strain was grown to mid-logarithmic phase under inducing conditions in YPR galactose (yeast extract (1%), peptone (2%), raffinose (2%), and galactose (0.2%)). Cells were harvested, resuspended in YPR containing no galactose, and allowed to grow for 22 h. Under these non-inducing conditions, Nfs1p was no longer synthesized from the GAL1 promoter. Mitochondria were isolated from these Nfs1p-depleted (nfs1) cells. Mitochondrial proteins (100 μg) were analyzed by SDS-PAGE followed by immunoblotting using anti-Nfs1p (A), anti-Aco1p (C), and anti-Tom40p (D) antibodies. B, mitochondrial proteins (100 μg) were separated by native PAGE, and aconitase activity was measured by an in-gel assay. E, mitochondria (100 μg of proteins) were incubated at 30 °C for 15 min with [35S]cysteine (10 μCi; 0.19 μM), ATP (4 mM), and GTP (1 mM), and insertion of Fe-35S clusters into endogenous apoAco1p was examined by native PAGE followed by autoradiography. Wild-type (WT) mitochondria served as control.

was undetectable in the Nfs1p-depleted (nfs1) mitochondria (Fig. 3E). These results suggest that as in intact cells, Nfs1p is also required for Fe-S cluster biogenesis of endogenous aconitase in isolated mitochondria.

It is important to note that these assays for Fe-S cluster biogenesis in isolated intact mitochondria reflect biochemical activities that differ from chemical Fe-S cluster reconstitution. Iron-sulfur clusters can be chemically assembled on several purified apoproteins including aconitase in vitro using ferrous salt and sulfide under anaerobic conditions. Under such conditions, [35S]sulfide, generated from Na2S, can also be exchanged with the existing unlabeled sulfides of active [4Fe-4S] or inactive [3Fe-4S] forms of aconitase clusters (33). By contrast, the Fe-S cluster-forming activities in isolated mitochondria are dependent on Nfs1p. Nucleotides (GTP and ATP) are required for cluster biogenesis in mitochondria, and anaerobic conditions are not needed, perhaps because isolated intact mitochondria deploy mechanisms to protect newly formed Fe-S clusters from oxidative damage. Iron-dependent Cluster Labeling of Endogenous Aconitase—Iron content of mitochondria purified from the D273-10B strain grown in rich media was found to be 3 nmol/mg of proteins as measured by a chemical assay (15). The cluster insertion experiments described above were performed in the absence of added iron. These assays most likely utilized a pool of stored iron in mitochondria for the formation of new Fe-35S clusters. However, the addition of ferrous salt further stimulated formation of Fe-35S clusters as judged by increased radiolabeling of endogenous apoAco1p (Fig. 4, compare lanes 1 and 4). These results suggest that the endogenous iron pool in isolated mitochondria may not be present at optimal levels. The nature and location of iron used for Fe-S cluster formation was further evaluated as follows. Isolated intact mitochondria were treated with metal chelators such as o-phenanthroline (membrane-permeable) or EDTA (membrane-impermeable), washed, and then incubated with [35S]cysteine and nucleotides (ATP and GTP) with or without added ferrous ascorbate. Radiolabeling of Aco1p was strongly inhibited by o-phenanthroline (compare lanes 1 and 2) and was recovered by added ferrous salt (compare lanes 2 and 5). These results suggest that o-phenanthroline, by virtue of its ability to cross the inner membrane, bound and immobilized iron from an endogenous pool, making iron unavailable for Fe-S cluster biosynthesis. The inhibitory effect was reversed by iron addition, allowing new Fe-35S clusters to be made and Aco1p to become radiolabeled. By contrast, EDTA pretreatment had no effect on cluster biogenesis because it cannot efficiently enter into the matrix to chelate the endogenous pool of iron in this compartment (compare lanes 1 and 3). Together, these results suggest that isolated mitochondria can utilize endogenous or imported iron for cluster biogenesis.

Insertion of Fe-S Clusters into Endogenous Aconitase and Activity of Halo-aconitase Thus Formed—We tested if the nucleotide-dependent insertion of Fe-S clusters into endogenous apoAco1p leads to formation of active holo-Aco1p, thereby exhibiting an increase in total aconitase activity. For this purpose, isolated wild-type mitochondria were incubated with unlabeled cysteine and ferrous salt in the absence or presence of added nucleotides (ATP and GTP), just as was done for the radiolabeling experiments. Mitochondria were then washed and solubilized with a non-ionic detergent, and total aconitase activity was measured using a spectrophotometric
assay. Fe-S clusters were inserted into endogenous apoAco1p only in the presence of added nucleotides (Fig. 2A), with a concomitant ~9% increase in total aconitase activity (Table 1). These results suggest that under our experimental conditions, isolated wild-type mitochondria contain at least ~9% of total aconitase protein in apo form that can be converted to active holo form.

**NTP Hydrolysis and Insertion of Clusters into Endogenous ApoAconitase**—Insertion of newly formed clusters into endogenous apoAco1p was observed in the presence of added ATP or GTP (Fig. 2A). These experiments, however, did not delineate the individual roles of ATP and GTP in cluster biogenesis. For example, a portion of added ATP can directly enter into the matrix via ADP/ATP carriers in the inner membrane. Another portion can be converted to GTP by the nucleoside diphosphate kinase activity in the soluble intermembrane space (11, 28). GTP thus generated may enter into the matrix via ADP/ATP carriers in the inner membrane. Another portion can be converted to GTP by the nucleoside diphosphate kinase activity in the soluble intermembrane space (11, 28). GTP thus generated may enter into the matrix via the GTP/GDP carrier (Ggc1p) in the inner membrane, thereby stimulating cluster biogenesis (see Fig. 6D, left panel).

To delineate the individual roles of NTPs (ATP and GTP) and to determine whether hydrolysis of NTPs is required for cluster biogenesis, assays were performed in the presence of poorly hydrolyzable NTP analogs. The radiolabeling of endogenous apoAco1p was observed in the presence of added ATP or GTP (Fig. 2A), with a concomitant ~9% increase in total aconitase activity (Table 1). These results suggest that under our experimental conditions, isolated wild-type mitochondria contain at least ~9% of total aconitase protein in apo form that can be converted to active holo form.

**GTP Acts in the Matrix to Facilitate Cluster Biogenesis**—Ggc1p is a GTP/GDP carrier in the mitochondrial inner membrane and allows exchange of cytosolic GTP for matrix GDP across this membrane. The Δaggc1 mutant exhibits decreased levels of matrix GTP and increased levels of matrix GDP (2). To investigate the effects of low matrix GTP on Fe-S cluster biogenesis in vivo, we measured aconitase activity in the Δaggc1 mutant. A defect in cluster biogenesis is expected to cause a defect in the insertion of clusters into aconitase, thereby leading to impaired activity. Aconitase activity was found to be greatly decreased in Δaggc1 (Fig. 6A, top panel) even though the mutant contained Aco1p protein at wild-type levels (bottom panel). Thus, the decreased aconitase activity in Δaggc1 was not due to impaired Aco1p protein synthesis, reduced import into mitochondria, or enhanced degradation of the protein in vivo. Alternatively, a critical step in mitochondrial Fe-S cluster biogenesis might require GTP and, thus, might be impaired in Δaggc1 mitochondria.

To further test this hypothesis, we investigated whether restoration of matrix GTP in Δaggc1 mitochondria can rescue defects in cluster biogenesis of aconitase. The enzyme Nm23-H4 is a nucleoside diphosphate kinase, which can convert ATP (and GDP) to GTP. The human Nm23-H4 was able to rescue iron regulatory phenotypes when targeted to the mitochondrial matrix of the Δaggc1 mutant (8). The greatly reduced aconitase activity in Δaggc1 was restored to a significant extent in Δaggc1 + Nm23-H4 cells (Fig. 6A, top panel). These results suggest that an adequate level of GTP in the mitochondrial matrix is required for efficient Fe-S cluster biogenesis of Aco1p in vivo. This notion was further substantiated by experiments with isolated mitochondria as follows.

Unlike in the case of wild-type mitochondria (Fig. 6B, lane 2), Fe-35S clusters were not inserted into endogenous apoAco1p in Δaggc1 mitochondria even in the presence of added ATP and GTP (Fig. 6B, lane 3). The greatly reduced level of matrix GTP in Δaggc1 mitochondria is insufficient for the process, and this defect cannot be rescued by the addition of GTP because these mutant mitochondria cannot transport GTP into the matrix. In contrast to Δaggc1 mitochondria, isolated Δaggc1 + Nm23-H4 mitochondria were able to efficiently insert newly formed Fe-35S clusters into endogenous apoAco1p in the presence of added ATP plus GTP (Fig. 6B, lane 4) or ATP alone (Fig. 6C, lane 3). As in the case of Δaggc1 mitochondria, GTP cannot enter into the matrix of Δaggc1 + Nm23-H4 mitochondria. However, unlike Δaggc1 mitochondria, ATP that enters into the matrix via ADP/ATP carriers (AAC) can be converted to GTP by matrix-localized Nm23-H4 in Δaggc1 + Nm23-H4 mitochondria (Fig. 6D). We conclude that GTP in the mitochondrial matrix plays a critical role in Fe-S cluster biogenesis of Aco1p in vivo.

### Table 1

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>Nucleotides</th>
<th>Total aconitase activity</th>
<th>µmol cis-aconitate formed/mg protein/min</th>
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<tr>
<td>Wild type</td>
<td>–</td>
<td>1.21 ± 0.01</td>
<td></td>
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<tr>
<td>Wild type</td>
<td>+</td>
<td>1.32 ± 0.02</td>
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<tr>
<td>aco1 (C4485)</td>
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<td>0</td>
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*Table 1: Nucleotide-dependent reconstitution of aconitase activity. Wild-type and aco1 (C4485) mutant mitochondria were incubated with unlabeled cysteine and reduced iron in the absence or presence of nucleotides (ATP and GTP), and total aconitase activity was measured.*
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FIGURE 6. In vivo targeting of Nm23-H4 to the matrix of Δggc1 mitochondria restores cluster biogenesis of aconitase. A, cells were treated with Zymolyase to generate spheroplasts. After lysis of spheroplasts with Triton X-100, proteins were separated by native PAGE, and aconitase activity was evaluated by an in-gel assay (top panel). Each lane corresponds to equal number of starting cells; volume (ml) of cell suspension × A600 was kept constant at 0.25 for each lane. Identical samples were also analyzed by SDS-PAGE followed by immunoblotting using anti-Aco1p antibodies (bottom panel).WT, wild type; Δg, Δggc1; Δg + Nm, Δggc1 mutant with the human Nm23-H4 targeted in vivo to the matrix. B and C, as indicated, mitochondria (200 μg of proteins) were supplemented with ATP (4 mM) plus GTP (1 mM) (panel B) or ATP (4 mM) alone (panel C). After the addition of [35S]cysteine (10 μCi; 0.19 μM), samples were incubated at 30 °C for 15 min, and radiolabeling of endogenous aconitase was analyzed by native PAGE followed by autoradiography. D, a schematic of matrix GTP-dependent cluster biogenesis in mitochondria. IMS, intermembrane space; IM, inner membrane; AAC, ADP/ATP carriers.

Aco1p activity was analyzed by native PAGE followed by autoradiography. The radioactive Fe-35S clusters were found to be efficiently inserted into imported Yah1p in the absence of GTPγS (lane 2) or presence of GTPγS (lane 3) and analyzed by native PAGE followed by autoradiography. The radioactive label with or without GTPγS was added after precursor import and resolation of mitochondria, and thus, the observed effects on cluster labeling should be independent of import efficiency. Newly formed Fe-35S clusters were found to be efficiently inserted into imported apoYah1p in the absence of GTPγS (lane 2), and the process was greatly inhibited in the presence of GTPγS (lane 3). Radiolabeling of the endogenous Yah1p in mitochondria was not visible (lane 1). Endogenous aconitase served as an internal control. These results suggest that as in the case of endogenous apoAco1p, efficient cluster biogenesis of imported apoYah1p also requires GTP hydrolysis.

Efficient Formation of Cluster Intermediates Requires GTP Hydrolysis—Fe-S clusters are usually assembled on scaffold proteins Isu1p/Isu2p and subsequently transferred to apoproteins. Lill and co-workers (24) have shown that overexpressed Isu1p can be isolated with an intact cluster labeled with 55Fe in vivo and that this cluster is stable to air. To test cluster assembly in isolated mitochondria, we generated a wild-type strain carrying a plasmid for constitutive overexpression of Isu1p with a C-terminal His tag. Isu1p-His6 was overexpressed by ∼12-fold compared with the wild-type level of Isu1p (data not shown). Nucleotide-depleted mitochondria with overexpressed Isu1p-His6 were incubated with [35S]cysteine and ferrous salt. Nucleotides were not added to prevent transfer of clusters from Isu1p to endogenous apoAco1p, thereby trapping cluster intermediates on Isu1p. Mitochondria were reisolated, solubilized with Triton X-100, and subjected to Ni-NTA agarose chromatography. Upon elution with imidazole, large amounts of radioactivity were found to be associated with purified Isu1p-His6, whereas for wild-type mitochondria containing Isu1p without a His tag, the form is then a substrate for addition of Fe-S clusters within mitochondria (27, 34).

To determine the GTP requirement for the cluster biogenesis of imported ferredoxin, experiments were performed in two steps because protein import into the matrix also requires nucleotides (28). In the first step bacterially expressed and purified apoYah1p precursor was imported into mitochondria (Fig. 7, lanes 2 and 3). In the second step, after washing to remove unimported Yah1p precursor molecules, mitochondria were incubated with [35S]cysteine and ATP in the absence (lane 2) or presence of GTPγS (lane 3) and analyzed by native PAGE followed by autoradiography. The radioactive label with or without GTPγS was added after precursor import and resolation of mitochondria, and thus, the observed effects on cluster labeling should be independent of import efficiency. Newly formed Fe-35S clusters were found to be efficiently inserted into imported Yah1p in the absence of GTPγS, and the process was greatly inhibited in the presence of GTPγS (lane 3). Radiolabeling of the endogenous Yah1p of mitochondria was not visible (lane 1). Endogenous aconitase served as an internal control. These results suggest that as in

Fe-S cluster biogenesis of Aco1p both in vivo and in vitro. It is important to note that a complete block in mitochondrial cluster biogenesis in vivo is expected to be lethal because several proteins require Fe-S clusters for their functions. GGC1 is not essential for cell viability. Although greatly reduced, Δggc1 mitochondria contain some GTP in the matrix (2), which may be sufficient for basal levels of cluster biogenesis in vivo for cell survival. Such a notion is consistent with greatly reduced but detectable levels of aconitase activity in Δggc1 cells (Fig. 6A) even though insertion of Fe-S clusters into endogenous apoAco1p in isolated Δggc1 mitochondria cannot be detected (Figs. 6, B and C).

GTP Hydrolysis Is Also Required for Efficient Cluster Biogenesis of Ferredoxin—Ferredoxin (Yah1p) contains a [2Fe-2S] cluster. In the case of endogenous apoAco1p, efficient cluster biogenesis of imported apoAco1p also requires GTP hydrolysis.
only a background level of radioactivity was detected in the imidazole eluate. Radioactive counts associated with imidazole eluates containing Isu1p were 8–10-fold more than those of control eluates lacking Isu1p, and this difference was considered as radioactive counts specifically associated with Isu1p and shown in Fig. 8A, bar 1. Most likely, this radioactivity represents stable Fe-35S cluster intermediates assembled on Isu1p. When GTPγS was included in the cluster assembly reaction, the radioactivity specifically associated with Isu1p was found to be inhibited by ~50% (Fig. 8A, bar 2). Under these conditions, the yield of Isu1p-His6 bound to Ni-NTA agarose remained unchanged (Fig. 8B). These results suggest that GTP hydrolysis is required for efficient assembly of Fe-S cluster intermediates on Isu1p.

**DISCUSSION**

Several Fe-S cluster-containing proteins reside in mitochondria, and these proteins play important roles in organellar functions. Here we show that isolated intact yeast mitochondria contain a nucleotide (GTP and ATP)-dependent machinery capable of forming new Fe-S clusters and inserting them into endogenous apoaconitase or imported apoferredoxin. A role for GTP in Fe-S cluster biogenesis has not been previously reported. We have shown that GTP acts in the mitochondrial matrix and must be hydrolyzed to facilitate Fe-S cluster biogenesis. Whereas the energy of hydrolysis of the γ-phosphate of GTP and ATP is the same, the addition of ATP cannot circumvent processes that require GTP hydrolysis for cluster biogenesis. Furthermore, the effects of matrix GTP on cluster biogenesis are not mediated by its effects on mitochondrial protein synthesis because cluster biogenesis is not affected in the presence of chloramphenicol, an inhibitor that blocks mitochondrial translation. These results suggest that a critical GTP-dependent step in cluster biogenesis is mediated by a GTPase in the mitochondrial matrix. Many GTP-utilizing proteins exist at the matrix side of the mitochondrial inner membrane (3), and one or more of these GTPases might participate in cluster biogenesis. None of the known components of the mitochondrial Fe-S cluster machinery has been shown to exhibit GTPase activity.
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Fe-S cluster biogenesis in the mitochondrial matrix occurs by a multistep process requiring multiple components. For brevity, cluster biogenesis can be subdivided into early, intermediate, and late stages. The early stage requires cytochrome desulfurase (Nfs1p) activity for the sulfur contribution from cysteine (15, 16), and this process involves the formation of persulfide sulfur (Nfs1p-SSH) bound to a conserved cysteine residue of the enzyme. Nfs1p forms a complex with Isd11p, an 11-kDa protein of the matrix, and this complex is proposed to be the physiological sulfur donor in vivo (35, 36). The cytochrome desulfurase activity of purified Nfs1p does not appear to require nucleotides (37). Whether formation/stabilization of the Nfs1p-Isd11p complex is facilitated in the presence of nucleotides remains to be determined.

The intermediate stage represents assembly of cluster intermediates on the Isu1p/Isu2p and/or Isa1p/Isa2p scaffold proteins. This process requires transport of cytoplasmic iron into the matrix via carrier proteins in the inner membrane (Mrrs3p/Mrs4p), chaperone functions of the yeast frataxin homolog (Yfh1p) to contribute iron, and redox components such as ferredoxin (Yah1p) and ferredoxin reductase (Arh1p) for the reduction of sulfur and other steps involved in transfer (for review, see Ref. 14). Iron import into the mitochondrial matrix does not require nucleotides, as judged by heme synthesis in the matrix (38). Our data suggest that GTP hydrolysis is required for efficient assembly of Fe-S cluster intermediates on Isu1p (Fig. 8). In prokaryotes, insertion of metal (iron or nickel) atoms into hydrogenases is likely mediated by GTPases (39, 40). It is, therefore, tempting to speculate that a GTPase in the mitochondrial matrix, through hydrolysis of GTP, is involved in the insertion of ferrous ions into scaffold proteins for the assembly of Fe-S cluster intermediates. Alternatively, the GTPase may participate in the trafficking of persulfide sulfur from the cysteine desulfurase to scaffold proteins. A precedent for such a possibility is so far lacking. More work is needed to identify the precise GTP-requiring step(s) involved in Fe-S cluster assembly on scaffold proteins.

The late stage involves transfer of Fe-S cluster intermediates from scaffold proteins to apoproteins. A Hsp70 chaperone Ssq1p may be involved in this process (24). The J-protein Jac1p and the scaffold protein Isu1p cooperatively stimulate the ATPase activity of Ssq1p (41). A role of ATP in the “late stage” of cluster biogenesis is, therefore, expected, although this has not been directly demonstrated. Other ATP-requiring steps in cluster biogenesis are also possible. For example, Hsp60 has been implicated in aconitase maturation (42). In an earlier study, mitochondrial detergent extract containing overexpressed biotin synthase was used as an Fe-S protein substrate to determine the energy requirement for its cluster assembly. In this assay samples were incubated with radiolabeled iron and unlabeled cysteine, and the radioactivity associated with biotin synthase was determined by immunoprecipitation. Depletion of endogenous ATP was found to decrease radiolabeled iron associated with the protein, suggesting a role of ATP in the process (43). Using endogenous aconitase of isolated wild-type mitochondria as a substrate, data presented here take these findings one step forward and demonstrate that ATP hydrolysis is required for cluster biogenesis. Whether ATP hydrolysis is specifically required for the transfer of cluster intermediates from scaffold proteins to aconitase remains to be determined. A role of GTP in this process is also possible. Cluster biogenesis in intact mitochondria as described here occurs in a “native” environment without any significant changes in the redox status of the organelle. Further characterization of this process should allow ascertainment of the nucleotide requirements for each stage of Fe-S cluster biogenesis.

Our observation that GTP hydrolysis is required for the cluster biogenesis of both aconitase [4Fe-4S] and ferredoxin [2Fe-2S] is intriguing. Recent studies suggest that pathways for cluster biogenesis of aconitase and ferredoxin might diverge (14, 44). There may exist a common GTP-dependent pathway for the assembly of [4Fe-4S] and [2Fe-2S] cluster intermediates on Isu1p/Isu2p that are destined for aconitase and ferredoxin, respectively. Subsequent transfer of these cluster intermediates to respective apoproteins might then follow different pathways. Although most Fe-S proteins contain the cubic [4Fe-4S] cluster as in aconitase or the rhomboic [2Fe-2S] cluster as in ferredoxin, the GTP requirement described here may or may not provide a general mechanism for all Fe-S cluster-containing proteins in mitochondria. Future research efforts will address these issues.

There is apparently a strong connection between GTP levels and iron metabolism in mitochondria. We previously observed that GTP in the mitochondrial matrix plays an important role in organellar iron homeostasis (8). Here we demonstrated that hydrolysis of matrix GTP is required for efficient cluster biogenesis. These findings together reconfirm an accepted notion in the field that mitochondrial Fe-S cluster synthesis is crucial for maintaining organellar and cellular iron homeostasis (45, 46). Although it has been shown that there should exist an Fe-S cluster regulatory protein controlling iron distribution, the identity of this regulatory protein is still unknown (47). Similarly, the GTPase involved in Fe-S cluster biogenesis and iron homeostasis is also unknown. It would be intriguing if the regulatory protein with Fe-S clusters is also a GTPase. The clusters of this hypothetical protein might provide a critical redox function required for new Fe-S cluster formation, and this process might be regulated through GTP hydrolysis by the GTPase function. A similar scenario has been discussed by Fontecave and co-workers (39) in their characterization of HydF. The HydF protein, required for metalla tion of an Fe-Fe hydrogenase, contains both GTPase activity and an Fe-S cluster cofactor.

Diminished activity of various mitochondrial Fe-S proteins (including aconitase and mitochondrial iron overload are prominent in the heart and central nervous system of patients with Friedreich ataxia. This is a neurodegenerative disease and is characterized by a deficiency in mitochondrial matrix protein frataxin. Studies with yeast and mouse models for this disease suggest that iron accumulation in mitochondria is a consequence of impaired mitochondrial Fe-S cluster biogenesis (48–51). Cluster biogenesis occurs in yeast and human mitochondria. Many of the proteins involved as well as the biochemical features of the process are conserved. Although the precise role of GTP in cluster biogenesis remains to be determined, it may also be a conserved function. Thus, conclusions from yeast studies reported here will be informative to human physiology.
REFERENCES
